

ANTI - INFLAMMATORY POTENTIAL OF VAISVANARA CHURNAM – AN AYURVEDIC POLYHERBAL FORMULATION IN CHOLESTEROL FED RATS**SANTHI KRISHNAN^a, I.S. ASWATHY^b, JASMINE PETER^c, VIDYA SABU^d AND A. HELEN^{e1}**^{abcde}Department of Biochemistry, University of Kerala, Kariavattom Campus, Kerala, India**ABSTRACT**

Ayurveda (Indian traditional medicine) has been used Vaisvanara Churnam (VC) for various inflammatory diseases such as rheumatoid arthritis through oral route of administration. Most of the non-steroidal anti-inflammatory drugs (NSAIDs) are non-specific and bring about gastro-intestinal complications. Synergistic potential of herbal medicines are enormous compared to individual ones. The objective of the present study is to monitor the effect of VC as an anti-inflammatory drug in cholesterol fed rats. Rats divided into five groups, control group, VC (450mg/kg body wt) given group, while other three groups were given high cholesterol diet (HCD) (1.5% cholesterol + 0.5% cholic acid). The fourth and fifth groups were given VC (450mg/kg) and rosuvastatin (RS) (10mg/kg body wt) respectively along with HCD, for 60 days. Results indicated that VC treatment in HCD-fed rats down-regulated the activities of inflammatory enzymes like COX, LOX, NOS, as well as pro-inflammatory cytokines IL-6, IL-1B while up-regulating IL-10, the anti-inflammatory cytokine. Histopathology of heart confirmed the results by showing marked edema in HCD group which was maintained to normal histology in the case of VC treatment. Vaisvanara Churnam, an ayurvedic polyherbal formulation may exert its anti-inflammatory activity by the inhibition of inflammatory mediators that play vital role in inflammatory disorders like atherosclerosis.

KEYWORDS: Inflammation, Atherosclerosis, Vaisvanara Churnam, Lipid Profile

Ayurveda, the Indian system of holistic medicine has its primary goal to know the exact cause of disease ailments and uprooting the disease from roots itself. There are various herbs identified that can effectively manage the problem of inflammation, hyperlipidemia and edema in Ayurveda. Though herbs take time to cure diseases but their results are long lasting without causing any adverse impact on health. Ayurvedic system of medicine uses herbal formulations for inflammation and the combined/synergistic effect of herbal forms are enormous than individual ones (Chorgade, 2007 & Anwikar and Bhitre, 2010).

Inflammation is a defence response of body to hazardous stimuli such as allergens or injury to tissues on the other hand uncontrolled inflammatory response is the main reason of a vast continuum of disorders including atherosclerosis, cancer, autoimmune disorders imposing a huge economic burden on individuals and consequently on society (Bagad et.al, 2013). Recent research advances strongly established the fundamental role for inflammation in mediating all stages of atherosclerosis from initiation through progression (Libby, 2002 & Bhaskar et.al, 2013). Various medicines for controlling inflammatory crisis; NSAIDS (Non-steroid anti inflammatory drugs) and immunosuppressants are non-specific thereby bring about gastro-intestinal complications as well as other adverse effects. Our Society need to apply natural anti-inflammatory factors within medication therapy to achieve enhanced pharmacological response and lowest degree of

side effects (Bagad et.al, 2013 & Ghasemian and Owlia, 2015).

Vaisvanara Churnam (VC) is a classical ayurvedic polyherbal formulation used for treating low appetite, constipation, bloating, rheumatoid arthritis and impaired digestion (Chakradatta Amavata chikitsa (15-18), Sahasrayogam, Astangahridayam). VC composed of Rock salt, Trachyspermum ammi, Trachyspermum roxburghianum, Zingifer officinale and Terminalia chebula. Components of this traditional medicine possess anti-inflammatory, analgesic and laxative properties. Immunity boosting property and improved digestion of VC is well explained in ayurvedic texts. The vata reducing property, laxative and carminative properties of VC indicated that the drug can be used in the treatment of Amavata (Rheumatoid arthritis), Gulma (Lump in Abdomen), Arsha (Haemorrhoids) and other Vata Roga. Due to the presence of rock salt and spices, VC can be used to treat phlegm and acts as expectorant and increases Pitta or digestive juice flow. Studies showed that both the alcoholic and aqueous extracts of Trachyspermum ammi seeds exhibited significant anti-inflammatory potential (Thangam and Dhananjayan, 2003 & Boskabady et. al, 2005). Earlier reports showed that Zingifer officinale (dry ginger) had ameliorative property on musculoskeletal disorders and rheumatism through the inhibition of cyclooxygenase and lipooxygenase pathway in synovial fluid (Srivastava and Musthafa, 1992). There are detailed studies regarding the phytochemical as well as pharmacognostical analysis of VC (Azra et.al, 2008). The

present study elucidated the scientific aspects about the anti-inflammatory potential of VC in cholesterol fed rats, since inflammation is one of the major pathogenesis for atherosclerosis.

MATERIALS AND METHODS

Chemicals and Solvents

All biochemicals used in the present study were purchased from Sigma Chemical Company, and other chemicals of analytical grade were obtained from SRL Chemicals.

Drug Preparation

All the ingredients were procured from local market and the in-house formulation of VC was prepared as per reference (Pattanayak et.al, 2010). All ingredients were taken and roasted in a stainless steel pan at a low temperature till it becomes free from moisture. The ingredients were made into fine powder individually in a pulverizer and passed through sieve & mixed together as per the medicinal ratio to obtain a homogeneous blend.

Animal Experiments

Adult male wistar rats weighing 150-180g were selected from animal house, Department of Biochemistry, Kariavattom campus and used with approval of animal ethics committee [IAEC-KU-17/2014-'15-BC-AH (29)]. The experimental design comprised of Group I - Control, Group II - Vaisvanara Churnam (VC) [450mg/kg], Group III - High Cholesterol Diet (HCD) [1.5% cholesterol + 0.5% cholic acid], Group IV - HCD+VC [450mg/kg], Group V -HCD+ RS (Rosuvastatin) [10mg/kg]. The dosage of 450mg /kg body weight of VC was fixed on the basis of Body Surface Area (BSA) (Shannon Reagan-Shaw et al, 2007) and given in 10ml hot water orally. Duration of the study was 60 days. At the 60th day, of the experiment, rats were subjected to euthanasia by means of intraperitoneal injection of thiopentone sodium (<40mg/kg body weight). Blood and tissues were collected in ice cold containers for various biochemical parameters. Blood samples were collected into tubes without anti-coagulant, kept at room temperature for 1hour. Serum was separated by centrifugation for 20 minutes at 1500g. Collected serum kept in fresh sealed tubes and stored in -80°C until analysed.

Biochemical Estimations

To study the anti-inflammatory effect of Vaisvanara Churnam on HCD-fed rats, the lipid profile in serum and atherogenic index were determined after

treatment for 60 days. Biochemical estimations for analysing the lipid profile comprise TC, TG, HDL-C, and LDL-C levels in serum were measured with a test kit method (Agappe Diagnostics). Cholesterol, HDL-C and TG levels were determined, whereas LDL-C levels were calculated using Friedewald's equation (Warnick G R et. al, 1990). The atherogenic index (AI) was calculated by the following formula: Atherogenic Index (AI) = LDL-C /HDL-C.

Monocyte Isolation

Isolation of monocytes was done as described by (Radhika et.al, 2007) Mononuclear cells were prepared from the whole blood of rats (Hutch et.al, 1996). 3ml volume of histopaque 1083 was placed in a 15ml tube and blood (3ml) was layered on the top of this density gradient. After centrifugation (400 x g for 30 minutes at room temperature) the blood cells were separated into two fractions: upper white layer consisting of mononuclear cells and majority of platelets formed at interface region. A lower layer was formed containing erythrocytes and granulocytes. The plasma layer was formed on the top was clear and contained no cells. Then plasma layer was removed and discarded. From buffy coat monocytes was carefully taken off by aspiration and washed with phosphate buffered saline (PBS). This process was repeated twice. After that pellet was resuspended in the PBS-Tween and was subsequently subjected to freeze thaw in 3 times. The resulting lysate used as enzymes source.

Assay of Inflammatory Enzymes

Assay of cyclooxygenase (COX) activity was done by the method of (Shimizu T et.al, 1984). Monocytes isolated from the control and the treatment groups, incubated with Tris-HCl buffer (pH-8), glutathione 5 mmol/ L and 5mmol/L for 1 min at 25°C. Arachidonic acid 200 µmol /L was added for initiating the reaction and terminated after 20 min incubation at 37°C by the addition of 10% trichloroacetic acid in 1N hydrochloric acid. COX activity was determined after the centrifugal separation and addition of 1% thiobarbiturate. The absorbance read at 530 nm.

Determination of lipoxygenase (LOX) activity was done by the method of (Axelrod B et.al, 1981). The reaction medium [2ml final volume contained Tris-HCl buffer (pH 7.4) 50mmol/L], enzyme protein and solution of sodium linoleate were prepared in a solubilised state. Determination of 5-LOX activity was performed by monitoring the absorbance at 234 nm which reflects the

formation of 5-hydroxyeicosatetraenoic acid. Nitric oxide synthase (NOS) was determined by the method described by (Salter. M and Knowles .R.G., 1997) and NOS activity is expressed as amount of citruline produced per milligram of protein. Protein was assayed by the method of (Lowry O.H et.al, 1951).

Enzyme Linked Immunosorbent Assay (ELISA)

Indirect ELISA was performed using specific antibody. Tissue lysate precoated onto ELISA plates served as the antigen. O-dianisidine was used as substrate and the absorbance of colored horseradish per-oxidase (HRP) product was measured by spectrophotometrically at 405nm with the aid of automated micro plate reader (Thermo Multiskan Spectrum). ELISA used in present study to quantify the amount of different cytokines such as IL-6, IL-1 β , IL-10 using specific antibodies, were purchased from Abcam and Santa Cruz Biotechnology Inc.

Histopathological Examination of Heart

After sacrificing the rats, the heart tissues were removed and washed properly in saline and kept in bouin's fixative solution. The tissues were then processed, sectioned at 5 μ m thicknesses and subsequently stained

with Ehrlich's haematoxylin and eosin for examination under light microscope.

Statistical Analysis

All the results were analysed using statistical program SPSS/PC+, Vr.11.0 (SPSS Inc., Chicago, USA) and expressed as mean \pm standard error of the mean. One way analysis of variance (ANOVA) was done for comparison testing of significant differences among groups. Pair-fed comparisons between the groups were carried out using Duncan multiple range tests. $P < 0.05$ was considered statistically significant.

RESULTS

Serum Lipid Profile And Atherogenic Index

The supplementation of cholesterol in the HCD group showed significantly increased TG, TC and LDL-C levels, and atherogenic index compared with the control, indicating that the rats became hypercholesterolemic, but the treatment of Vaisvanara Churnam (VC) significantly lowered their levels. HDL-C concentration in the serum of HCD-fed rats showed a significant decrease compared to the control; however treatment of VC significantly increased the HDL-C levels.

Table: 1 Changes in the serum lipid profile and atherogenic index

Lipid Profile	I	II	III	IV	V
TC(mg/dL)	65.26 \pm 2.43	63.81 \pm 2.36	176.87 \pm 6.55 ^a	89.88 \pm 3.34 ^{a,b}	94.01 \pm 3.5 ^{a,b}
LDL-C (mg/dL)	28.31 \pm 1.1	24.88 \pm 0.973	123.78 \pm 4.85 ^a	54.69 \pm 2.15 ^{a,b}	59.72 \pm 2.29 ^{a,b}
HDL-C (mg/dL)	39.43 \pm 1.46	41.84 \pm 1.23	19.58 \pm 0.72 ^a	30.65 \pm 1.14 ^{a,b}	24.69 \pm 0.91 ^{a,b}
TG (mg/dL)	91.55 \pm 3.41	88.76 \pm 3.3	218.42 \pm 8.13 ^a	97.88 \pm 3.64 ^{a,b}	101.65 \pm 3.78 ^{a,b}
Atherogenic index	0.718	0.5946	6.322 ^a	1.784 ^{a,b}	2.419 ^{a,b}

I –Control, II – Vaisvanara Churnam (VC), III – High Cholesterol Diet (HCD), IV – HCD+ VC (450mg/kg),

V- HCD+ Rosuvastatin (RS). Values expressed as average of 6 values \pm SEM in each group.

TC, Total Cholesterol; LDL-C, LDL-Cholesterol; HDL-C, HDL-Cholesterol; TG, Triglycerides

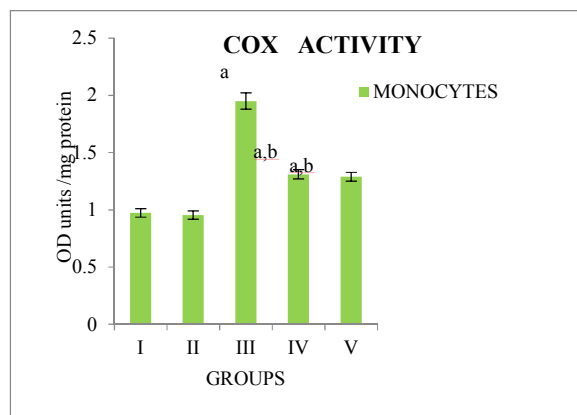
a-Mean value was significantly different from that of group I ($p < 0.05$).

b-Mean value was significantly different from that of group III ($p < 0.05$).

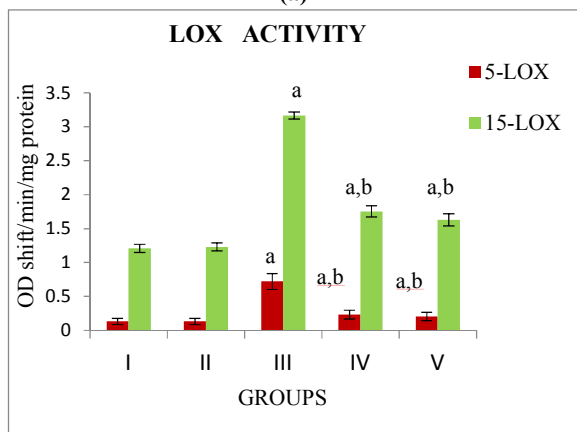
Effect of Vaisvanara Churnam (VC) on Inflammatory Enzymes

To study the anti-inflammatory effect of VC in vivo model system, the activities of inflammatory marker enzymes cyclooxygenases (COX), lipoxigenases (5-LOX and 15-LOX), and nitric oxide synthase (NOS) were done by isolating mononuclear cells. The results

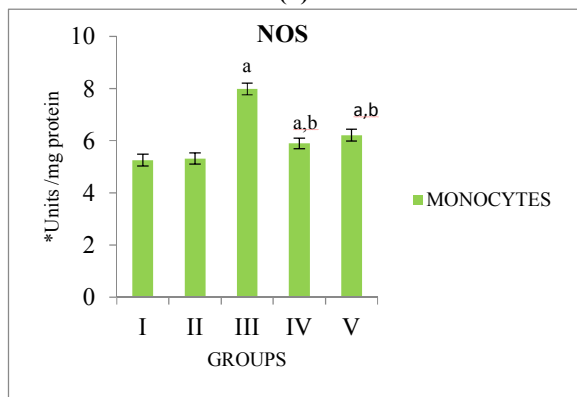
were represented in Figure.1. The cholesterol fed group exhibited significant increase in the inflammatory enzyme activities compared to control group. VC treatment at a dose of 450mg/kg body weight significantly ($P < 0.05$) inhibited the cholesterol induced inflammation.



(a)



(b)



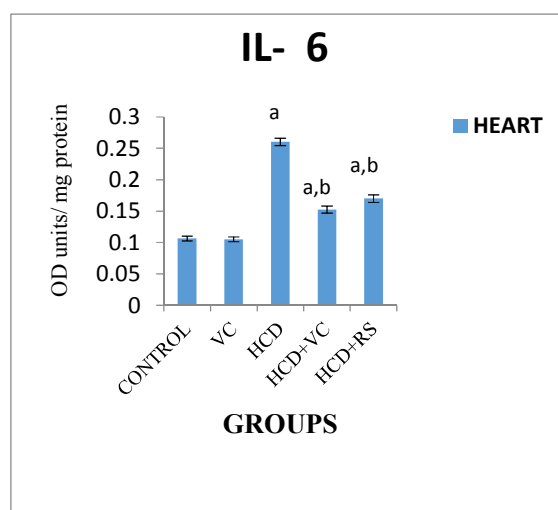
(c)

Figure 1: A-COX activity, B-5 and 15-LOX activities, C- NOS activity.

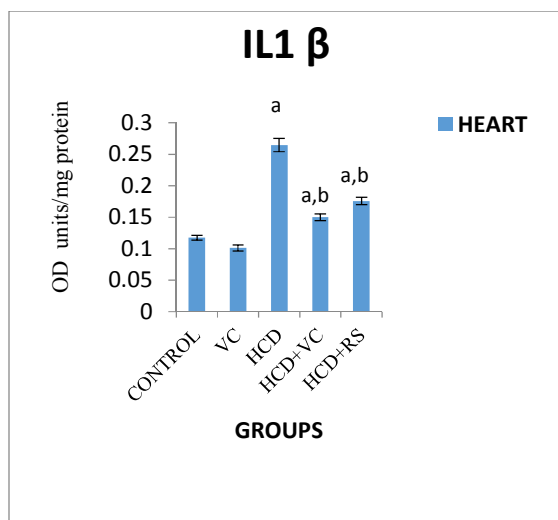
I –Control, II – VC, III – High Cholesterol Diet (HCD)
 IV – HCD + VC, V- HCD+ Rosuvastatin.
 Values expressed as average of 6 values ± SEM in each group
 a -Statistical difference compared with group I (p< 0.05).
 b- Statistical difference compared with group III (p<0.05).
 *Units =1nmol of NO produced /min at 37°C.

Effect of VC Treatment on the Pro-Inflammatory and Anti-Inflammatory Cytokine Levels

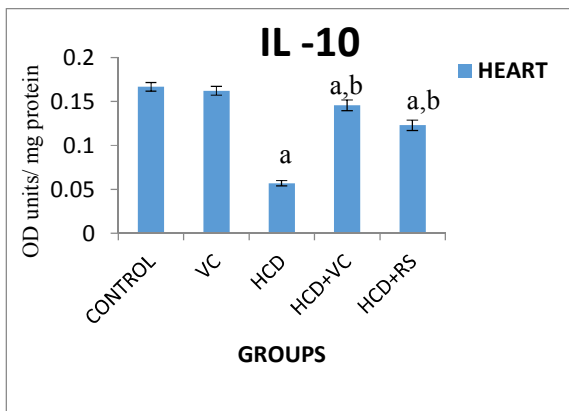
The pro-inflammatory cytokine levels IL-6, IL-1β in the heart of HCD fed rats were found to be significantly higher than that of control. VC treatment significantly (P<0.05) decreased the levels when compared to HCD-fed group. This data was comparable to the standard drug, Rosuvastatin. The level of anti-inflammatory cytokine IL-10 in HCD-fed group was significantly lower as compared to control group while VC treated group showed significantly enhanced IL-10 level. The results shown in Figure: 2.



(a)



(b)



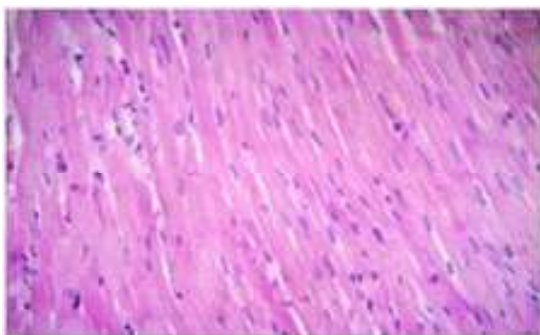
(c)

Figure 2: Values of graphs A, B and C are expressed as average of 6

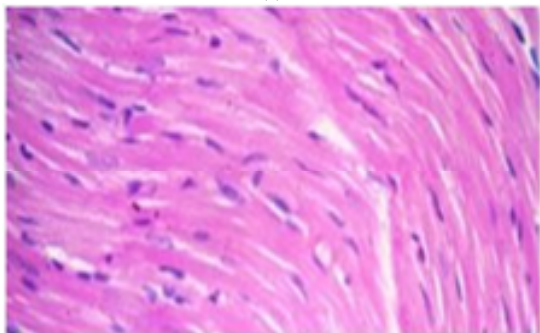
values \pm SEM in each group. a- Statistical difference compared with group I ($P < 0.05$); b- Statistical difference compared with group III ($P < 0.05$).

Histopathological Examination of Heart

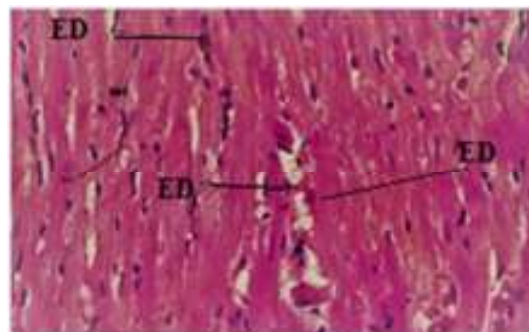
Histopathologic results of heart from the HCD-fed rats showed several marked edema (ED) as well as severe necrotic changes. No evidence of necrosis or haemorrhage seen in the VC treated HCD fed rats myocytes. Some muscle fibers of Rosuvastatin treated HCD fed rats showed slight edema (ED) formation.



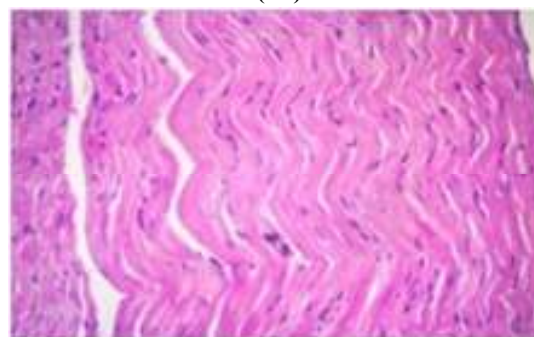
(I)



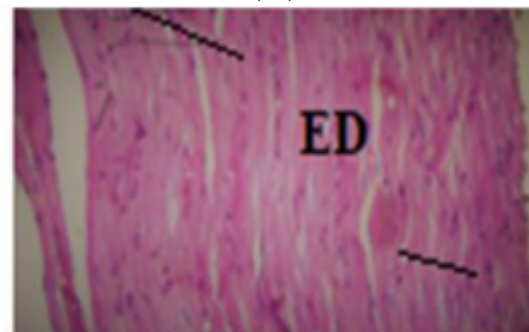
(II)



(III)



(IV)



(V)

Figure 3: Haematoxylin and eosin – stained cross-sections of heart. Group I - the heart of control rat; group II – VC given group (positive control); group III – HCD (High Cholesterol Diet) (negative control); group IV – HCD +VC (450mg/kg b.wt); group; V – HCD + RS (10mg/kg b.wt). ED - edema indicating inflammation in atherosclerosis.

DISCUSSION

Atherosclerosis, the underlying cause of coronary heart disease and stroke is widely regarded as an inflammatory disease process (Ross, 1993). There is a prominent chronic inflammatory component in atherosclerosis that drives the lesion progression in artery wall. (Han and Boisvert, 2015). Cholesterol rich environments contribute the progression of atherosclerosis due to the production of several inflammatory mediators.

The pro-inflammatory cytokines such as IL-6, TNF- α , IL-1 β are important among mediators that help to maintain the progression of inflammation throughout the tissue. They are synthesized by activated macrophages, T-cells, endothelial cells etc. These cytokines are inducers for the production of other pro-inflammatory cytokines and inducible pro-inflammatory enzymes like COX-2 (Ballard et. al, 1988). Excess tissue cholesterol stimulates the biosynthesis of IL-6, IL-1 β , which helps in the propagation and maintenance of the inflammatory response (Kang et.al., 1992).

The serum lipid profile results revealed that the rats became hypercholesterolemic upon high cholesterol diet feeding. Previous studies in our laboratory supported this experimentally induced hypercholesterolemia. However the progressive treatment of Vaisvanara Churnam along with HCD shows hypolipidemic effect. Cyclooxygenase and lipoxygenase enzyme activities in inflammation during atherosclerosis are mediated by a variety of eicosanoids with pro- and anti- atherogenic effects that may vary during the evolution of the plaque. Increased COX activity has been implicated in atherosclerosis (Burleigh et.al, 2005). Studies have shown that 15-LOX highly expressed in macrophages which plays key role in the oxidation of circulating LDL (Belkner et. al, 1993). Present studies supported this data, that higher 15-LOX activity in cholesterol fed rats may enhance the oxidation of LDL, leading to atherogenesis.

Activities of inflammatory enzymes cyclooxygenase, 5-lipoxygenase and 15-lipoxygenase in the mononuclear cells were significantly higher in cholesterol induced rats indicating the increased production of prostaglandins (PG) and leukotriene (LT) production. VC administration significantly reduced the activities of these inflammatory enzymes may be due to the active constituents of VC. The main composition of VC is Terminalia chebula and from the ethanolic extract of fruits of T. chebula contains chebulagic acid, a benzopyran tannin acts as a COX-LOX dual inhibitor (Reddy et al. 2009 & Athira et.al, 2013). Previous study proved that chebulagic acid from the immature seeds of T. chebula significantly suppressed the onset and progression of collagen induced arthritis in mice (Nair et. al, 2010). Vaisvanara Churnam (VC) treatment deteriorated the activities of inflammatory enzymes in cholesterol fed rats. This provides scientific evidence regarding the beneficial role of VC as anti-inflammatory agent may be due to the inhibition of activities of these enzymes leading to the inhibition of PG and LT synthesis. Several studies

have discovered the underlying molecular mechanism behind the anti-inflammatory effect of Trachyspermum roxberghianum (TR) seeds which is another constituent of VC. Its powerful anti-inflammatory and chemopreventive role arises due to the blocking of cyclooxygenase enzyme and 5-lipoxygenase enzymes while suppressing the action of pro-inflammatory cytokines like IL-1 β , TNF- α etc. This may be due to the presence of pharmacologically bioactive terpenes like Thymoquinone (TQ), Bergapten present in TR seeds (Paul et.al, 2013) which are responsible for the significant anti-inflammatory potential of VC.

Role of elevated IL-6 levels contributed to the exacerbation of atherosclerosis. In the present study the levels of IL-6 significantly increased in HCD rats. This higher level of IL-6 rapidly elicits cellular immune responses (Kopf et. al, 1994). This can further activate vascular endothelial cells, upregulate the expression of various chemokines and adhesion molecules facilitate leukocyte recruitment directly to the site of inflammation (Lipsky, 2006). All these processes finally contribute the transition of acute inflammation to chronic inflammation (Kaplanski et. al, 2003). However, VC treatment inhibited the IL-6 production thereby decrease the intensity of inflammatory responses.

Increased IL-1 β which is an pro-inflammatory cytokine, in HCD significantly amplifies the inflammatory response, recruiting more inflammatory cells, stimulating metalloproteinase activities, and ultimately inducing inflammatory cell death (pyroptosis) in leukocytes and resident cells (Terkeltaub et.al, 2009 & Hurme et.al, 1998) while treatment of VC for 60 days brought the level of IL-1 β to normal to some extent. This significantly prevents the recruitment of cells to the site of inflammation.

IL-10 protects against atherogenesis by its anti-inflammatory property, its effects on macrophage polarization, and its effects on modulating lipid metabolism. The ability of IL-10 to suppress the activation of macrophages during inflammation is extensively studied (Libby, 1995). The present study determined anti-inflammatory effect of IL-10 that strongly attributed to the attenuation of atherogenesis by the administration of VC in HCD fed rats. The most notable capability of IL-10 is, it can inhibit the release of several pro-inflammatory cytokines such as IL-1 β , TNF α and IL-8 from monocytic cells and induce the production of IL-1 receptor antagonist (Terkeltaub, 1999 & Uyemura et.al, 1996). By VC treatment, level of pro-inflammatory cytokines were

significantly reduced in cholesterol fed rats that in turn leads to the suppression of series of mediators in the inflammatory cascade. A significant elevation of IL-10 level was observed in the VC treated HCD rats, clearly exhibit anti-inflammatory potential of this classical drug.

Nitric Oxide (NO) plays multifaceted role in the pathogenesis of inflammation and represents distinct physiologic and pathologic effects during all phases of inflammation (Rick Lyons, 1995). The enzyme, nitric oxide synthase that generates NO leads to edema formation in rat myocytes (Schwartz et.al, 2002). Current study revealed that nitric oxide synthase activity was significantly higher in cholesterol induced rats that pointed out the overproduction of NO, ultimately leads to edema in cholesterol induced group. The VC treatment suppressed the activity of nitric oxide synthase thereby prevents tissue lesion formation. This is supported by the histopathologic examination which revealed that prominent myocytic changes occurred in the presence of excess tissue cholesterol in HCD. HCD fed rats showed edema as well as necrotic changes thereby lost the normal cardiac myocytes. It is not evidenced in myocytes of VC treatment which helps to maintain the normal histology. But rosuvastatin, a standard cholesterol lowering drug showed slight edema in myocytes.

CONCLUSION

Vaisvanara Churnam administration proved to maintain equilibrium between pro-inflammatory cytokines and anti-inflammatory cytokines thereby protecting the body from inflammatory disorders. Anti inflammatory effect exerted by VC may be due to the synergistic action of the components present; thereby enhancing the anti-inflammatory cytokines while inhibiting pro-inflammatory cytokines secretion. VC may be useful for alternative therapeutic treatment of clinical conditions associated with atherosclerosis.

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