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Research Article

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ANTIATHRITIC ACTIVITY OF ARUMUGA CHENDOORAM IN FREUND'S COMPLETE ADJUVANT INDUCED ARTHRITIS IN RATS

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ABSTRACT

The aim of the study was to investigate the antiarthritic activity of Arumuga chendooram in Freund's complete adjuvant induced arthritis in rats. Group I served as normal rats. On day zero, group II to IV rats were injected into the sub plantar region of the left hind paw with 0.1ml of Freund's complete adjuvant. This consists of Mycobacterium butyricum suspended in heavy paraffin oil by thorough grinding with motor and pestle to give a concentration of 5mg/ml (This dose confirmed in our lab followed by different concentrations (1 to 10mg/ml)). Administration of standard indomethacin (3 mg/Kg body weight) and Arumuga chendooram treated to group III and Group IV rats respectively were started on the first day and continued for 21 days. Group II rats served as control rats (arthritis rats). Arthritis markers were analyzed end of the experimental periods. Supplementation of Indomethacin and Arumuga Chendooram restored the levels of Nitric oxide, CRP, Homocystine, TNF- α , IL-6, cortisol and RF. N-acetyl- β -glucosaminidase and β -glucuronidase activity were restored in Indomethacin and Arumuga Chendooram treated group. Results of the study concluded that Arumuga Chendooram possesses significant antiarthritic activity. The possible mode of antiarthritic activity of Arumuga Chendooram due to regulation of inflammatory mediators.

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INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, inflammatory, systemic autoimmune disease (Smolen and Steiner, 2003). Several prevalence and incidence studies of RA have been reported during the last decades, suggesting a considerable variation of the disease occurrence among different populations (Riise *et al.*, 2001; Andrianakos *et al.*, 2003). A study carried out in Middle East estimates a prevalence of affects about 1% of the general population; RA prevalence in Egypt represents about 0.2% (Alamanosa and Drosos, 2005). The disease is characterized by aggressive synovial hyperplasia

(pannus formation) and inflammation (synovitis) (Fournier, 2005). Animal disease models that reproduce the pathology of human RA are of great interest as vehicles for the testing of potential therapeutics designed for treatment. These models are required to evaluate the safety, effectiveness, and toxicity of many new potential RA treatments. Rodent populations with RA-like disease are created by collagen-induced arthritis (CIA) and complete Freund's adjuvant (CFA) which behaves similar to rheumatoid Arthritis within the cells, creating synovitis and erosions (Hegen *et al.*, 2008). Lorenzo *et al.* (2008) reported that rodent adjuvant arthritis, as an experimental model, resembles RA in histological pathology, pannus formation and a

number of angiogenic mediators, including cytokines and growth factors. The similarities in joint pathology between AA and RA could be exerted for screening of new drugs for treatment of RA disease.

Conventional medicine includes treatment with steroids, nonsteroidal anti-inflammatory drugs (NSAIDs) as well as biological agents as tumor necrosis factor alpha (TNF- α), interleukin-1 beta (IL-1 β) antagonists (Fleischmann *et al.*, 2004), and disease-modifying anti-rheumatic drugs (DMARDs) (Choy *et al.*, 1998). These treatment agents are associated with unpleasant side effects such as gastrointestinal disturbances (Scheiman, 2001) and have shown only limited success against all forms of arthritis (Chandrashekar *et al.*, 2002). As Alternative therapies are popular among people with rheumatoid arthritis, herbal products are receiving increasing public interest. However, alternative medicine should complement, not replace conventional care. Therefore, the present study was to investigate the Antiarthritic activity of Arumuga chendooram in Freund's complete adjuvant induced arthritis in rats.

MATERIALS AND METHODS

Collection of plant materials

Animals

Male albino rats of Wistar strain approximately weighing 180-220 were used in this study. They were healthy animals procured from Sri Venkateswara enterprises, Bangalore, India. The animals were housed in spacious polypropylene cages bedded with rice husk. The animal room was well ventilated and maintained under standard experimental conditions (Temperature $27\pm 2^\circ\text{C}$ and 12 hours light / dark cycle) throughout the experimental period. All the animals were fed with standard pellet diet (Gold Mohur, Mumbai, India) and water *ad libitum*. They were acclimatization to the environment for 1 week prior to experimental use. The experiment was carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India.

Preparation of Arumuga Chendooram

The Siddha medicine Arumuga chendooram was prepared as per the procedures of IMCOPS, Chennai. In the first stage of the preparation of Arumuga chendooram. Five parts of purified mercury (Suththi seitha rasam), nine parts of purified sulphur (Suththi seitha kanthakam), seven parts of purified lode stone (Suththi seitha kantham), twelve parts of purified iron filing (Suththi seitha ayapodi), four parts of rock salt (Induppu) and eight parts of desiccated borax (Poriththa venkaram) were ground with sufficient quantity of aloe juice (*Kumari charu*

for five days continuously. This was then made into small cakes and dried. It was then sealed in discs and burnt for 24 hours. If the colour of the chendooram does not appear as dark purple the grinding and burning are usually repeated. Equal to pH and then attractive particle interactions predominate which may influence the drug delivery.

Experimental Design:

Freund's Complete Adjuvant induced Arthritic Model

Adult Wistar male rat with an initial body weight of 180 to 220g were taken, and divided into four groups each containing six animals. Group I served as normal rats. On day zero, group II to IV rats were injected into the sub plantar region of the left hind paw with 0.1ml of Freund's complete adjuvant. This consists of *Mycobacterium butyricum* suspended in heavy paraffin oil by thorough grinding with motor and pestle to give a concentration of 5mg/ml (This dose confirmed in our lab followed by different concentrations (1 to 10mg/ml)). Administration of standard indomethacin (3 mg/Kg body weight) and Arumuga chendooram treated to group III and Group IV rats respectively were started on the first day and continued for 21 days. Group II rats served as control rats (arthritis rats). The degree of inflammation was measured by a mercury displacement method. The edema formation and the percentage of inhibition were calculated as follows.

$$\text{Percentage of inhibition} = \frac{V_c - V_t}{V_c} \times 100$$

Where V_c is the edema volume of the control group and V_t is the edema volume of the treated group.

Collection of blood sample

At the end of the experimental period, the animals were anaesthetized using chloroform vapour prior to dissection. Blood samples were collected from the tail vein into a micro centrifuge tube containing 50mM ethylenediamine tetra acetic acid (EDTA) for the determinations of biochemical analysis.

Biochemical analysis

The N-acetyl- β -glucosaminidase activity was determine by the method of Walker and Pugh, (1960). The β -glucuronidase activity was determined earlier by the method of Fishman *et al.*, (1948). The total corticosterone from the homogenate of brain was estimated by following the method of Silber *et al.*, (1958) as modified by Katyase and Pandya (2005). NO concentration in the serum was measured by the method of Sastry *et al.*, (2002).

Measurement of TNF- α , IL-6 and Homocysteine

Plasma TNF- α levels were determined using an enzyme linked immunosorbent assay (ELISA) kit from R&D Systems Inc., Minneapolis, USA. Antibodies specific for rat TNF- α and IL 6 were coated onto the wells of the microtiter strips and the samples including standards of known rat TNF- α was pipetted into the wells, incubated and washed. Intensity of the colour was determined at 450 nm with a correction wavelength of 540 nm. Homocysteine (Hcy) was quantitatively estimated in plasma by Enzymatic Immunosorbant Assay (ELISA). Homocysteine Microplate Enzyme immunoassay provided by BIO-RAD (BIO-RAD, USA). Protein bound Hcy was reduced by dithiotheritol to free Hcy and enzymatically converted to S-Adenosyl-L-Homocysteine (SAH) in a separate procedure prior to the immunoassay.

Rheumatoid factor

The latex turbidimetry method was used in the present study using RF turbilatex kit of SPINREACT Company. Calibration was carried out for linear range up to 100 IU/ml the reading of RF factor of all the groups obtained was compared with the control animals.

C-Reactive Protein

The C-reactive protein (CRP) concentration level in the plasma were quantified using a commercial kit according to the standard protocol recommended by the manufacturer (Assay Pro, USA, and eBioscience, USA). In the C-reactive protein assay, 25 μ L of the standards was added to the relevant wells, and 25 μ L of the diluted samples

were added in duplicates. Biotinylated rat CRP (25 μ L) was added to all wells, gently mixed, and kept for incubation for 2 h. After incubation, the plate was washed using wash buffer and 50 μ L of diluted streptavidin peroxidase conjugate was added and incubated for 30 min. The plate was washed and filled with 50 μ L chromogen substrate and left for 10 min for color development. A stop solution was added, and the absorbance produced was immediately read at 450 nm. The CRP concentration of each of the samples was calculated in mg/mL based on the standard curve obtained.

RESULTS AND DISCUSSION

Effect of Arumuga chendooram on arthritis markers in experimental rats

Nitric oxide, CRP, Homocystine, TNF- α , IL-6, cortisol and RF levels were found to be significantly higher in arthritic rats as compared with normal rats. Supplementation of Indomethacin and Arumuga Chendooram restored the levels of Nitric oxide, CRP, Homocystine, TNF- α , IL-6, cortisol and RF. The restoration with Arumuga Chendooram treatment was statistically significant in Group IV (Table 1). N-acetyl- β -glucosaminidaseb and β -glucuronidase activity were higher in Group II than Group I. N-acetyl- β -glucosaminidaseb and β -glucuronidase activity were restored in Indomethacin and Arumuga Chendooram treated group (Table 1).

Table 1 Arthritis markers of Freund’s adjuvant induced arthritis in experimental rats

Parameters	Group I	Group II	Group III	Group IV
RF (IU/ml)	8.21 \pm 0.55	48.46 \pm 3.2 [#]	24.22 \pm 2.43*	12.32 \pm 0.83*
CRP (mg/dl)	2.74 \pm 0.21	4.65 \pm 0.35 [#]	3.21 \pm 0.25*	2.94 \pm 0.23*
TNF- α (pg/ml)	10.64 \pm 0.72	15.74 \pm 1.07 [#]	11.42 \pm 0.77*	7.42 \pm 0.50*
IL-6 (pg/ml)	33.21 \pm 2.25	98.44 \pm 6.69 [#]	72.94 \pm 5.59*	52.32 \pm 3.87*
Homocystine (μ g/ml)	7.86 \pm 0.53	13.45 \pm 0.91	12.04 \pm 0.83***	9.89 \pm 0.67*
Serum Cortisol (ng/ml)	8.24 \pm 0.56	11.89 \pm 0.80	10.64 \pm 0.69***	8.85 \pm 0.60*
N-acetyl- β -glucosaminidase (U/min/ml)	31.95 \pm 2.17	47.24 \pm 3.21 [#]	42.45 \pm 2.87***	32.14 \pm 2.18*
β -glucuronidase (mU)	1.36 \pm 0.09	2.87 \pm 0.19 [#]	2.56 \pm 0.19**	.1.68 \pm .11*
NO (μ M/L)	27.36 \pm 1.86	58.41 \pm 3.97 [#]	52.68 \pm 3.25***	32.87 \pm 2.23*

Values were expressed as mean \pm SD for six rats in each group.

* Significantly different from Group II [#]*p< 0.001; ** p< 0.01; *** p< 0.05

[#] Significantly different from Group I

Biologic markers, commonly termed “biomarkers,” are biologic characteristics (eg, of blood or joint fluid) that can be objectively measured and serve as indicators of normal or pathologic processes or as measures of the response to therapy. In patients with rheumatoid arthritis (RA) the term is commonly applied to diagnostic or prognostic indicators and to measures used to assess disease activity, such as acute phase reactants. The US National Institutes of Health has defined a biological marker (biomarker) as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” (Biomarkers Definitions Working Group, 2001). The inflammatory process is a combination of many pathways like a synthesis of prostaglandin, interleukin or other chemo toxin, adhesive protein receptor action, platelet-activating factors. All can act as chemotactic agonists. Inflammation initiates with any stress on the membrane or by other trigger or stimuli, these activate hydrolysis of membrane phospholipid by phospholipase A into arachidonic acid, which further substrate for cyclooxygenase and lipoxygenase enzyme and byproduct of these are prostaglandins PGE₂, PGH₂ and leukotrienes like LTC₄, LTB₄ etc. Several cytokines also play essential roles in orchestrating the inflammatory process, especially interleukin (IL), CRP, tumor necrosis factor- α (TNF- α) and nitric oxide (NO). IL-6 and TNF are considered principal mediators of the biological responses to bacterial lipopolysaccharide (LPS, also called endotoxin). They are secreted by monocytes and macrophages, adipocytes, and other cells (Iwalewa *et al.*, 2007; Gallin and Snyderman, 1999).

In our study, markedly increased levels of sera RF, CRP, IL-6, TNF- α and Homocysteine were found in AIA mice. The present results agree with the observation of Gonzalez-Gay *et al.* (2005) who stated that RA is caused by number of pro-inflammatory molecules released by macrophages. These include reactive oxygen species and eicosanoids such as prostaglandins, leukotrienes and cytokines (IL-1, IL-6, and TNF α) (Bharadwaj *et al.*, 1999). Goldring and Gravalles (2000) also reported that characteristic feature of arthritic joints is the persistence of pro-inflammatory cytokines such as TNF- α and IL-6 produced by the inflamed synovium as well as by chondrocytes in the affected joints.

The present results of cytokines can be confirmed by Suffredini *et al.* (1999) who reported that the pro-inflammatory cytokines especially IL-1, TNF α , and IL-6, increase the production of several plasma proteins such as CRP. Arumuga Chendooram treatments significantly inhibited IL-6 and TNF- α

produced by synoviocytes which derived from arthritic tissue in rats decreasing the levels of serum CRP levels as compared with the RA group. Dai *et al.* (2003) suggested that TNF- α considered as an important factor in promoting mechanisms leading to inflammation, whereas IL-6 led to cartilage and bone destruction. Inhibition of IL-6 reduced the extent of inflammation and bone destruction in adjuvant induced arthritis (Feige *et al.*, 2000).

Inflammation is known to result in increased production of nitric oxide (NO) and prostaglandins. NO is an important mediator of diverse physiologic and pathologic processes, including arthritis. Joint inflammation in autoimmune adjuvant induced arthritis is dependent on the enhanced production of NO. NO is ideally suited as a potent inflammatory mediator because of its strong reactivity with oxygen, superoxide, and iron containing compounds (McCartney *et al.*, 1999). Corticosteroids, such as cortisol and cortisone, have been used for the control of inflammation for many years. The action of these corticosteroids seems in part to depend upon their ability to stabilize cell membranes (Belliveau *et al.*, 1982). The low level of corticosterone in arthritis rat plasma may in some way reflect the reduced the inflammatory response.

The concentration of total homocysteine (tHcy) in plasma or serum is an established marker of common diseases. RA is associated with increased co-morbidity and mortality resulting from CVD. A significant component in the pathogenesis, preventing treatment of heart disease involves homocysteine. Elevated homocysteine levels observed in our study were in agreement with a previous study (Wallberg *et al.*, 2002), where higher homocysteine levels were associated with inflammatory markers. Homocysteine facilitates the generation of hydrogen peroxide and causes oxidative damage to low density lipoproteins. Though a significant increase in serum homocysteine levels was observed, the definite role for this marker can be attributed only, if other risk factors which mediated CVD events in RA are also assessed (Pallinti *et al.*, 2009).

Cortisol is an end product of hypothalamic-pituitary-adrenal (HPA) stimulation. Often called an “axis,” this biologic system is stimulated by any inflammatory, emotional, or physical stress (Strittmatter *et al.*, 2005). One of cortisol’s important actions is its anti-inflammatory function. Cortisol can be considered the “brake” for the immune system, preventing it from overreacting to infections, injuries and trauma. Cortisol may rise in any acute pain episode, including a breakthrough pain flare in a patient with chronic pain (Nakagawa and Hosokawa, 1994). Plasma cells produce antibodies e.g., rheumatoid factor (RF) that contribute to these

complexes. Serum rheumatoid factor (RF) is the immunological expression of an individual's immune system reaction to the presence of an immunoglobulin molecule that is recognized as "non-self." This response to the "non-self" immunoglobulin results in the presence of immune complexes. These, in turn, bind complement and may eventually lead to synovium, cartilage, and bone destruction. Higher the levels of serum rheumatoid factor, higher are the development of inflammation (Shivanand Pandey *et al.*, 2010). Arumuga Chendooram treated animal showed significantly lesser serum RF when compared to disease control animals. CRP is an acute-phase protein and has been identified as an important biomarker for various inflammatory, degenerative, and neoplastic diseases (Pepys and Hirschfield, 2003). Elevated levels of CRP have been found in the blood during almost all diseases associated with active inflammation or tissue destruction, particularly in RA (van Leeuwen and van Rijswijk, 1994; Kushner, 1991) Sustained increase in serum CRP levels suggests a lasting production and stimulation of acute-phase proteins during disease progression. This report agreement with Shivanand Pandey *et al.* (2010) studies.

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 and beta D glucuronidase have been implicated in the loss of collagen in RA induced by FCA. In the present investigation sustained elevation of these enzymes was found in RA induced rats. Treatment with Arumuga Chendooram 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 Arumuga Chendooram treated rats demonstrate therapeutic effect of Arumuga Chendooram on the arthritic animals. Regarding the effect of Arumuga Chendooram on cellular enzymes, the data presented indicated a normalization in serum activities NAG and -glucuronidase activity. It has been shown that Arumuga Chendooram has the ability to elevate PGE1. The biological effects of PGE1 in various cells are mediated by activation of adenylate cyclases with subsequent elevation of intracellular cyclic adenosine monophosphate (cAMP) levels (Owen, 1986). Moreover, western blot analysis showed that NAG synthesis was repressed by increasing the levels of intracellular cAMP (Silva *et al.*, 2004).

CONCLUSION

Our data suggested that Arumuga Chendooram possesses significant antiarthritic activity. The possible mode of anti-arthritic activity of Arumuga Chendooram appears to be restored the arthritic markers like RF, CRP, IL-6, TNF- α , Homocystine and enzyme activity N-acetyl- β -glucosaminidase and β -glucuronidase activity through regulation of inflammatory mediators.

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