

Original Article

Appraisal of Hydro-alcoholic Fruit Extract of *Citrullus colocynthis* on Type II Collagen-induced Arthritis Mediated Diabetes in Rats

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Abstract

Context – Rheumatoid arthritis (RA) is a chronic inflammatory systematic auto-immune disease affecting the synovial joints which is associated with progressive disability, premature death followed by socioeconomic burden. *Citrullus colocynthis* in its previous study has been reported for its anti-inflammatory activity on animal models.

Objective – The present study was designed to evaluate the effect of *Citrullus colocynthis* on type II collagen induced Arthritis mediated diabetes in Wistar rats.

Materials and methods – The Collagen induced arthritis model was established and the animals were randomly divided into five groups. Each group was orally administered with the extract (250 mg/kg and 500 mg/kg). Treatment was started on the 14th day and persisted for 25 days. The symptoms of Collagen induced Arthritis and the extract treatment was compared and investigated.

Results – Extract (500 mg/kg) showed a significant decrease of IL-6 and TNF – α followed by a decrease in the blood glucose levels when compared with the positive control. Extract treatment diminished the swelling of the hind limbs and monocyte infiltration in blood vessels in a Collagen induced arthritis animal model.

Conclusion – The results indicate that *Citrullus colocynthis* extract could be used to improve arthritis by reducing the inflammatory factors such as TNF – α and IL -6. However further experiments are required to determine the influence of *Citrullus colocynthis* extract on signal transduction in animal models.

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Introduction

Rheumatoid arthritis is a common skeletal autoimmune disease characterized by synovial inflammation which leads to irreversible joint destruction (1). It has affected about 24.5 million

people till 2015 (2). In India it affects about 0.92% of the adult population. Early diagnosis and treatment can prevent the disability which unfortunately does not happen in any case especially in India. Every year the case increases especially among the female population. The onset might be after delivery in female population. Stress contributes to a major part of the onset of the disease. About 5% of the first degree relatives are in danger for creating Rheumatoid arthritis. Cigarette smoking, coffee consumption and long term use of oral contraceptives increase the development of RA (3). In general it is characterized by loss of cartilage and tendons and inflammation in the synovial joints. The pathophysiological mechanism of RA is not clear, it is known that various immune cells like B and T lymphocytes, osteoclasts, fibroblast and chondrocyte are involved in the chronic inflammation during RA pathogenesis (4). Current treatment therapies including steroids, non-steroidal anti-inflammatory drugs and biological agents like tumor necrosis factor and interlukin 1 beta antagonists have shown only limited effect against RA (5). These therapies are helpful in controlling symptoms associated with acute RA, but unfortunately their effects on chronic and enduring effects are always not satisfactory. The side effects of the drug therapy are significant and include GIT disturbances and cardiovascular diseases (6).

Citrullus colocynthis commonly called as bitter apple is a perennial plant native to the dry areas of North Africa, Egypt, Turkey, India, Pakistan and Ethiopia (7). It contains carbohydrates, proteins, amino acids, tannins, saponins, phenolics, flavonoids, curcubitacins, cardiac glycosides some trace elements and other chemical constituents (8) (9) (10). Traditionally the roots have been used in inflammation of the breasts, joint pains, externally it is used in ophthalmia and in uterine pains. The fruit and roots are mixed with water and applied to boils and pimples. The root paste is applied to enlarged abdomen of the children (7). It possess anti-inflammatory (11), anti-bacterial (12), anti-oxidant (13) and anti-cancer activity (14). The literature reviews have reported the anti-oxidant and diabetic activity of the plant also. Since not much study is was done to evaluate the biological activity of the fruit. In our study we established the CIA rat model and treated the CIA rats with different doses of the fruit extract

to investigate the anti-arthritis activity of *Citrullus colocynthis*.

Materials and Methods

Collection and authentication

The fruits of *Citrullus colocynthis* was collected from the river side of Kanchipuram district Tamil Nadu, India by the end of August 2015. The fruits were authenticated by Prof. P. Jayaraman. Ph.D, Director, Plant Anatomy Research Centre, Tambaram. The authentication number is PARC/2015/3813.

Preparation of the extract

The fruits were shade dried and powdered coarsely. The power was passed through a sieve of number 22. A quantity of the powder was defatted with pet ether then successfully extracted with 70% hydroalcohol at room temperature. The yield was calculated. The extract was filtered and dried in a desiccator. The prepared extract was used for phytochemical screening.

In vitro anti-arthritis activity

The in vitro anti-arthritis activity of petroleum ether and hydro-alcoholic extract of *C. colocynthis* was evaluated by protein denaturation method with diclofenac sodium as the standard drug. The percentage inhibition of protein denaturation was calculated with the following formula

$$\% \text{ Inhibition} = 100X (A_t / A_c - 1)$$

Where A_t = Absorbance of the test, A_c = Absorbance of the blank

Animals

Wistar albino rats (150–200 g) of either sex was used. The animals were obtained from Kings Institute, Guindy. The animals were maintained under controlled temperature ($23\pm20^\circ\text{C}$) and relative humidity (40–60%) with the standard environmental conditions of 12/12 light and dark cycle in the animal house. The experimental protocol was approved by IAEC/162/2015.

Induction of Arthritis and drug administration

Type II collagen was emulsified with incomplete Freund's adjuvant (IFA) at a 1:1 ratio. Rats were intra-dermally injected with 2 mg/kg of collagen–IFA suspension at the base of the tail (day 0). A boost injection with 1 mg/kg of the collagen –IFA suspension was given on day 7 in the same manner (15) (16) (17) (18). Thirty Wistar rats of both the sex were randomly divided into 5 groups with each group consisting of six rats. Group I – normal control (CMC), Group II – Positive control (Arthritis induced), Group III – Low dose (250 mg/kg), Group IV – High dose (500 mg/kg), Group V – Hydrochloroquine (4 mg/kg). Treatment was started from 14th day after induction and continued till 28th day. On 29th day blood samples were collected by retro-orbital puncture for the estimation of cytokines and blood glucose estimation. The ankle joints and pancreas were excised washed thoroughly with saline for the presence of the any tissues and fixed in 10% formalin for the histopathological studies (19).

Assessment of Arthritis in rats

After the second immunization the rats were checked for the development of arthritis based on the extent of edema in their paws. The incidence and severity of arthritis was evaluated by observing changes in their arthritis scores every 2 days, measuring hind paw volumes every 4 days and measuring body weight every 3 days. The observed severity of the arthritis was assessed by a semi qualitative score which as follows 0 – normal with no macroscopic signs of arthritis or swelling, 1 – mild but distinct redness and swelling of the ankle or apparent redness and swelling of the individual digits, regardless of the number of affected digits, 2 – moderate redness and swelling of the ankle, 3 – redness of the entire paw including the digits and 4 – maximally inflamed limb with the involvement of multiple joints.

Measurement of paw volume

Paw volumes of the animals from the respective groups were recorded by using a digital plethysmometer on day 1, 14, 18, 22, 26 and 30 days after collagen induction.

Estimation of TNF – α and IL – 6

The levels of TNF – and IL – 6 were determined with the help of ELISA kits in accordance with the manufacturers instructions (20) (21).

Histopathology

Rats were sacrificed at day 31 after the extract treatment. The ankle joints were harvested fixed in 10% formalin, embedded in paraffin, sectioned followed by Haematoxylin and eosin staining to investigate the joint pathology. Histologic severity was scored using four parameters namely – joint inflammation, chondrocyte death, cartilage erosion and bone erosion.

Statistical analysis

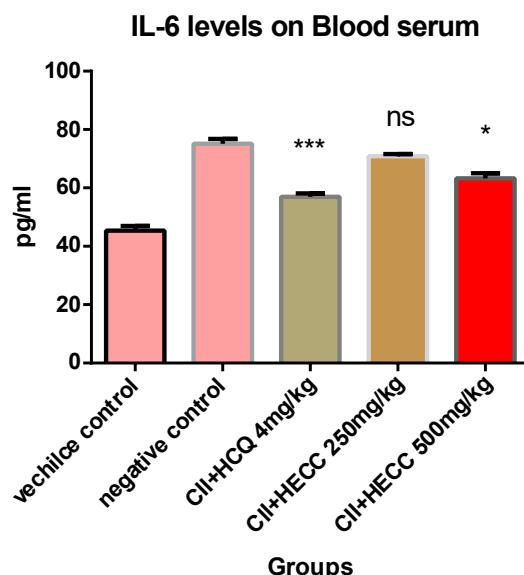
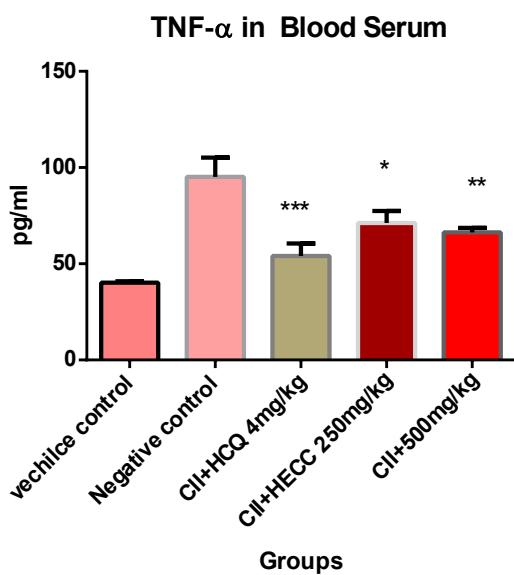
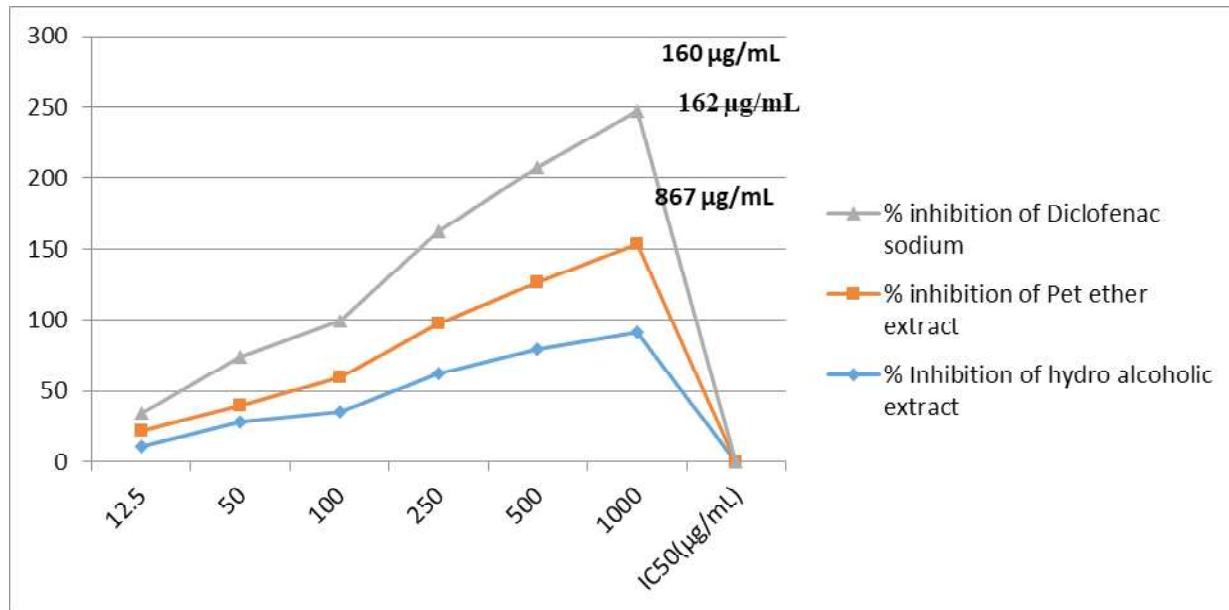
All the values were expressed as Mean \pm SEM. The data was analyzed by one way ANOVA followed by Dunnet's test. All the statistical analysis were performed using Graph Pad Software (Version 6.0).

Results

The anti-inflammatory potency of the Pet ether and hydro-alcoholic extract of *C.colocynthis* was estimated by *in vitro* anti-arthritic activity. The IC₅₀ value was found to be 867 μ g/mL for pet ether extract, 162 μ g/mL for hydro-alcoholic extract and standard drug diclofenac it was 1160 μ g/mL respectively. Based on the values the hydro-alcoholic extract was selected for *in vivo* studies.

Estimation of TNF – α

TNF- α values are given in the form of Group V shows a significant decrease in the TNF- α ($P<0.01$), where as Group IV shows only less significant decreased in TNF- α levels when compared with Control group. Group III shows a maximum significant decrease in the level of TNF- α ($P<0.001$). The result suggest that higher dose of test extract shows maximum decreasing level of TNF- α . Values are expressed in Mean \pm SEM. Comparison were made between Group II (Negative control) vs Group III, IV, V.

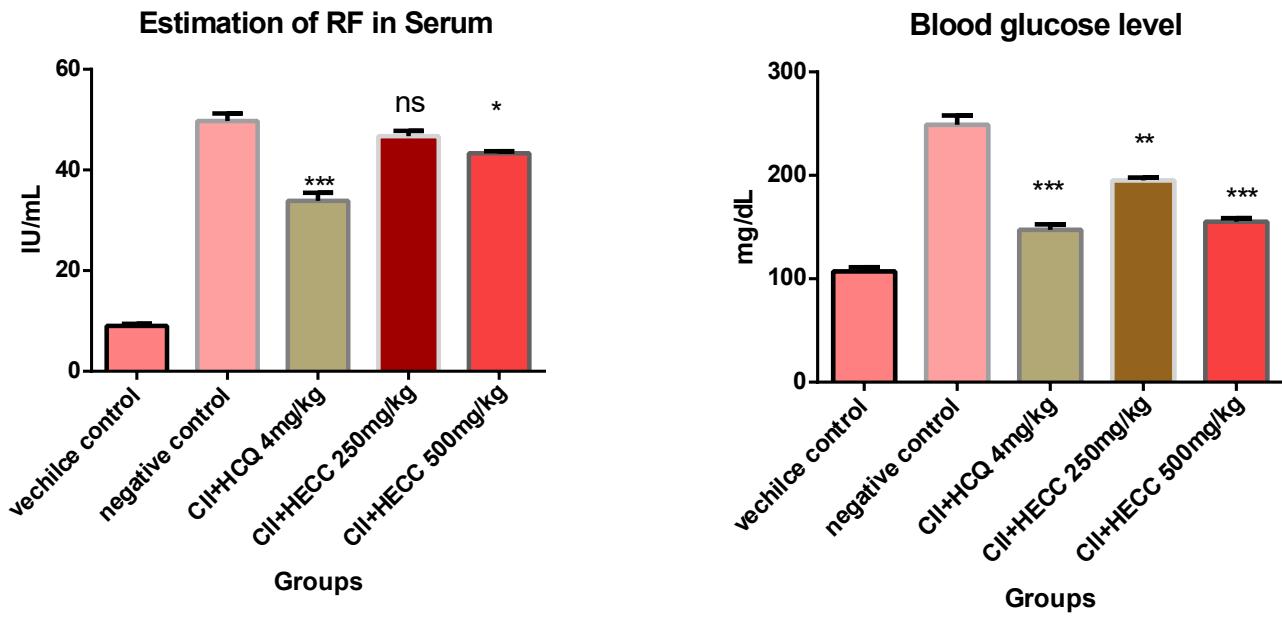


Estimation of IL-6

IL-6 values are depicted in the bar diagram. There was no significant difference in Group IV, whereas Group V shows a significant difference with control group ($P<0.05$). Group III shows a better response to the IL-6 ($P<0.001$). From this result higher dose of test extract having a minimal inhibition on IL-6, whereas low dose doesn't have any response on IL-6. Values are expressed in Mean \pm SEM. Comparison were made between Group II (Negative control) vs Group III, IV, V.

Estimation of Rheumatoid factor

Rheumatoid factor is one the important biomarker for RA. However it has on little impact on DM. RF positive will increase the lipoprotein which is directly increase the metabolic disorder and lead to the DM. Group V shows the significant decrease in RF when compare to control group ($P<0.05$), whereas Group IV shows no significant decrease in RF in blood serum. Values are expressed in Mean \pm SEM. Comparison were made between Group II (Negative control) vs Group III, IV, V.



|Histopathology of Ankle Joints



Fig. 1 Normal Fig. 2. Arthritis induced Fig. 3 Low dose Fig. 4. High dose Fig. 5 Standard

Histopathology of Pancreas



Fig.6.Normal Fig.7 Arthritis induced Fig.8 Low dose Fig.9 Standard Fig 10. High dose

Estimation of blood glucose level

In control Group blood glucose was increased upto 250 mg/dL, group V shows significant decrease in Blood glucose level ($P<0.001$) which is equal to Group III, whereas Group IV shows less significant value ($P<0.01$).

Discussion

RA is a autoimmune disease that causes inflammation of the joints which is portrayed by hyperplasia of the synovial tissues, vascular exclusion and the formation of granulation tissues (22) (23). In the pathogenesis of RA, the aggravation

that begins in the synovial layer or the membrane spreads to the ligament and the bone tissues prompting annihilation of the joint structure. Inflammatory mechanisms also initiate angiogenesis which further leads to joint deformity, rigidity and finally loss of function (24). RA is a chronic disease where patients experience episodes of occurrence and mitigation. Although individual cases differ the morbidity rate of RA may reach to about 60–70% (25). Studies give knowledge into the underlying mechanism of RA to justify the novel remedial medications. Till now it has been archived that various mRNA and micro RNA molecules are engaged in the pathogenesis of RA (26), followed by numerous inflammatory cytokines, including TNF- α and IL-1, which may eventually participate in damaging the inflammatory responses observed in RA(27)(28).

In this study effect of *C. colocynthis* fruits extract on arthritis and its mediated diabetes mellitus was estimated. Chronic arthritis condition leads to the higher risk of developing impaired glucose metabolism that may eventually progress to type 2 diabetes mellitus (29). Cytokines like TNF- α and IL-6 plays a major role in RA and DM. In chronic arthritis condition levels of TNF- α and IL-6 is elevated. On this condition these elevated cytokines will lead to the DM many ways. TNF- α plays a dual role in the pathogenesis of RA and Diabetes mellitus. TNF- α will lead to the Insulin receptor resistance. *In vitro* experiment have been shown that TNF- α can inhibit the signal from the insulin receptor by tyrosine phosphorylation of the both insulin receptor and insulin receptor substrate. Strong evidence from the studies like animal models and cell culture methods it states that TNF- α have a vital role in the alteration of glucose metabolism in adipose tissue and liver (30).

Increasing level of TNF- α in blood levels leads to release of leptin, an another inflammatory chemokines which is produced by the adipose tissues, this leptin decrease the insulin secretion and leads to IR (31). TNF- α may function as a mediator of insulin resistance because this cytokines can damage the insulin receptor and insulin receptor substrate (IRS) and then this cytokines can stimulate the expressions of SCOS (Suppressor of cytokines

signal) which binded either IRS1 or IRS2 and mediate the insulin damage (32). As a result, insulin could not take glucose into the muscle cells and adipose tissue therefore glucose levels will be increased in blood plasma. As its compensation the β -cells of the pancreas will produce excessive amount of insulin and leads hyperinsulinemia (33).

IL-6 is a important cytokine with a key role on nonimmune and immunoregulation events in immune system. Circulating IL-6 will increase insulin resistant states. The effect of IL-6 on insulin receptor is higher than TNF- α . IL-6 will inhibit the insulin receptor (IR) signal transduction as well as it will inhibit the synthesis of glycogen (34). When increased level of IL-6 in body will affect both the proximal and distal events in hepatic IR signal transduction (35). Increased IL-6 levels in one of the major marker for the RA condition. Group II showed a significant elevated levels of IL-6. Group V shows a decreased level of IL-6, but Group IV shows no significant changes in the level of IL-6 (36) (37).

In this present study level of TNF- α was increased significantly in control group, whereas extract treated group shows significant decrease level of TNF- α in dose dependent manner which near to the standard group. So it was concluded that extract of *C. colocynthis* was inhibited the elevated levels of TNF- α in RA condition (38) (39). Rheumatoid factor levels were increased in CII induced arthritis control group. When animal were treated with extract it shows significant decrease of RF in Group V but Group IV shows no significant changes in the level of IL-6(40)(41)(42).

Blood glucose level were increased significantly in Group II, it shows that elevated level of cytokines such as TNF- α and IL-6 leads to the DM (40). From this it was confirmed that arthritis will mediate the DM. After treatment, blood glucose was decreased in dose dependent manner, Group III also shows a significant decrease in the level of blood glucose levels. From this result by suppressing the cytokines in arthritis condition will leads to the minimize the chances of occurring DM in RA.

Histopathological changes of joints of the

experimental groups are showed a marked difference. Group I showed the histopathology of normal ankle joints. Group II negative control arthritic ankle joint showed prominent abnormalities from the normal ankle joints, like edema formation, degeneration with partial erosion of cartilage. Standard drug treated rat joint showed normal bone marrow. Other two groups (Group IV and V) showed cellular infiltrates on the articular surface with less cartilage destruction.

In Pancreas histopathology group shows normal cells arranged in lobules with prominent nuclei. Group II (arthritis control) the islets are largely occupied by a uniform eosinophilic material and few atrophic cells. Group III, Beta cells were seen normal, in Group IV the islets were present with heavy lymphocytic infiltration in and around it. Group V cells were present with large proportion of islet cells, though with smaller volume as compared with Group I. infiltration and no eosinophilic deposits were seen.

Consolidating all above the result we concluded that arthritis will mediate diabetes mellitus via many mechanism. Cytokines like TNF- α and IL-6 will mediate the arthritis and its causes DM. By suppressing the cytokines in the arthritis diabetes

mellitus can be prevented. This effect of the extract might be due to the presence of Cucurbitacin compound. In addition recently they have found Cucurbitacin which is main active constituent of *C. colocynthis* has the potency to inhibit the TNF- α induced cytokine in human synoviocyte MH74 cells (43).

Conclusion

The results of this study indicate that the administration of the *Citrullus colocynthis* extract reduced the elevated blood inflammation indicator including IL-6 and TNF- α type II collagen IgG in serum. Moreover the infiltration of inflammatory cells into the synovial membrane and swelling of the hind limb improved with the *Citrullus colocynthis* treatment. In addition hematological parameters and toxicity marker of the kidneys did not change by the administration of *Citrullus colocynthis* extract. This we suggested that *Citrullus colocynthis* extract may act as therapeutic agent against rheumatoid arthritis. However further experiments are required to explore how the extract influences the anti-inflammatory mechanism or inflammation signaling pathways including NF- κ B or NACHT and PYD domains containing protein 3 (NLRP3).

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