

Full Length Research Paper

A study of the inhibitory effects of *Citrullus colocynthis* (CCT) using hydro-alcoholic extract on the expression of cytokines: TNF- α and IL-6 in high fat diet-fed mice towards a cure for diabetes mellitus

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To promote the proper use of herbal medicine and to determine their potential as sources for new drugs, it is essential to study medicinal plants, which have a folkloristic reputation in a more intensified way. *Citrullus colocynthis* (CCT) is used in Iranian traditional medicine as a healing agent for reducing obesity-related diabetes troubles. We proposed that CCT may perform its effects through inhibition of inflammatory cytokines secreted in obesity conditions. Control group was fed with normal diet (N-D) for 42 days alone or plus 50 mg/kg hydro-alcoholic (H-A) extract of CCT. The obese mice were given high fat diet (H-F-D) for 42 days alone or plus CCT extract. Food intake and body weight were recorded each week and expression of TNF- α , IL-6 and IL-10 in serum were assayed by ELISA technique after every two weeks. CCT extract reduced body weight by 4.02% (ns- $p > 0.05$) and food intake by 3.52% (ns- $p > 0.05$), but dramatically decreased expression of TNF- α 44.83 (** $p < 0.001$), IL-6 30.23 (** $p < 0.001$) and marginally increased IL-10 5.31 (ns- $p > 0.05$) in obese mice. This study demonstrated that, although CCT extract did not show anti-obesity effects, it could have an anti-inflammatory effect through down regulation of obesity-associated pro-inflammatory cytokines.

Key words: *Citrullus colocynthis*, inflammatory cytokines, obesity, TNF- α , IL-6, IL-10.

INTRODUCTION

Herbal medicine represents one of the most important fields of traditional medicine all over the world. Different extracts from traditional medicinal plants have been tested to identify the source of the therapeutic effects (Cragg et al., 1997). *Citrullus colocynthis* (L.) Schrad., Cucurbitaceae (colocynth or wild-gourd or bitter-apple), is a non hardy, herbaceous perennial vine, branched from the base. In south-eastern of Iran, CCT locally known as Abujahl watermelon is a well recognized plant in the traditional medicine and was used by people in rural areas as a purgative, anti-diabetic, and insecticide.

Moreover, traditional medicine in Iran has for centuries used the fruits of CCT for the treatment of diabetes and hemorrhoids. Obesity is an abnormal condition of accumulating lipid in the adipose tissue. It is known that it is caused by various environmental and genetic factors and, one of the main environmental factors causing obesity is the high fat diet (H-F-D) which has come into wide use today. Obesity can be a risk factor for many diseases, including insulin-dependent *Diabetes mellitus*, hyperlipemia, and hypertension. Hence it is very important to prevent obesity for a healthy life (Bray, 2004; Kopelman, 2000). Recently, obesity has increased at an alarming rate and is now a worldwide health problem. It is widely accepted that obesity results from disequilibrium between energy intake and expenditure and obesity, is known to be a strong risk factor for type II diabetes associated with insulin resistance (Larsson et al., 1981;

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Hartz et al., 1983). One of the novel strategies for anti-obesity is to exploit the natural products from traditional medicinal plants in form of plant extracts or functional foods. However, information in pharmacology and action mechanisms of natural compounds present in oriental remedies are limited. The active ingredients of many plant species are isolated for direct use as drugs, lead compounds or pharmacological agents (Fabricant and Farnsworth, 2001; Grover et al., 2002). In the United Arab Emirates, CCT belongs to the mostly used plants in folk medicine, because of the anti-inflammatory activity (Wasfi et al., 1995). Traditional plant medicines or herbal formulations might offer a natural key to unlock diabetic complications (Ivorra et al., 1989; Nammi et al., 2003). CCT contains active substances such as saponins, alkaloids and glycosides and it is used as anti-diabetic, antihypertensive and antioxidant (Abdel-Hassan et al., 2000; Ziyyat et al., 1997; Gebhardt, 2003). Some studies have shown that CCT can exert insulinotropic and immunostimulating effects (Wasfi, 1994; Nmila et al., 2000; Bendjeddou et al., 2003; Al-Ghaithi and El-Ridi, 2004; Dallak et al., 2009). The mechanisms by which chronic inflammation can evoke diabetes are not clear. However, it is known that the synthesis and release of the main pro-inflammatory cytokines, TNF- α , IL-1 and IL-6, are associated with diabetes. Adipose tissue is one of the main sources of inflammatory mediators and so one of the major risk factors for becoming diabetic. Adipose tissue is a significant source of endogenous TNF- α and the expression of this cytokine in adipose tissue is elevated in obesity (Peraldi and Spiegelman, 1998). This abnormal expression of TNF- α in adipose tissue plays a critical role in peripheral insulin resistance in obesity. The increased expression of TNF- α is significantly correlated with the hyperinsulinemia in the presence of normoglycemia. It has been demonstrated as a marker of insulin resistance (Hofmann et al., 1994). The cytokine IL-6 has been implicated as a pathogenetic factor in the early events leading to diabetes. IL-6 is secreted by subcutaneous adipose tissues, and levels of this cytokine correlate well with the BMI of humans (Mohamed-Ali et al., 1997). The anti-inflammatory properties of IL-10 include inhibition of pro-inflammatory cytokine production from macrophages and lymphocytes and promotion of the IgG antibody response (Moore et al., 2001). Therefore, activated innate immunity and inflammation are relevant factors in the pathogenesis of obesity associated diabetes (Abdel-Hassan et al., 2000; Frohlich et al., 2000; Satoh et al., 2003; Pickup, 2004). The objective of this study is to determine whether CCT extracts have a considerable effect on the expression of adipose tissue cytokines, in order to clarify their biological activity for the treatment of obesity-associated diseases.

Therefore, the effect of CCT extract to suppress inflammation that is associated with obesity-related diabetes is investigated.

MATERIALS AND METHODS

Plant materials and extraction

Fresh plants were collected from south-east Iran, especially Sistan region in large quantities. The plant was identified by Dr. Sh. Najafi, Department of Biology, Zabol University. Fruits were thoroughly washed using deionized water, and mopped with tissue paper and air-dried in a shade to prevent the decomposition of chemical constituents. All seeds were separated manually from the pulp of the fruits. The dried pulp of fruits was homogenized with a grinder (Muleinex) to fine powder before extraction. The pulp powder from individual CCT (250 g) was extracted three times at room temperature with 100 ml of water/ethanol mixture (80/20, v/v) for 6 h each round according to the approach of Halliwell and Gutteridge (1985) and Yoshikawa et al. (2007). Ethanol-soluble portions were pooled from the 300 ml filtrate. The oven (45 to 50 °C) dried ethanol extract (10 g) was dissolved in freshly prepared normal saline (0.9%) to a final stock solution (10 mg/ml), which was used later to administer 150 μ l (50 mg/kg) of the extract to mice in the treatment group.

Animal treatment

Male BALB/c mice, at four weeks of age procured from Pasteur Institute, Tehran, Iran were used. Forty eight (n = 48) mice weighing 21 to 25 g maintained under 12 h light/dark cycle (7 am on, 7 pm off), at 23 to 25 °C and humidity 50 to 70%. Mice were allowed to acclimatize in our animal facility laboratory for three days before being randomly assigned into four groups (n = 12 for each group). Mice had free access to tap water throughout the study. All experimental protocols were approved by the Institutional Animal Ethics Committee prior to the beginning of the experiments. The mice were housed in standard metal cages (12 mice /cage) and were fed either a standard diet containing 11.7% fat calories (containing 50% wheat, 21% corn, 20% soybean, 8% concentrated proteins and 1% salts and vitamins) or a H-F-D containing 40% fat calories (saturated fat from anhydrous milk fat) and 0.2% cholesterol (Reeves et al., 1993) with or without 50 mg/kg of CCT extract for a period of 6 weeks. On daily basis, food containers were removed every morning at 9:00 am and returned to with fresh food at 6:00 pm. After the herbal treatment experiments, all mice were killed, four mice per group fortnightly at 10:00 am after overnight fasting. Body weight was recorded every two weeks.

Toxicity assay

For mortality assay, the mice were treated with different concentrations of H-A extracts of CCT (25, 50, 75, 100 mg/kg) for distinct periods (28, 35, 42 days). The percentage of live mice was taken as a measure of animal viability. Only mice fed 25, 50 mg/kg extract lived until 42 days (100%), while higher doses showed toxic effect with decreased animal viability. Finally the highest dose with maximum viability (50 mg/kg) was used in our experiments.

Experimental design and sample preparation

After the animals have attained 3 weeks of age, they were divided into three real experimental groups labeled A-C and one control groups. In group A, animals fed N-D with an adequate amount of saline (the solvent of CCT) for 42 days. In group B, animals fed H-F-D with an adequate amount of saline (the solvent of CCT) for 42 days (obese mice). In group C, N-D fed animals received 50 mg/kg of H-A extract of CCT by gavage for 42 days. In group D, H-F-D fed

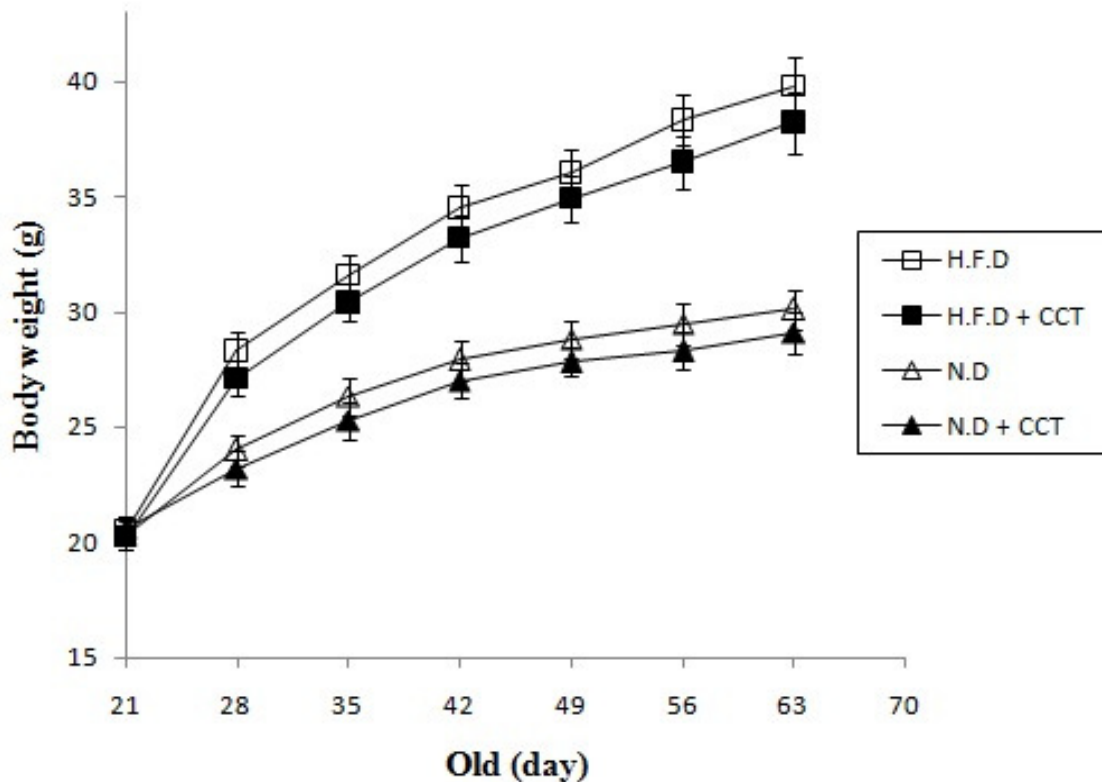


Figure 1. Effect of H-A extract of CCT on body weight in mice fed a normal and H-F diet. Group N-D received normal diet (N-D) by solvent of CCT (0.9% saline) as a standard group; group N-D+CCT received N-D followed by 50 mg/kg of H-A. Extract of CCT orally; group H-F-D received high fat diet (H-F-D) followed by solvent of CCT (0.9% saline) as an obesity or inflamed group; group H-F-D+CCT received H-F-D followed by 50 mg/kg of H-A extract of CCT orally. Values are the mean \pm S.E.M. of 14 mice in each group. In all groups p -value are not significant ($p>0.05$).

animals received 50 mg/kg of H-A extract of CCT by gavage for 42 days. Food intake and body weight were recorded every week. At the selected time intervals (every two week in each groups), mice blood was collected by cardiac puncture and shed on suitable tubes. Serum was obtained by centrifugation at 3000 rpm/20 min and stored at -80°C . The concentrations of TNF- α , IL-6 and IL-10 in serum were measured with ELISA kits, according to the manufacturer's manual.

Quantification of total serum cytokines

The concentrations of each cytokine in the serum were determined using commercially available ELISA kits (eBioscience, San Diego, CA, USA). Each well of a microplate was coated with 100 μl of capture antibody, and incubated overnight at 4°C . After washing (five times) with buffer (1x PBS, 0.05% Tween-20) and blocking with assay diluent, serum and standard cytokines were added to individual wells and the plates were maintained for 2 h at room temperature. The plates were washed (5 times), then biotin conjugated detecting mouse antibody was added to each well and incubated at room temperature for 1 h. The plates were washed again and further incubated with avidin-HRP (horseradish peroxidase) for 30 min before detection with 3,3',5,5'-tetramethylbenzidine (TMB) solution. Finally, reactions were stopped by adding stop solution (1M H_3PO_4), and absorbance at 450 nm was measured with an ELISA reader (Molecular Devices,

Sunnyvale, CA, USA). The amount of cytokine was calculated from the linear portion of the generated standard curve.

Statistical analysis

Data on all parameters are expressed as group means \pm SEM ($n = 14$ animals/group). Differences between the experimental groups were analyzed using the Student's t -test. Differences among groups at different time-points (body weights) were analyzed by repeated measurement of analysis of covariance (ANCOVA) using baseline weight as the covariate (SAS version 9.1.3, SAS Institute, Inc., Cary, NC). In all analyses, p -values <0.05 were considered to be statistically significant.

RESULTS

Body weight and food intake

The results of body weight and food intake are shown in Figures 1 and 2, respectively. Although rats fed with the H-F-D continued to show increased body weight (32.23%, $***p < 0.001$) and food intake versus N-D group until the end of the study, CCT extract marginally reduced

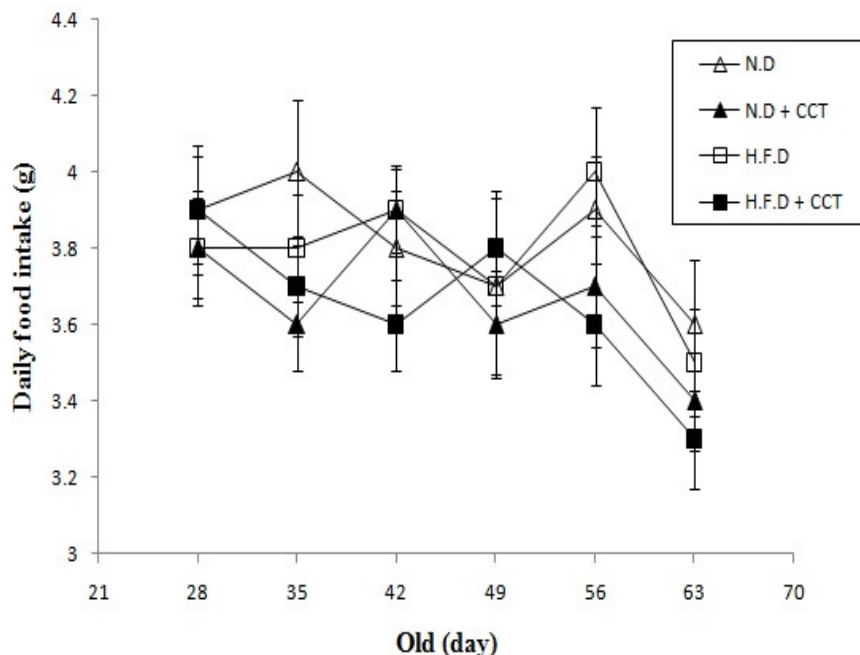


Figure 2. Effect of H-A extract of CCT on daily food intake in mice fed a normal and H-F diet. The group abbreviations (N-D, N-D+CCT, H-F-D, H-F-D+CCT) are the same with Figure 1. Values are the mean \pm S.E.M. of 14 mice in each group. In all groups p -value are not significant ($p > 0.05$).

body weight by 4.02 and 3.32% (ns - $p > 0.05$) and food intake by 3.52 and 3.93% (ns - $p > 0.05$) in obese and normal mice respectively. The body weight of the CCT-treated group did not show significant differences from the control group in each diet (ns - $p > 0.05$). In the H-F-D diet groups with or without CCT treatment, diarrhea was not reported during the experiment. The intake was determined by the difference between the initial weighed of administered food and the weight of food left at the end of each week for a period of 6 weeks. Food efficiency was not increased in the H-F-D group compared with the normal group, and treatment of CCT extract reduced that value not significantly in both diet groups. Treatment with CCT reduced the daily food intake relative to the control group in each diet (ns - $p > 0.05$) and total food consumption during the whole experimental period was not very different among groups, being 22.9 ± 2.1 g (N-D), 22 ± 0.9 g (N-D \pm CCT), 22.1 ± 1.6 g (H-F-D), and 21.9 ± 0.1 g (H-F-D \pm CCT), respectively.

Measurements of soluble cytokines

In order to gain further insights in the regulatory effects of CCT on obesity-related inflammation *in vivo*, we first fed mice with HFD to up-regulate the expression of inflammatory cytokines. To investigate the effect of CCT extract in the time course of the cytokine secretion in obese BALB/c mice, both anti-inflammatory (IL-10) and

pro-inflammatory (IL-6 and TNF- α) cytokines were determined. The results showed that all the levels of IL-10, IL-6, and TNF- α secretion in the obese mice increased, although slightly fluctuating, in a time-dependent manner during the six-week incubation period (Figures 3, 4 and 5). Consistent with fatty accumulation, HFD-fed mice showed a dramatic increase in serum TNF- α (92.26%, $***p < 0.001$) and IL-6 (137.4%, $***p < 0.001$) levels and a moderate increase in serum IL-10 levels (23.21%, $*p < 0.05$) in comparison with control mice (Figures 3, 4 and 5). *In-vivo* down-regulation of TNF- α and IL-6 was confirmed by the CCT extract, with decrease of IL-6 and TNF- α secretion by 44.83 ($***p < 0.001$) and 30.23 ($***p < 0.001$), respectively in comparison to the obese mice after six weeks administration (Figures 3 and 4). Serum levels of IL-10 were not significantly different among treatments and CCT extract slightly increased IL-10 by 5.31% (ns - $p > 0.05$) and 6.55% (ns - $p > 0.05$) in obese and normal mice respectively (Figure 5). Furthermore, CCT extract decreased the expression of TNF- α and IL-6 by 22.56% ($*p < 0.05$) and 32.51% ($*p < 0.05$), respectively, in normal mice (Figures 3 and 4).

DISCUSSION

The concept of inflammation in relation to metabolic conditions, such as obesity and insulin resistance started

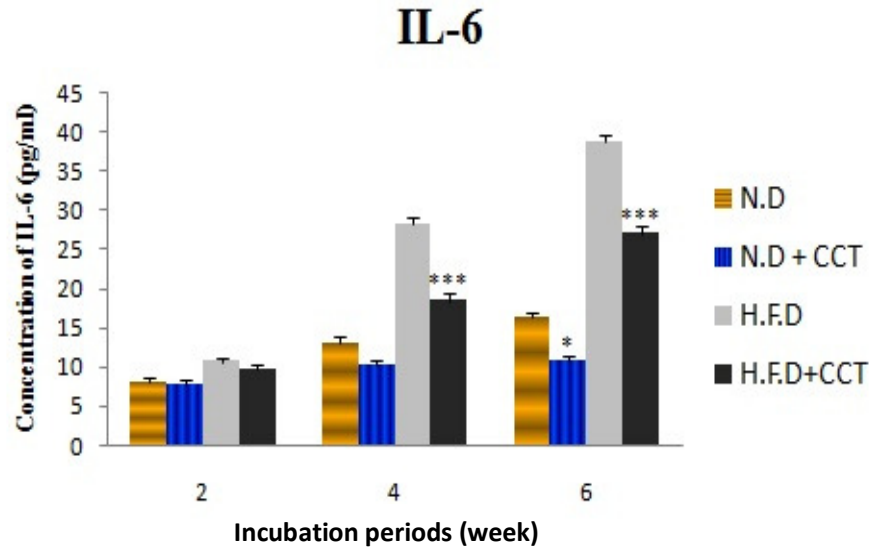


Figure 3. Effect of oral administration of pulp extract of CCT on serum IL-6 levels in different groups of mice. The group abbreviations (N-D, N-D+CCT, H-F-D, H-F-D+CCT) are the same with Figure 1. Data are presented as the mean \pm S.E.M. * p <0.05 and *** p <0.001 vs HF diet group.

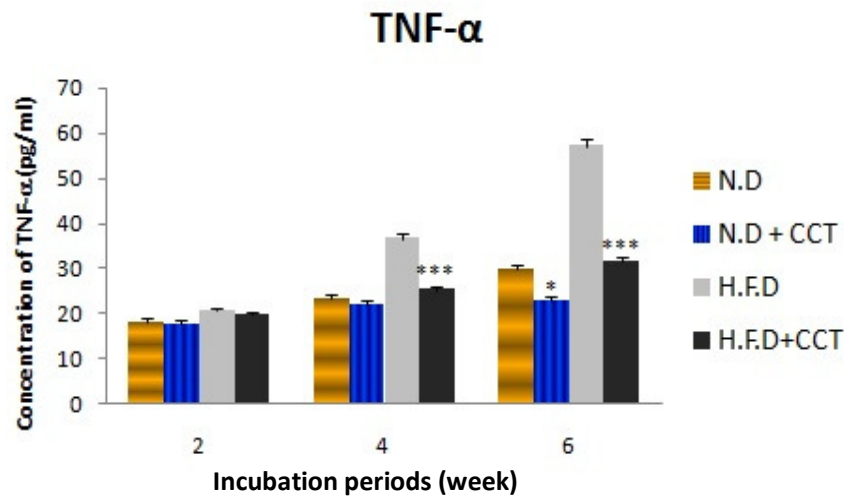


Figure 4. Effect of oral administration of pulp extract of CCT on serum TNF- α levels in different groups of mice. The group abbreviations (N-D, N-D+CCT, H-F-D, H-F-D+CCT) are the same with Figure 1. Data are presented as the mean \pm S.E.M. * p <0.05 and *** p <0.001 vs HF diet group.

with a publication by Hotamisligil, which demonstrated that adipocytes constitutively express the proinflammatory cytokine TNF- α and that TNF- α expression in adipocytes of obese animals (*ob/ob* mouse, *db/db* mouse and *fa/fa* Zucker rat) is markedly increased (Hotamisligil et al., 1993). Numerous studies in the past have demonstrated that the biochemical signaling

pathways of diabetes form a complex, interconnected network. Inhibition of one part of the network may result in compensation through another pathway. Because botanicals contain a variety of organic chemical complexes, they usually act on multiple targets. Activated innate immunity and inflammation are relevant factors in the pathogenesis of diabetes, with convincing data that

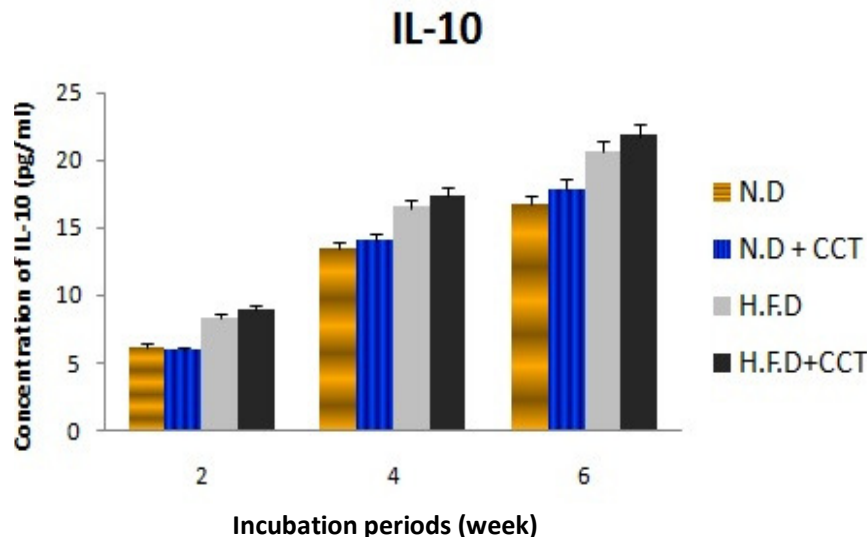


Figure 5. Effect of oral administration of pulp extract of CCT on serum IL-10 levels in different groups of mice. The group abbreviations (N-D, N-D+CCT, H-F-D, H-F-D+CCT) are the same with Figure 1. Data are presented as the mean \pm S.E.M.

diabetes includes an inflammatory component (Pickup and Crook, 1998; Frohlich et al., 2000; Crook, 2004; Pickup, 2004). The adipose tissue of obese humans contains an increased number of macrophages, and once activated, these macrophages secrete a host of cytokines, such as TNF- α , IL-6, and IL-1. The adipose tissue-resident macrophages are responsible for the expression of most of the tissue TNF- α and IL-6. The expression of macrophage markers in human adipose tissue was high in subjects with obesity and insulin resistance, and was also correlated with the expression of TNF- α and IL-6 (Wellen and Hotamisligil, 2003; Strissel et al., 2007). Several studies have demonstrated that high levels of TNF- α are associated with insulin resistance in adult animals and humans (Zinman et al., 1999; Skoog et al., 2002; Nilsson et al., 1998; Winkler et al., 1998), and inhibition of TNF- α can improve insulin sensitivity in animals (Cheung et al., 1998). Circulating levels of IL-6 are elevated in people with type II diabetes (Pickup et al., 1997), correlate positively with insulin sensitivity (Bastard et al., 2000) and may predict the development of diabetes (Pradhan et al., 2001). Levels of IL-6 in adipose tissue also coincide with insulin sensitivity *in vivo* and *in vitro* (Bastard et al., 2002). IL-10 is a well-known anti-inflammatory cytokine and plays an important role in suppressing the inflammatory response in many *in vitro* or *in vivo* experimental models. It has been reported that IL-10 inhibits the release of pro-inflammatory cytokines IL-6 and TNF- α by human monocyte/macrophage in response to polymethylmethacrylate (PMMA, spherical 1-10 μ m)

particle challenge *in vitro* (Trindade et al., 2001). By a variety of methods, the production or action of IL-10 has been suggested to be deficient in both human patients and experimental animals of type I diabetes (Szelachowska et al., 1998; Alleva et al., 2000). For example, IL-10-deficient NOD mice demonstrate accelerated diabetes (Balasa et al., 2000). Conversely, treatment of NOD mice with recombinant IL-10 prevents the development of diabetes (Pennline et al., 1994). Hence, depending on the time and site of administration, IL-10 can exert distinct effects on diabetes, a phenomenon that has been traditionally dubbed "paradoxical" (Balasa and Sarvetnick, 1996). The mechanism of H-F-D-induced obesity is still unclear, but long-term exposure to a H-F-D can increase body weight and adiposity in human and animals (Portillo et al., 1999; Astrup et al., 1994). The studies presented in this dissertation showed that H-F-D alone can modulate pro-inflammatory mediators and inflammatory responses that are initiated by TNF- α or IL-6 in mice. A potential advantage of phytochemicals is that they may act through multiple pathways and reduce the development of resistance by cells. This model of pharmacognosy recognizes the advantage of administering the whole plant product to maximize activity (Trease and Evans, 1970). Over-extraction of a specific chemical constituent may remove this therapeutic gain. The challenge for modern pharmacognosy is to ensure that the optimum mixture of chemical constituents is maintained when a product is purified. Usually, such assurance will require a combination of chemical and biologic assays. Traditional

practice has been to combine multiple natural health products and, scientifically such combination may provide a therapeutic advantage. The literature on CCT is quite heterogeneous, particularly in regard to the portion of the fruit being evaluated for physiological and toxic effects, solvent used for extraction, dosage of the lyophilized extract administered, route of administration and acute or chronic effect of the extract. Though motivated by delineating the therapeutic potential(s) of this fruit, this heterogeneity limits comparisons of various findings. TNF- α and IL-6 act through classical receptor-mediated processes to stimulate both the c-Jun kinase (JNK) and the I κ B kinase (IKK)/nuclear factor- κ B (NF- κ B) pathways, resulting in up-regulation of potential mediators of inflammation that can lead to insulin resistance. As increase in release of TNF- α and IL-6 from macrophages and adipose tissue might have a role in the development of insulin resistance, we have proposed that CCT may reduce obesity-related diabetes troubles through down regulation of TNF- α and IL-6 independently from effect on up-regulation of IL-10. To our knowledge, this is the first study to report the associations of CCT extract with expression of these cytokines. To conclude, herbal therapy will provide an added advantage over the currently available conventional therapies and this study will be helpful for future studies towards innovations in medicinal plants therapies for obesity associated diabetes in our part of the world.

Conclusion

The prevalence of diabetes worldwide is increasing rapidly in association with the increase of obesity. Complications are a major fear of patients with diabetes. The current study reports on the immunomodulatory effects of the H-A extract of the pulp fraction of CCT in an animal model of obesity. According to this model, obese mice have increased pro-inflammatory cytokines and, therefore, a higher risk for type II diabetes. CCT extract alone did not show significant effects in body weight and food intake in mice. However, the results have demonstrated that the CCT extract exhibits anti-inflammatory activity by decreasing TNF- α and IL-6 levels, while maintaining the steady state of the anti-inflammatory cytokine IL-10 in obese mice. Abnormalities in innate immunity might participate in the development of diabetic complications and CCT can have a modulator effects in these dysfunctions, especially in obesity associated diabetes. Finally, we proposed that CCT may inhibit the release of NF- κ B-dependent cytokines including TNF- α and IL-6. The role of CCT bioactive components in the modulation of inflammation and the signaling pathways involved require further study.

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