EVALUATION OF THE EFFECTS OF SALVIA HYPOLEUCA ON THE EXPRESSION OF CYTOKINES: IL-6, IL-10 AND TNF-α IN HIGH FAT DIET-FED MICE TOWARDS A CURE FOR DIABETES MELLITUS

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Summary

Salvia hypoleuca is used in Iranian traditional medicine as a healing agent for reducing obesity-related diabetes troubles. We proposed that Salvia hypoleuca may perform its effects through inhibition of inflammatory cytokines secreted in obesity conditions. Control group was fed with normal diet for 42 days alone or plus 50 mg/kg hydro-alcoholic extract of Salvia hypoleuca. The obese mice were given high fat diet for 42 days alone or plus Salvia hypoleuca 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. Salvia hypoleuca 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 Salvia hypoleuca 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

Nowadays, medicinal plants receive attention to research centers because of their special importance in safety of communities. The use of herbal medicine for the treatment of diseases and infections is as old as mankind. The curative properties of medicinal plants are due to the presence of various complex chemical substances of different composition which occur as secondary metabolites (1,2). 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 (3). The genus Salvia, one of the most important genuses of Lamiaceae family, is widely used in flavoring and folk medicine all around the world (4). Fifty-eight species of this genus are documented in the Flora of Iran; 17 of them are endemic (5). The plants of the genus Salvia, which consist about 900 species (6) are generally known for their multiple pharmacological effects such as analgesic and anti-inflammatory (7), antioxidant (8), hepatoprotective (9), hypoglycemic activities (10), and antiischemia (11,12). Salvia hypoleuca is a well recognized plant in the traditional medicine and is used as a purgative, anti-diabetic, hemorrhoids, and insecticide. 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 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 (13,14). 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 (15,16). 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 (17,18). In the some part of asia, Salvia belongs to the mostly used plants in folk medicine, because of the anti-inflammatory activity (19). Traditional plant medicines or herbal formulations might offer a natural key to unlock diabetic complications (20,21). CCT contains active substances such as saponins, alkaloids and glycosides and it is used as anti-diabetic, antihypertensive and antioxidant (22-24). 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 (25). 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 (26). 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 (27). 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 (28). Therefore, activated innate immunity and inflammation are relevant factors in the pathogenesis of obesity associated diabetes (22, 29-31). The purpose of this study is to elucidate whether Salvia hypoleuca 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 Salvia hypoleuca extract for prevention of inflammation that is associated with obesity-related diabetes is investigated.

Materials And Methods

Plant materials and extraction

S. hypoleuca was collected from Guilan province (Iran), and authenticated at Medicinal Plants & Drugs Research Institute,Shahid-Beheshti University, Tehran, Iran. Its leaves and fruits were dried, under shade and powdered. The extract was prepared by maceration method (80% ethanol in 300 g/l for 48 h), and was filtered with a filter paper.

Ethanol was removed by a rotary evaporator. The extract was dissolved in normal saline and administrated orally into rats.

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 high fat diet 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 Salvia hypoleuca 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 Salvia hypoleuca extracts (100, 150, 175, 200 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 100, 150 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 (150 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 and one control group. In group A, animals fed normal diet with an adequate amount of saline (the solvent of extract) for 42 days. In group B, animals fed high fat diet with an adequate amount of saline (the solvent of extract) for 42 days (obese mice). In group C, normal diet fed animals received 150 mg/kg of Salvia hypoleuca extract by gavage for 42 days. In group D, high fat diet fed animals received 150 mg/kg of extract 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.

Evaluation 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 H3PO4), 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 ± SEM (n = 8 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 mice fed with the high fat diet continued to show increased body weight (32.23%, ***p< 0.001) and food intake versus normal diet group until the end of the study, salvia hypoleuca extract marginally reduced 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 salvia hypoleuca extract treated group did not show significant differences from the control group in each diet (ns-p> 0.05). In the high fat diet groups with or without extract 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 high fat diet group compared with the normal group, and treatment of extract reduced that and value not significantly in both diet groups. Treatment with extract 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, 22.9 ± 2.1 g (normal diet), 22 ± 0.9 g (normal diet + extract), 22.1 ± 1.6 g (high fat diet), and 21.9 ± 0.1 g (high fat diet + extract).

Measurements of soluble cytokines

In order to gain further insights in the regulatory effects of Salvia hypoleuca extract on obesity-related inflammation in vivo, we first fed mice with high fat diet to up-regulate the expression of inflammatory cytokines. To investigate the effect of 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, high fat diet 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 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 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, the 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).

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

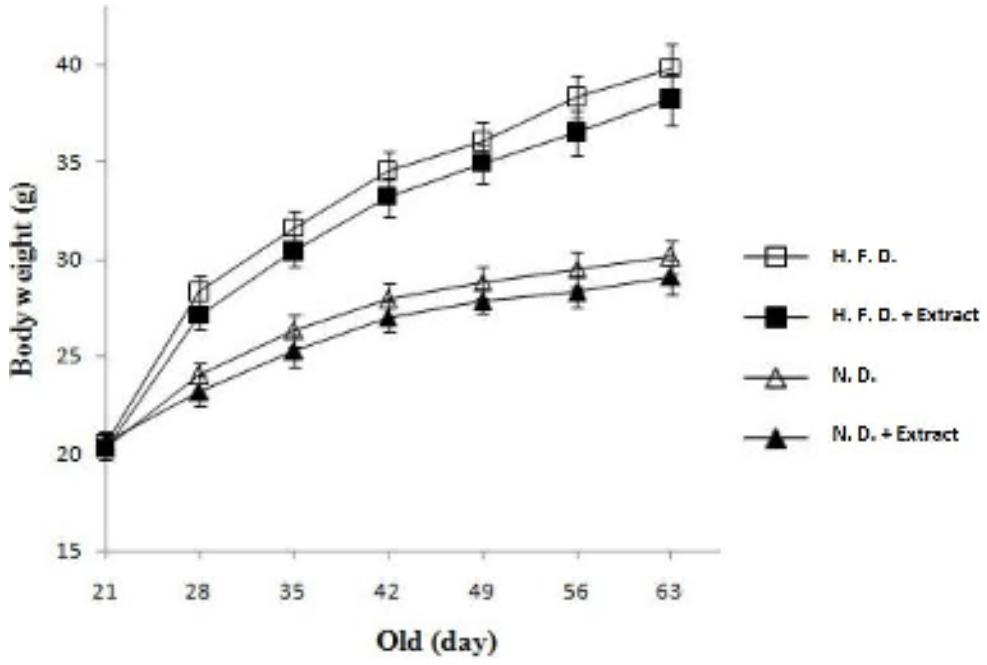


Figure 2. Effect of Salvia hypoleuca extract on daily food intake in mice fed a normal and high fat diet. The group abbreviations (N.D. (Normal Diet), N.D. + Extract, H.F.D. (High Fat Diet), H.F.D. + Extract) are the same with Figure 1. Data are presented as the mean ± S.E.M. *p<0.05 and ***p<0.001 vs H.F.D. group.

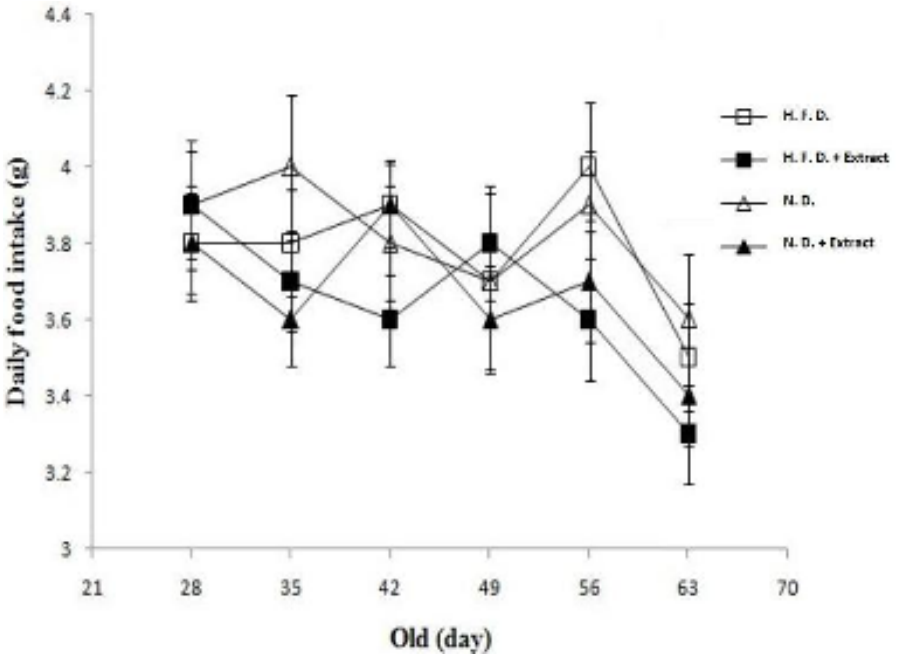


Figure 3. Effect of Salvia hypoleuca on serum IL-6 levels in different groups of mice. The group abbreviations (N.D. (Normal Diet), N.D. + Extract, H.F.D. (High Fat Diet), H.F.D. + Extract) are the same with Figure 1. Data are presented as the mean ± S.E.M. *p<0.05 and ***p<0.001 vs H.F.D. group.

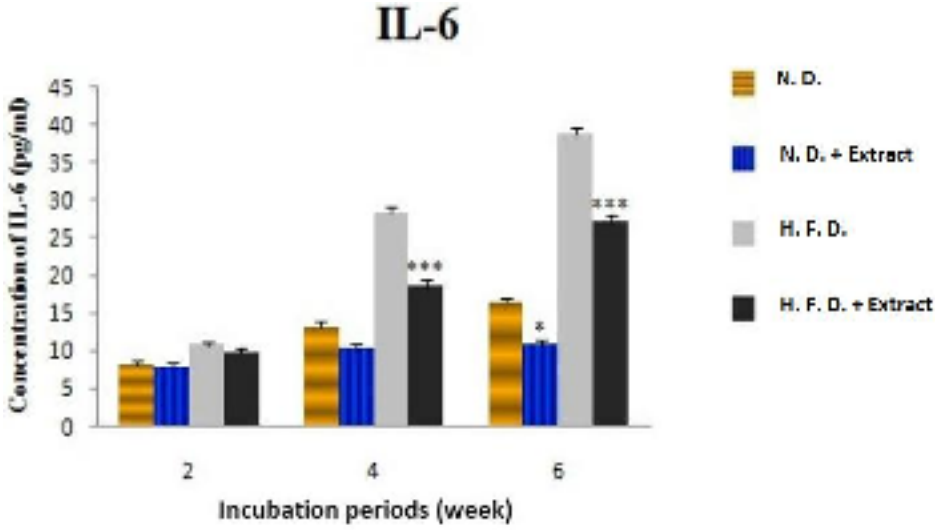


Figure 4. Effect of Salvia hypoleuca extract on serum TNF-α level in different groups of mice. The group abbreviations (N.D. (Normal Diet), N.D. + Extract, H.F.D. (High Fat Diet), H.F.D. + Extract) are the same with Figure 1. Data are presented as the mean ± S.E.M. *p<0.05 and ***p<0.001 vs H.F.D. group.

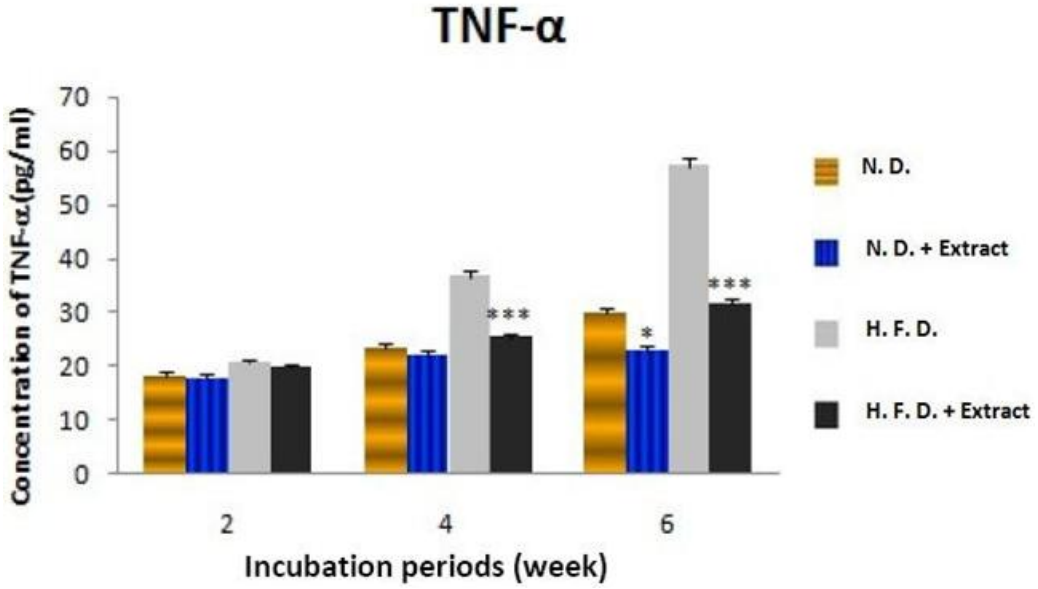
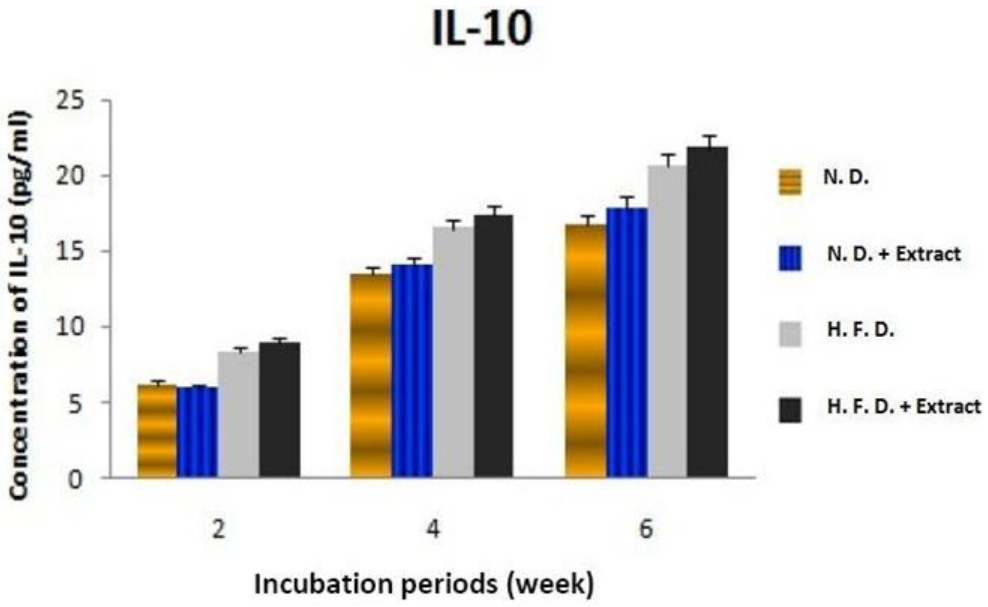


Figure 5. Effect of Salvia hypoleuca extract on serum IL-10 levels in different groups of mice. The group abbreviations (N.D. (Normal Diet), N.D. + Extract, H.F.D. (High Fat Diet), H.F.D. + Extract) are the same with Figure 1. Data are presented as the mean ± S.E.M*p<0.05 and ***p<0.001 vs H.F.D. group.



Discussion

The concept of inflammation in relation to metabolic conditions, such as obesity and insulin resistance started 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 (32). 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 diabetes includes an inflammatory component (29-31). 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 (32-34). Several studies have demonstrated that high levels of TNF-α are associated with insulin resistance in adult animals and humans (35-38), 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 (31), correlate positively with insulin sensitivity (39) and may predict the development of diabetes (40). Levels of IL-6 in adipose tissue also coincide with insulin sensitivity in vivo and in vitro (39). 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 μm) particle challenge in vitro (41). 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 (42,43). For example, IL-10–deficient NOD mice demonstrate accelerated diabetes (44). Conversely, treatment of NOD mice with recombinant IL-10 prevents the development of diabetes (45). 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” (46). The mechanism of high fat diet-induced obesity is still unclear, but long-term exposure to a high fat diet can increase body weight and adiposity in human and animals (47,48). The studies presented in this dissertation showed that high fat diet alone can modulate proinflammatory mediators and inflammatory responses that
are initiated by TNF-α or IL-6 in mice. TNF- α and IL-6 act through classical receptor-mediated processes to stimulate both the c-Jun kinase (JNK) and the IκB kinase (IKK)/nuclear factor-κB (NF-κB) 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 Salvia hypoleuca 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 Salvia hypoleuca extract with expression of these cytokines. 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. In 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 Salvia hypoleuca extract of on 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. The extract alone did not show significant effects in body weight and food intake in mice. However, the results have demonstrated that the 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 Salvia hypoleuca can have a modulator effects in these dysfunctions, especially in obesity associated diabetes. Finally, we proposed that Salvia hypoleuca may inhibit the release of NF-κB-dependent cytokines including TNF-α and IL-6. The role of Salvia hypoleuca bioactive components in the modulation of inflammation and the signaling pathways involved require further study.

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