



The effects of *Cynodon dactylon* on the immune response of *NMRI-MICE* after challenge with *REV1*

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ABSTRACT

Cynodon dactylon is used in Iranian traditional medicine as a healing agent for reducing the complications of diabetes mellitus. We proposed that *Cynodon dactylon* may perform its effects through moderating humoral and cellular immune responses. We aimed to determine the possible effects of hydroalcoholic extract of *Cynodon dactylon* on humoral and cellular immune responses following the Rev1 challenge in the mouse model. 20 NMRI-male mice were randomly grouped in two equal groups and immunized with Rev1 [0.1 ml Rev1+0.9 PBS]. Mice in the treatment group orally received 400 mg/kg hydroalcoholic extract of *Cynodon dactylon* every day from the beginning of the study for 2 weeks. Blood samples were obtained from the animals 5 days after the last injection. Moreover, 48 hr before bleeding time, Rev1 [0.1 ml Rev1+0.9 PBS] was injected into the left hind foot pad of mice. The levels of anti-Rev1 antibody and the specific cellular immune responses were measured by microhemagglutination test and footpad thickness, respectively. Moreover, susceptibility of macrophages respiratory burst and proliferation of immune cells were measured in order with Nitroblue tetrazolium [NBT] and Microculture Tetrazolium Assay [MTT]. The concentrations of IL-1, TNF α , IL-6, and IL-10 in the serum were determined using commercially available ELISA kits. We found a significant increase in anti-Rev1 antibody levels and simultaneously a significant decrease in the level of cellular immunity [DTH] in the treatment group compared to the control group. Lymphocyte proliferation index in splenocytes was significantly increased in the treatment group. However, the level of respiratory burst in phagocytic population of splenocytes dramatically decreased in the treatment group compared to the control. A significant decrease in IL-6, TNF- α , IL-1 and increase in IL-10 serum levels were also seen in the treatment group. *Cynodon dactylon* extract could have an anti-inflammatory effect through downregulation of pro-inflammatory cytokines and can be considered as the moderating immune system compound.

Keywords: *Cynodon dactylon*, Humoral immunity, Cellular immunity

INTRODUCTION

Herbal drugs include plants, herbal complexes and herbal products or plant or even a combination of plants which were used thousand years before the advent of modern drugs and also used currently [1]. Herbal plants are used all over the world in different methods both in allopathic and traditional systems [2]. According to the World Health Organization [WHO], 80% of people worldwide, use traditional treatment [3]. Moreover, 60% of drugs confirmed by the Federal Drug Association [FDA] during 1984-1994 were isolated from natural sources, especially plants [4]. *Cynodon dactylon*, also known as Bermuda grass, is a perennial grass native to the warm temperate and tropical regions [4]. In Northwest Iran, *C. dactylon* is known as "Chayer" and the aqueous extract of its rhizomes is widely used in the treatment of cardiovascular disorders such as atherosclerosis and heart failure because of its hypolipidemic and cardiac tonic effects [5,6]. In recent studies the anti-diabetic, anti-microbial [7], hypolipidemic [8], hepatoprotective [9] and anti-emetic [10] effects of *Cynodon dactylon* have been established.

We aimed to investigate the effects hydroalcoholic extract of *Cynodon dactylon* on humoral and cellular immunity in NMRI mice after challenge with Rev1.

MATERIALS AND METHODS

Plant materials and extraction

C. dactylon was purchased from a traditional herbal market and the genus and species was authenticated at the Herbarium of Botany in Urmia University, Urmia, northwest Iran. The rhizomes of the plant were dried in shade and coarsely ground to powder using an automatic grinder. 200 g of the powder was mixed in 2 L of distilled water and placed on a magnet stirrer at a temperature of 50°C for three days. The mixture was then filtered three times using the Wattman's paper. The solution was finally evaporated to dryness for 12 hrs at 70°C. The total amount of the crude extract obtained was 34 g. The extract was diluted with water in order to be given orally by gavage needle[11].

Six to eight-week-old male NMRI mice were purchased from the animal house of Urmia University of Medical Sciences. The animals were housed for one week before the experiment, maintained under constant temperature [22-24°C] and 12 hr light/dark cycle and received food and water ad libitum. Animal welfare was observed in compliance with the regulations of Iran's Ministry of Health, and approved by the Medical Ethics Committee of the University for Animal Studies.

Experimental design, Immunological challenge and evaluation

Mice were randomly allocated to two control and treatment groups of 10 mice each. Each group had 10 animals. Mice in the treatment group orally received 400 mg/kg hydroalcoholic extract of *C. dactylon* every day from the beginning of the study for 2 weeks. When the experiment began, animals were intraperitoneally immunized twice with one week interval by Rev1 [0.1 ml Rev1+0.9 PBS]. Blood was extracted from their hearts 5 days after last injection and the levels of anti-Rev1 antibody were measured by microhemagglutination test as described previously [12]. Moreover, 48 hr before bleeding time, Rev1 was administered subcutaneously into the left hind foot pad of each mouse and simultaneously the same volume of Phosphate-buffered saline [PBS] was injected into the right foot pad as negative control. Footpad thickness was measured before bleeding time with a dial caliper and the mean percentage increase in footpad thickness was measured according to the following formula: $[\text{Thickness of left footpad}] - [\text{Thickness of right footpad}] * 100 / [\text{Thickness of right footpad}]$.

Quantification of total serum cytokines

The serum concentrations of each cytokine was 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], and 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 mins 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.

Splenocytes proliferation

Proliferation potential of lymphocytes in splenocyte population was evaluated by MTT assay[12]. The splenocytes were plated in 96-well flat-bottomed plates in RPMI 1640 medium supplemented with 10% fetal calf serum [1×10⁵ cells/100 µl/well] and stimulated with 50 µl PHA solution [1 mg/ml] or medium alone. After 72 hr incubation, cultures were pulsed with 20 µl of the MTT solution [5 mg/ml] for 4 hr at 37°C. Then 150 µl DMSO was added and shaken vigorously to dissolve formazan crystal. The optical density [OD] at 550 nm was measured using microplate reader [Dynatech, Denkendorf, Germany]. The experiments were done in triplicate sets. The results were expressed as the proliferation index according to the ratio of OD₅₅₀ of stimulated cells with MOG35-55 to OD₅₅₀ of non-stimulated cells.

Respiratory burst in splenocyte population

Respiratory burst of phagocytic cells in splenocyte population was checked using NBT dye reduction as described previously with some modification[13,14]. In brief, 100 µl of suspension of splenocytes were mixed with 0.1 ml of *Staphylococcus aureus* suspension [10⁸ cell/ml] and 0.1 ml of 0.1% NBT in PBS [pH=7.4]. The mixture was incubated at room temperature for 15 mins and subsequently kept at 37°C for an additional 15 mins. The reduced dye was extracted in dioxan and quantitated at 520 nm.

Statistical analysis

Data were analyzed by Student's *t* test and presented as mean \pm SD. P values less than 0.05 were considered statistically significant.

RESULTS

Footpad thickness after challenge with REV1 was performed as an indicator for evaluation of delayed type of hypersensitivity [DTH] reaction. Mice in the treatment group showed significantly lower DTH responses than the control mice. Conversely, mean antibody titer in the treatment group was significantly higher than the mean antibody titer in control mice [table 1].

Table 1: The footpad thickness percentage and the titer of anti-Rev1 antibody of examined animals in control and treatment group

Groups	Percent footpad thickness	Titr anti-Rev1 antibody
control	46.123 \pm 1.265	23.456 \pm 5.216
treatment	16.111 \pm 2.324	169.526 \pm 7.145
P value	<0.01	<0.001

Splenocyte proliferation and respiratory burst showed a significant increase and decrease, respectively, in mice treated with *C. dactylon* compared with the control group [table 2].

Table 2: The results of NBT and MTT assays for animals in control and treatment groups

groups	NBT	MTT
control	1/365 \pm 0/036	0/465 \pm 0/133
treatment	0/528 \pm 0/030	1/326 \pm 0/116
P value	<0/001	<0/001

The serum levels of IL-6, TNF- α , and IL-1 were checked using the ELISA kit according to the manufacturer's instructions [figure 1].

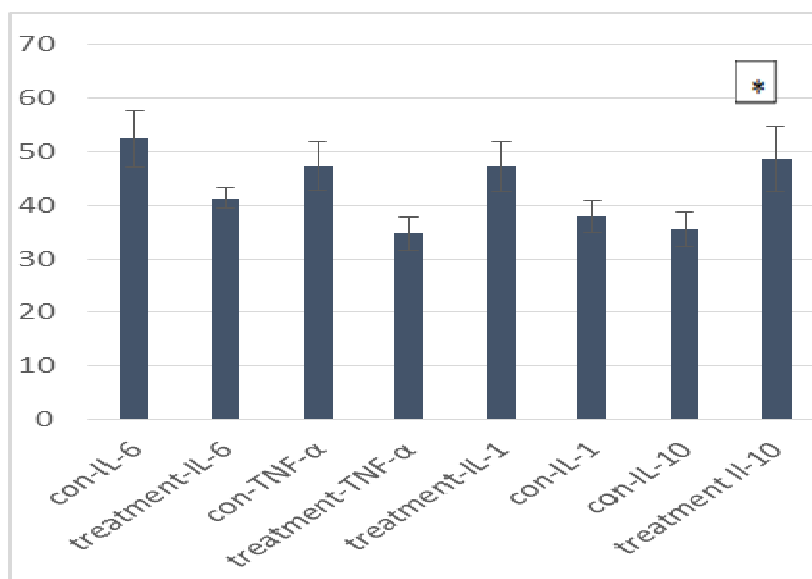


Figure 1: Effect of *C. dactylon* on the serum level of IL-6, TNF- α , IL-1 and IL-10 compared with the control group [*: P<0.05]

DISCUSSION

C. dactylon has a key role in ethno-medicinal practices and traditional medicinal systems. It is extremely useful in a wide variety of diseases and disorders. Various pharmacognostic and pharmacological properties of this extract have been previously investigated by researchers, supporting its medicinal. Recently, several studies have shown that *C. dactylon* plays an essential role in improving inflammation, candidal and bacterial infections, lipidemic and glycemic disorders, reproductive system and fertility, type II diabetes mellitus, and breast cancer [15]. We studied the effects of the hydroalcoholic extract of *C. dactylon* on humoral and cellular immunity in NMRI mice after challenge with Rev1. *C. dactylon* contains 28.17% enzymes, 11.79% ash, 10.47% Proteins. Ash contains 0.77% calcium, 0.58% phosphorus, 0.34% manganese, 0.23% sodium, 2.08% potassium. Dry grass contains per 400 grams 36.16% carbohydrate, 6.04 % proteins. It contains phenolic phytochemicals viz. ferulic, syringic, paracoumaric, vanillic,

para hydroxyl benzoic and orthohydroxy phenyl acetic acid[16,17]. Flavonoids and glycosides were found to be present in the aqueous extract of *C. dactylon* while alkaloids, glycosides and flavonoids were reported to be present in the ethanol extract of the plant [18]. Other compounds like vitamin C, β carotene, fats, palmitic acid etc. have also been reported[19]. Analysis of leaves of *C. dactylon* by GC-MS technique revealed that *C. dactylon* leaves contain glycerin [38.49%], 9, 12-Octadecadienoyl chloride,[Z,Z]-[15.61%], hexadecanoic acid, ethyl ester [9.50%], ethyl -d-glucopyranoside [8.42%], linoleic acid, ethyl ester [5.32%], and phytol [4.89%][16]. Saponins, tannins, flavonoids, phenols in the ethanolic extract of *C. dactylon* have significant antioxidant activity by inhibiting DPPH and Nitric oxide free radical[20].

In this study, the reduction in the respiratory burst in the phagocytic cells of the spleen cells in the treatment group may be due to the antioxidant activity of *C. dactylon* compounds. Mangathayaru and co-workers studies show that oral administration of the *C. dactylon* juice at 250 and 500 mg/kg in BALB/c mice increased humoral antibody response upon antigen challenge, statistically significant increase in antibody titre in the haemagglutination antibody assay and plaque forming cell assay[21]. 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[22]. In this study, It seems that the increase in the proliferation of immune cells, the levels of anti-Rev1 antibody and reduction in production of pro-inflammatory cytokines [IL-1, IL-6, TNF- α] could be attributed to the production of IL-10 in the treatment group. T cell-mediated immunity has a substantial role in determining the extent of organ specific autoimmune diseases[23-25]. DTH is one of the typical response patterns of T cell-mediated immunity[25]. The first requirement for DTH reaction is the priming of a special effector class of antigen specific T cells[26]. DTH response has long been believed to be mediated by Th1 cells[27, 28]. Despite the significant increase in the proliferation of spleen cells in the treatment group in our study compared to the control group, likely reduction in the respiratory burst could explain the decrease in the intensity of the DTH response. It is also possible that the increased proliferation of T cells were as a result of deviation of T cell polarization in a non-Th1 direction.

CONCLUSION

C. dactylon may inhibit the release of cytokines including TNF- α , IL-6 and IL-1 and increase IL-10 production. These results showed the significant anti-inflammatory and immunomodulatory activity of *C. dactylon* bioactive components. The modulation of inflammation and the signaling pathways involved require further study.

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