J.Vet.Res. 62,4:119-123,2007

Evaluation of the effects of *Zataria multiflora*, *Geranium pelargonium*, *Myrth* and *Lemon* essences on immune system function in experimental animals

Khosravi,A.R.[1]*, Franco,M.[2], Shokri,H.[1], Yahyaraeyat,R.[1]

[1]*Mycology Research Center, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran*

[2]*Department of Pathology, School of Medicine and Immunology Division, UNESP, Botucatu, Brazil*

(Received 23 October 2005 , Accepted 22 April 2006)

Abstract: **The effects of some Iranian herbal essences have been evaluated on the function of immune system using experimental animals. Rabbits received *Zataria multiflora*, *Geranium pelargonium*, *Myrth*, *Lemon* essences and normal saline (control group), 6 times with 6 days of interval. Five days after the last injection of the essences, *Candida albicans* antigens were injected into all the animals. Phagocytosis and killing assays and lymphocyte transformation test (LTT) were carried out on blood samples. The cellular immunity was significantly stimulated against *C. albicans* antigens and Con-canavalin A (Con-A) mitogen in animals that injected subcutaneously with *Z. multiflora* and *G. pelargonium* in comparison with the control group, whereas *Myrth* essence had no considerable effect and *Lemon* essence suppressed the cellular responses. *Zataria multiflora*, *Myrth* and *Lemon* essences stimulated innate immunity when injected subcutaneously, whereas *G. pelargonium* essence had no significant effect. Humoral responses to *Candida* antigens were significantly decreased in animals injected with *Lemon* essence as compared to other essences ($p< 0.05$).**

Key words: *Candida albicans*, zataria, Geranium, immunostimulation, innate immunity.

Introduction

During the last 25 years, the frequency of life-threatening infections has increased dramatically among cancer patients, transplant recipients, patients with AIDS and patients receiving broad-spectrum antibiotic, corticosteroid and cytotoxic drugs (Anaissie, McGinnis and Pfaller, 2003). Numerous risk factors have been known that can impair mechanisms of host defense, predisposing them to serious diseases (Kournikakis *et al*., 2003). For these reasons, the widespread efforts were made to identify immune modulator agents to combat these infections. Among these agents, herbal medicine has been noticed by some investigators (Patwardan and Hopper, 1991; Wagner, 1999; Constantine, 2000; Ang-Lee, 2001; Ernst and Pittler, 2002). Herbal medicines have been used since ancient times as drugs for the treatment of a range of diseases (Brown, 1996; Wagner, 1997; Masshour and Frishman, 1998; Gardiner and Kemperky, 2000) and represented stimulatory effects on the function of innate and acquired immunity (De Simone, 1993; Barbour, Hamadeh and Hilan, 1996; Lai and Roy, 2004). The goal of this study was to identify the effects of some Iranian herbal essences including *Z. multiflora*, *G. pelargonium*, *Myrth* and *Lemon* on the function of immune system.

* **Corresponding author's email: khosravi@ut.ac.ir, Tel: 021- 61117151, Fax: 021-66933222**

Materials and Methods

Herbal Essences

Four essences including *Z. multiflora*, *G. pelargonium*, *Myrth* and *Lemon* were obtained from Barij Essence Company (Kashan, Iran).

Table 1. The results of lymphocyte transformation test (LTT) in rabbits.

Antigen	Lymphocyte	Mean ± Standard deviation	Stimulation Index
Con-canavalin A (Con-A)	Group A_1	712.3±10.7	5.9
	Group A_2	414.1±28.7	3.1
	Group B_1	618.6±42.5	4.6
	Group B_2	428±94.1	3.9
	Group C_1	411.7±18.4	2.9
	Group C_2	385.2±12.6	2.8
	Group D_1	455.1±44.7	1.6
	Group D_2	312±15.3	2.1
	Group E_1	485.2±11.2	3.7
	Group E_2	498.9±49.2	3.8
Candida albicans	Group A_1	619.3±12.1	4.5
	Group A_2	362.7±14.2	2.9
	Group B_1	567.9±18.7	4
	Group B_2	312.7±11.5	2.2
	Group C_1	428.6±43.2	3.9
	Group C_2	381±69.9	2.7
	Group D_1	419.7±99.5	1.2
	Group D_2	307.2±97.4	1.9
	Group E_1	316.6±12.1	2.3
	Group E_2	328.2±18.4	2.5

Animals

Fifty male and female rabbits, 1000-1800 g, were obtained from Razi Institute. Animals were maintained in 5 rabbit cages, under standard conditions, in the Center of Laboratory Animals Maintenance of Faculty of Veterinary Medicine. The animals were fed daily with an approved protocol. The animals were divided into 10 groups of 5 rabbits; each group was administrated as follows:

A_1: rabbits were injected subcutaneously with *Z. multiflora* essence

A_2: rabbits were gavaged orally with *Z. multiflora* essence

B_1: rabbits were injected subcutaneously with *G. pelargonium* essence

B_2: rabbits were gavaged orally with *G. pelargonium* essence

C_1: rabbits were injected subcutaneously with *Myrth* essence

C_2: rabbits were gavaged orally with *Myrth* essence

D_1: rabbits were injected subcutaneously with *Lemon* essence

D_2: rabbits were gavaged orally with *Lemon* essence

E_1: rabbits were injected subcutaneously with sterile saline

E_2: rabbits were gavaged orally with sterile saline

The whole rabbits were injected subcutaneously and orally with doses of essences, 0.2 g / Kg, 6 times with 6 days of interval. Control group received sterile saline with the same dose and same routes. Bleeding of animals was done in 2 stages: 1 day before administering essences and 3 days after the last administration of essences. Blood was collected from marginal vein of the rabbits and a portion of blood was kept into heparinized tubes and the rest was stored into sterile tubes for obtaining serum.

Preparation of *C. albicans* Antigens: *Candida albicans* standard strain (CI2) was cultured on sabouraud glucose broth (Merck Chemical Co.,

Table 2. The results of killing and phagocytosis assays using animals' neutrophils.

Colony count (*Candida*)	Group A_1	Group A_2	Group B_1	Group B_2	Group C_1	Group C_2	Group D_1	Group D_2	Group E_1	Group E_2
0 - 9	1	0	0	0	1	0	0	0	0	0
10 - 49	3	1	2	0	2	0	3	0	0	0
50 - 99	1	1	1	0	2	0	2	1	1	1
≥ 100	0	3	2	5	0	5	0	4	4	4

Darmstadt, Germany). The yeasts were washed 3 times with sodium phosphate buffer (PBS) and disrupted by sonication method (dr. hielscher, GmbH, UP 200s). The antigens were suspended with PBS and centrifuged at 26000 × g for 2.5 hours. The supernatant was separated and concentrated using dialysis bag (cut off: 0.2, Sigma Chemical Co., St. Louis, USA). The antigens were maintained at freezing conditions. Five days after the last administration of essences, a certain dose of the antigens was inoculated subcutaneously, 3 times and intramuscularly, the last time, to all rabbits. After one week, in order to evaluate the humoral immunity, bleeding was carried out again.

ELISA Test: ELISA test was used for the detection of specific anti *Candida*-IgG antibody using *Candida* antigens, rabbit sera, before and after immunization and anti rabbit IgG conjugated with horse radish peroxidase.

Lymphocyte Transformation Test (LTT): Heparinized blood samples were mixed with RPMI (50:50, Sigma Chemical Co., St. Louis, USA) and then transferred to tubes containing Ficoll (Sigma Chemical Co., St. Louis, USA). After centrifugation, cloudy layer containing lymphocytes was separated, diluted with RPMI and the volume of lymphocytes was reached up to 1 ml. Percentage of live cells was determined using Trypan Blue (Sigma Chemical Co., St. Louis, USA). Then, lymphocytes were counted by neobar glass. Next stages were carried out according to standard methods and using Con-A mitogen.

Phagocytosis and Killing Assays: Killing of *C. albicans* by the animal neutrophils was tested as follows: 1) Dilution of neutrophils in 1 ml of sample: diluted suspension of 2000 cell /ml was prepared using saline, RPMI and neobar glass; 2) 20 µl of each serum was added to the suspension of saline and RPMI of the same animal; 3) Suspension of 2000 cell /ml of *C. albicans* cells was prepared using sterile saline; 4) 1 ml of neutrophil suspension of each animal was added to 1 ml of *C. albicans* suspension and then the total suspension, which contained neutrophil / *C. albicans*, was incubated at 37°C for 2 hours; 5) After incubation, 500 µl of each suspension was cultured on sabouraud glucose agar and maintained at 30°C for 72 hours and then *candida* colonies were counted.

Statistical Analysis: Chi-square (X^2) and Student t- tests were used to assess the statistical analysis.

Results

Table 1 shows that subcutaneous administration of essences stimulated the LTT responses, whereas oral administration had no effect. *Zataria multiflora* and *G. pelargonium* essences elevated LTT responses to *Candida* antigens and Con-A mitogen, whereas *Lemon* and *Myrth* essences suppressed LTT responses to mentioned antigens. In respect to stimulation index, significant differences were observed between group A_1, B_1 and other groups ($p<0.001$). As shown in Table 2, subcutaneous administration of *Z. multiflora*, *Myrth* and *Lemon* essences had more stimulatory effects on phagocytosis of *C. albicans* by neutrophils in comparison with *G. pelargonium* essence and subcutaneous administration had more enhancing effect on phagocytosis than the oral procedure. Regarding oral administration, there was an effect on phagocytosis only in group A_2. There were

Table 3. The results of ELISA test using animals' serum and *Candida* antigens.

Animal Groups	OD (nm)	
	Pre immunization	Post immunization
Group A_1	0.167	1.88
Group A_2	0.364	0.931
Group B_1	0.516	1.110
Group B_2	0.380	0.475
Group C_1	0.442	0.825
Group C_2	0.595	0.580
Group D_1	0.484	0.100
Group D_2	0.292	0.212
Group E_1	0.422	0.552
Group E_2	0.325	0.425

significant enhancing effects on humoral responses in A_1, A_2, B_1, B_2 and C_1 groups in comparison with other groups ($p<0.05$), whereas C_2, D_1 and D_2 groups had suppressive effects (Table 3).

Discussion

In recent years, different factors have been known that can compromise individual's immune defenses, predisposing them to serious and fatal infections (Anaissie, McGinnis and Pfaller, 2003). These factors including cancers, organ transplantation, chemotherapy, corticosteroid therapy, broad-spectrum antibiotic therapy, immunodeficiency syndromes and other underlying diseases have been increased remarkably (Kournikakis *et al*., 2003). Regarding the above mentioned points, the use of immune modulators either as a prophylaxis and/or as part of a treatment regimen may represent a broad spectrum approach to protect human or animals when exposed to a pathogenic challenge. Immune modulator agents can increase the specific and nonspecific components of the immune system (De Simone, 1993; Barbour, Hamadeh and Hilan, 1996; Lai and Roy, 2004). One class of extensively studied immune modulators is herbal medicines, which has been noticed by some investigators (Patwardan and Hopper, 1991; Wagner, 1999; Constantine, 2000; Ang-Lee, 2001; Ernst and Pittler, 2002). Immunity can be boosted by immune-enhancing herbs such as *Garlic*, *Echinacea*, *Licorice* and *Ginseng* (Yoshida, 1997; Li, 2000; Burger, 2001; McIntyre, 2002). In this study, the effects of Iranian herbal essences including *Z. multiflora*, *G. pelargonium*, *Myrth* and *Lemon* were studied on the function of immune system. The results of this study indicate that in animals, which were injected subcutaneously with *Z. multiflora* and *G. pelargonium*, cellular immunity responses to *Candida* antigens and Con-A mitogen were significantly stimulated in comparison with the control group, whereas *Myrth* essence had no considerable effect and *Lemon* essence suppressed significantly cellular immune responses. Subcutaneous injection of *Z. multiflora*, *Myrth* and *Lemon* essences stimulated the innate immunity (killing and phagocytosis), whereas *G. pelargonium* essence had no significant effect on this part of immunity. In comparison with other essences, humoral responses to *Candida* antigens were significantly decreased in animals, which were injected subcutaneously with *Lemon* essence. It has been found that subcutaneous injection of essences had more stimulatory effects on immune system than the oral administration route. In this study, the animals were challenged with 0.2 g/kg of each essence and it seems that the study should be continued with different doses in the future. According to the results, *Z. multiflora* and then *Myrth* and *G. pelargonium* seem to be immunostimulatory agents since they have positive effects on innate and acquired immunity.

Acknowledgments

This work was supported by the Research Council of the University of Tehran.

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مجله تحقیقات دامپزشکی، ۱۳۸۶، دوره ۶۲، شماره ۴، ۱۲۳-۱۱۹

ارزیابی اثرات اسانس های آویشن شیرازی، ژرانیوم پلارگونیوم، مورد ولیمو بر روی عملکرد سیستم ایمنی در حیوانات آزمایشگاهی

علیرضا خسروی[1]* مارسلو فرانکو[2] حجت اله شکری[1] رامک یحیی رعیت[1]

[1] مرکز تحقیقات قارچ شناسی، دانشکده دامپزشکی دانشگاه تهران، تهران - ایران.

[2] بخش پاتوبیولوژی، دانشکده پزشکی بوتاکاتو - برزیل.

(دریافت مقاله: ۱ آبان ماه ۱۳۸۴، پذیرش نهایی: ۲ اردیبهشت ماه ۱۳۸۵)

اثرات تعدادی از اسانسهای گیاهی ایرانی بر عملکرد سیستم ایمنی حیوانات آزمایشگاهی ارزیابی شدند. خرگوشها اسانسهای آویشن شیرازیِ، ژرانیوم پلارگونیوم، مورد، لیموو نرمال سالین (گروه شاهد) ۶ بار به فواصل ۶ روز دریافت کردند. آنتی ژنهای کاندیدا آلبیکنس ۵ روز پس از آخرین تزریق اسانسها به تمام حیوانات تجویز شدند. آزمایشهای فاگوسیتوزیس، کشتار سلولی و تغییر شکل لنفوسیتی بر روی نمونه های خون انجام گردید. در حیواناتی که بصورت زیر جلدی با آویشن و ژرانیوم تزریق شده بودند ایمنی سلولی به‌طور معنی داری در برابر آنتی ژنهای کاندیدا آلبیکنس و میتوژن کونکاناوالین-A در مقایسه با گروه شاهد تحریک شدند در حالیکه اسانس مورد تاثیر قابل توجهی نداشت و اسانس لیمو ایمنی سلولی را سرکوب کرد. تزریق زیر جلدی اسانس های آویشن، مورد ولیمو باعث تحریک ایمنی ذاتی شد در حالیکه اسانس ژرانیوم اثر معنی داری نداشت. پاسخ های هومورال در برابر آنتی ژنهای کاندیدا در حیوانات تزریق شده با اسانس لیمو نسبت به سایر اسانسها ($p<0.05$) بطور معنی داری کاهش یافته بودند.

واژه‌های کلیدی: کاندیدا آلبیکنس، آویشن، ژرانیوم، تحریک ایمنی، ایمنی ذاتی.

*) نویسنده مسؤول: تلفن: ۶۱۱۱۷۱۵۱-۰۲۱ نمابر: ۶۶۹۳۳۲۲۲-۰۲۱ Email:khosravi@ut.ac.ir