Iranian Journal of Pharmaceutical Research (2003) 51-55
Received: November 2002
Accepted: February 2003

Original Article

The Effects of *Allium hirtifolium* Boiss. on Cell-Mediated Immune Response in Mice

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Abstract

Based on experiments showing immunomodulatory effects of garlic (*Allium sativum* L.), we sought to see whether *Allium hirtifolim* Boiss., belonging to the same genus of garlic, has any effect on immune responses.

Hydroalcoholic extract and polyphenolic fraction of *A. hirtifolium* bulbs were prepared. Allicin (diallyl thiosulfinate), a biologically active component, was identified in plant bulbs by thin layer chromatography and determined by a spectrophotometery method at 412 nm. To study the effects of *A. hirtifolium* on acquired immunity, groups of Balb/c mice (n = 8-12) were used. Sheep red blood cell (SRBC) was injected (sc, $1x10^8$ cells/ml, 0.02 ml) and 5 days later hydroalcoholic extract (10-2000 mg/kg) and polyphenolic fraction (100-1000 mg/kg), betamethasone (4 mg/kg) or normal saline were given ip. After 1 h SRBC was injected to footpad (sc, $1x10^8$ cells/ml, 0.02 ml) and footpad swelling was measured up to 72 h. To see the effects of *A. hirtifolium* on intrinsic immunity the same procedure was used, but animals just received one injection of SRBC after ip injection of tested compounds. Our results showed that the amount of total thiosulfinates and allicin in *A. hirtifolium* bulbs were 0.53% and 0.37%, respectively. Betamethasone inhibited paw thickness in both models. Hydroalcoholic extract and polyphenolic fraction of *A. hirtifolium* significantly reduced footpad thickness in both models ($p < 0.05$). These findings showed that both hydroalcoholic extract and polyphenolic fraction of *A. hirtifolium* decreased acquired immunity response in a dose-dependent manner. However, only polyphenolic fraction of *A. hirtifolium* showed a dose-dependent effect on intrinsic immunity.

Keywords: *Allium hirtifolium*; Acquired Immunity; Allicin; hydroalcoholic extract; Polyphenolic fraction.

Introduction

The use of plant products as immunomodulator is still in a developing stage. There are several herbs used in the indigenous system of medicines that can modulate the body's immune system. A variety of plant derived materials such as polysaccharides, lectins, peptides, flavonoids and natural sulfur compounds have been reported to modulate the immune system (1-3).

The use of garlic and onion as dietary constituents and drugs for many disorders dates back to early civilization. It has been shown that these plants and their constituents have immunomodulatory effects (2).

Allium hirtifolium Boiss. (Alliaceae family), an Iranian traditional herb and condiment spice, is well known in Iranian folk medicine and its bulbs (corms) have been widely used for treating rheumatic and inflammatory disorders (4). It belongs to the same biological genus as *Allium sativum* L. (garlic) and other onions (5). To the best of our knowledge, there is no previous report on the pharmacological effects of *A. hirtifolium.* In this study, we sought to see

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whether *A. hirtifolium* has any effect on cellular immune system.

Experimental

Plant material

The bulbs of *A. hirtifolium* were collected from plants growing wild in Darre-bid mountain area of Khansar in Isfahan province (center of Iran) on June 1998. The plant specimen was identified by the herbarium department of Iranian Research Institute of Forests and Rangelands, Isfahan, Iran. A voucher specimen of the plant is deposited in the herbarium of the Faculty of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran.

Preparation of extracts

Preparation of the hydroalcoholic extract

Air-dried and powdered bulbs of the plant (100 g) were percolated with 400 ml of EtOH-H_2O (75:25) for 48 h. Then it was filtered and evaporated in a rotating evaporator under reduced pressure until dryness (6-7).

Preparation of the polyphenolic fraction

Polyphenolic fraction of air-dried and powdered bulbs of the plant (100 g) were extracted in two steps, firstly with EtOH-H_2O (9:1) and secondly with EtOH-H_2O (1:1). At each step sufficient solvent was added to make liquid slurry and the mixture was left for 48 h. The two extracts were combined and evaporated to about 1/3 of the original volume. The resultant aqueous extract was cleared by extraction in a separating funnel with chloroform and then evaporated to dryness under reduced pressure in a rotating evaporator (8).

Qualitative and quantitative analysis of allicin

Using a thin layer chromatography (TLC) method the presence of allicin (diallyl thiosulfinate) was evaluated in plant bulbs. Dichloromethane extract of *A. hirtifolium* was tentatively identified on silica gel GF_{254} precoated plate. Dichloromethane extract of fresh chopped garlic was used as an authentic sample for comparison. Toluene-ethyl acetate (100-30) was used as mobile phase and UV detection was performed at 254 nm. Further characterizations were achieved by vanillin-glacial acetic acid reagent and R_f value (9-10). Determinations of total thiosulfinates and allicin contents of *A. hirtifolium* bulbs were done according to a spectrophotometric method (11-12).

Animals

Six to eight weeks old Balb/c male mice were purchased from Razi Institute (Tehran, Iran). They were maintained in a temperature- and light-controlled environment with free access to standard rodent chow and water.

Sheep red blood cell-induced paw thickness

To study the effects of *A. hirtifolium* on acquired immunity, groups of Balb/c mice (n=8-12) were used. Sheep red blood cell (SRBC) was injected subcutaneously (sc) on the shaved back with $1x10^8$ cells/ml, (0.02 ml) on day 0. The mice were challenged on day 5 by injecting 1×10^8 SRBC (20 µl, s.c.) into the right hind footpad. Footpad thickness was measured with an engineer's caliper up to 72 h after antigen challenge, and the degree of footpad swelling was calculated as: percent increase = [(right footpad thickness after antigen challenge/uninjected left footpad thickness after antigen challenge) – 1] x 100. To block the effector phase of the SRBC-induced paw thickness hydroalcoholic extracts (10-2000 mg/kg), polyphenolic fractions (100-1000 mg/kg), betamethasone (4 mg/kg) or normal saline were given intraperitoneally (i.p.) 1 h before antigen challenge on day 5. To see the effects of *A. hirtifolium* on intrinsic immunity animals received one injection of SRBC on day 0 on the footpad (s.c.), one hour after ip injection of the tested compounds (13-16).

Statistical analysis

SIGMASTAT™ (Jandel Software, San Raphael, CA) was used to perform statistical tests. Analysis of variance followed by Mann-Withney test was used to examine the differences among groups. Significance was assumed at 5% level.

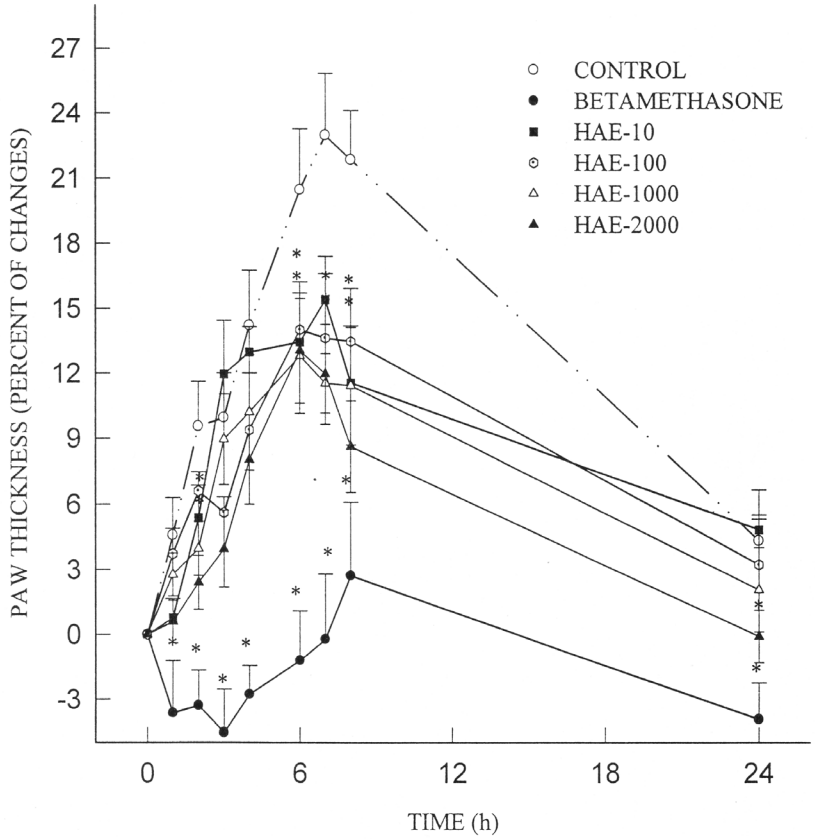

Figure 1. Effect of hydroalcoholic extracts (HAE) of *A. hirtifolium* on the intrinsic immunity. Animals received 20 µl SRBC in the right paw. The left paw of each mouse received vehicle alone. Tested compounds were given (10, 100, 1000 and 2000 mg/kg, i.p) 1 h before antigen challenge. Paw thickness was measured up to 72 h after antigen challenge, and results are shown as percentage increase in paw thickness ± sem. Groups of 8-12 mice per condition were used.

Results and Discussion

Evaporation and solvent removal of hydroalcoholic extract and polyphenolic fraction of *A. hirtifolium* gave semi-solid masses yielded 34% and 27%, respectively. Allicin is responsible for the pungent smell of garlic and onions and it has been shown to possess a variety of pharmacological effects. Also, it is the most abundant compound, representing about 70% of the overall thiosulfinates present or formed upon crushing of garlic cloves (2, 11-12). Based on our TLC experiment, the R_f of allicin was 0.45. After treatment with the reagent, allicin was distinguished by observing the characteristic violet-brown zone in garlic and *A. hirtifolium*. The yield of total thiosulfinates and allicin in bulbs of *A. hirtifolium* were 0.53% and 0.37%, respectively.

Delayed-type hypersensitivity (DTH) is a well-defined in-vivo model of cell mediated response. DTH reaction can be quantified by measuring the amount of paw thickness after injection of antigen (13-16). SRBC injection increased paw thickness with maximum response at 6-8 h (Figures 1-4). Betamethasone (4 mg/kg), a well-known immunosuppressive drug, inhibited paw thickness in both intrinsic and acquired immunity models, indicating the accuracy of the method that was used in this experiment (Figures 1-4). Hydroalcoholic extracts (100-2000 mg/kg) and polyphenolic fractions (100-1000 mg/kg) of *A. hirtifolium* bulbs significantly reduced paw thickness in both models ($P < 0.05$) (Figures 1-4). Although there were differences among the inhibitory responses of hydroalcoholic extracts of *A. hirtifolium* on intrinsic immunity, there was not a clear dose-dependent response. For polyphenolic fractions of *A. hirtifolium* a dose-dependent decrease in the intrinsic immunity was seen. These findings showed that both hydroalcoholic extracts and polyphenolic fractions of *A. hirtifolium* decreased acquired immunity response in a dose dependent manner. The immunomodulatory activities of *A. hirtifolium* could depend on various chemical

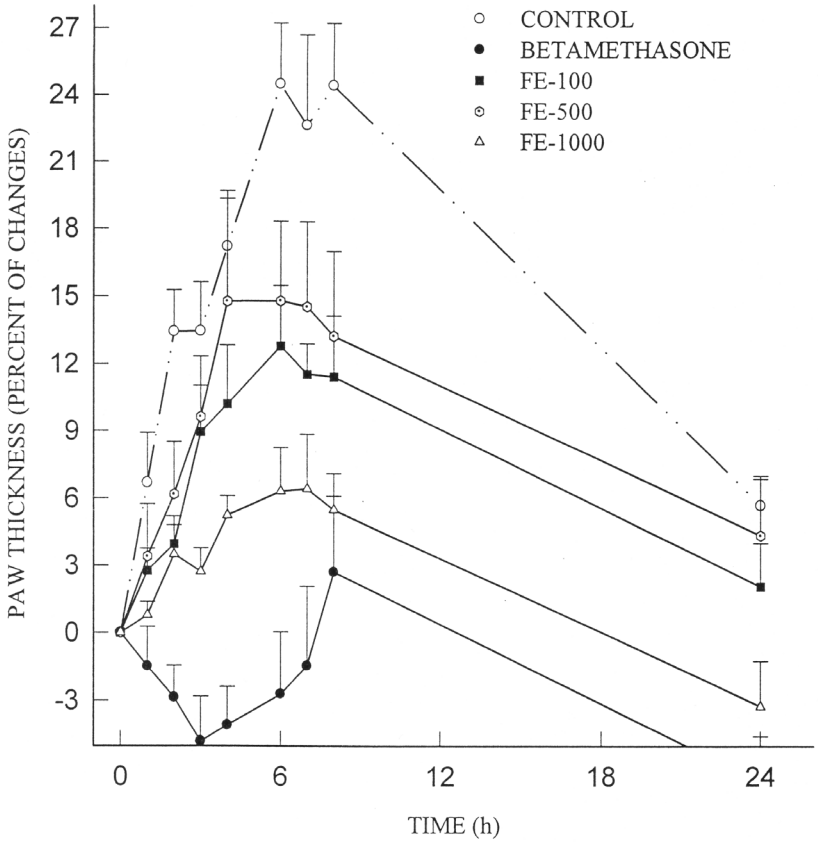

Figure 2. Effect of polyphenolic extracts (PPE) of *A. hirtifolium* on the intrinsic immunity. Animals received 20 µl SRBC in the right paw. The left paw of each mouse received vehicle alone. Tested compounds were given (100, 500 and 1000 mg/kg, i.p.) 1 h before antigen challenge. Paw thickness was measured up to 72 h after antigen challenge, and results are shown as percentage increase in paw thickness ± sem. Groups of 8-12 mice per condition were used.

compounds, above all on sulphur-containing compounds like allicin and polyphenolic compounds like flavonoids. Sulphur-containing compounds may interfere with the function of the gamma-glutamyl cycle as well as inhibitors of some of the enzymes having a SH-groups at the active site. Recently these compounds were found to inhibit the tumor cell metastasis in experimental animals, which may be partially due to the immunostimulation of stem cells (2). It has been also demonstrated that some flavonoids inhibit the release of reactive oxygen species from human polymorphonuclear leukocytes (PMNs). Such an effect has been attributed to lipoxygenase, myeloperoxidase or even NADPH-oxidase inhibition. Flavonoids also inhibit the phosphorylation of proteins that mediate the activation of PMNs induced by phorbol myristate acetate (PMA), and they appear to be able to inhibit the release of β-glycuronidase, probably by acting on phospholipase A_2 (1). Further pharmacological and phytochemical studies are needed to identify the constituents of *A. hirtifolium* and evaluate precisely their immunomodulatory activities and mechanisms.

Acknowledgment

This study was supported by a grant from the research council of Isfahan University of Medical Sciences, Isfahan, Iran. Authors are thankful to Mr. M. Norouzi, herbarium department of Iranian Research Institute of Forests and Rangelands, Isfahan, Iran for identification of the plant material.

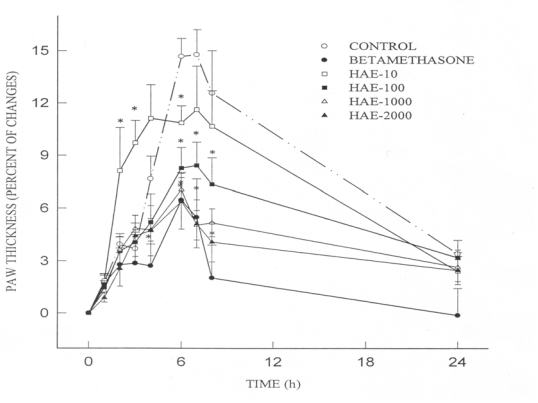

Figure 3. Effect of hydroalcoholic extracts (HAE) of *A. hirtifolium* on the acquired immunity. Animals received 20 µl SRBC in the shaved back. After 5 d, a hypersensitivity response was elicited by injecting SRBC to the right paw. The left paw of each mouse received vehicle alone. Tested compounds were given (10, 100, 1000 and 2000 mg/kg, i.p.) 1 h before antigen challenge. Paw thickness was measured up to 72 h after antigen challenge. Results are shown as percentage increase in paw thickness ± semGroups of 8-12 mice per condition were used.

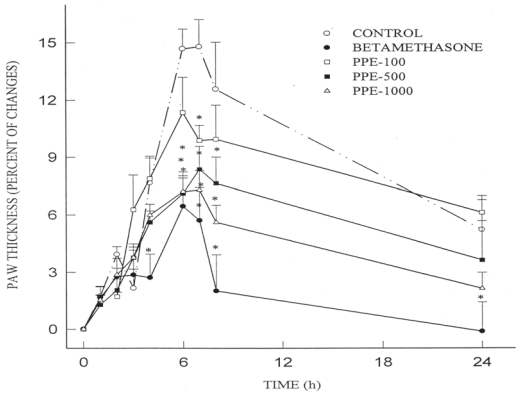

Figure 4. Effect of polyphenolic extracts (PPE) of *A. hirtifolium* on the acquired immunity. Animals received 20 µl SRBC to the shaved back. After 5 d, a hypersensitivity response was elicited by injecting SRBC to the right paw. The left paw of each mouse received vehicle alone. Tested compounds were given (100, 500 and 1000 mg/kg, i.p.) 1 h before antigen challenge. Paw thickness was measured up to 72 h after antigen challenge. Results are shown as percentage increase in paw thickness ±sem. Groups of 8-12 mice per condition were used.

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