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Effect of total flavonoid extract of *Tanacetum parthenium* L. (feverfew) pollen grains on immune system responses in Balb/C mice

Masoomeh Jannesar[1, 2], Ahmad Majd[3], Maryam Sharif Shoushtari[1, 4*], Mona Oraei[1]

[1]*Immunology, Asthma and Allergy Research Institute, Tehran University of Medical Sciences, Tehran, I.R. Iran*

[2]*School of Biology, University of Tehran, Tehran, I.R. Iran*

[3]*Department of Biology Faculty of Science, NorthTehran Branch, Islamic Azad University, Tehran, I.R. Iran*

[4]*Department of Biology, Faculty of Science, science and research Branch, Islamic Azad University, Tehran, I.R. Iran*

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Abstract

Tanacetum parthenium L. has been used in traditional medicine especially for reducing fever and as an insect repellant. This plant has some pharmacological properties, such as anticancer, anti- inflammatory, and it's also cardiotonic, antispasmodic and has emmenagogue properties and it can also be used as an enema for worms. In this study total flavonoid extract of *T.parthenium* pollen grains was evaluated for Immunomodulatory activity in male Balb/C mice by two methods: 1. Delayed type hypersensitivity (DTH) test, 2. Lymphocyte proliferation assay by the method MTT ((3-4, 5-dimethyl tetrazolyl-2) 2, 5 diphenyl) tetrazolyum bromide). Forty five Balb/C mice were classified in nine groups and treated with eight doses (1, 5, 10, 20, 30, 50, 70 and 100 mg/Kg) of total flavonoid extract. DTH response was significantly ($P<0.01$) increased at doses of 20, 30, 50 and 70 mg/Kg. The highest DTH response was observed at doses of 50 and 70 mg/Kg of the extract. A significant increase ($P<0.01$) in lymphocyte immune response was observed at all doses of the extract but the best response was found at the dose of 30 mg/Kg of extract. The results obtained in this study indicated that total flavonoid extract of *T.parthenium* pollen grains has significant immunomodulatory activity.

* **Corresponding Author:** Maryam Sharif Shoushtari ✉ sharifsh56@gmail.com

Introduction

The Asteraceae is the largest and most cosmopolitan family of flowering plants. Plants in this family were widely utilized in the past and are still used as medicinal herbs (Attard and Cuschieri, 2009). Twenty six different species from the genus of Tanacetum are reported thus far growing in various regions of Iran (Mozaffarian, 1996). *T.parthenium* is being used in folk medicine of Europe and Iran to alleviate the symptoms of migraine, arthritis and psoriasis, and to inhibit blood platelet aggregation, also it is used as an insect repellant, and as an antibacterial, antioxidant and antifungal herb (Johnson, 1984, Goren *et al.*, 1996, Bandonienė *et al.*, 2002).

Pollen grains, the male reproductive cells of flowers, are rich in secondary metabolites. Although pollen grains are known to have allergic effects but during ancient times, people all over the world used pollens for their medical properties (Graikou *et al.*, 2011). Pollens are used in human diets for their high concentrations of healthy compounds such as carotenoids, flavonoids and polyphenols (Baltrusaityte *et al.*, 2007, Kroyer and Hegedus, 2001, Moreira *et al.*, 2008). Flavonoids (natural polyphenol compounds) are one of the secondary metabolites that are present with high concentrations in pollen grains, legumes, fruits and vegetables. These compounds have been reported to have multiple biological effects including antiviral, immunomodulatory, anti-inflammatory, antimutagenic and anticarcinogenic activities (Howe *et al.*, 1990, Verma *et al.*, 1988, Havsteen, 2002, Picard, 1996). Immunomodulators are biological response modifying compounds that affect the immune response in either a positive or negative fashion. If an immunomodulator results in enhancement of immune reactions it is named as immunostimulator (Hadden and Smith, 1992).

Some studies have indicated that pollen grains have high concentrations of flavonoids with immunomodulatory activities (Verma *et al.*, 1988, Baltrusaityte *et al.*, 2007, Medeiros *et al.*, 2008). Medicinal plants such as *T.parthenium* are rich sources of immunomodulators (Veena and Mishra, 2011). Medicinal effects of *T.parthenium* are attributed to the leaves and flowers mainly due to the presence of sesquiterpene lactones and flavonoids (Pareek *et al.*, 2011). There is no scientific data on immunomodulatory activity of the *T.parthenium* pollen grains. The present paper reports the immunomodulatory activities of various doses of total flavonoid extract in *T.parthenium* pollen grains in Balb/C mice that can provide potential alternatives to conventional chemotherapies for a variety of diseases.

Materials and methods

Plant material

T.parthenium was collected from Chitgar Park located in Tehran, Iran in April 2011. Pollen grains were separated from plant flowers and then dried. Pollens were purified up to 90٪ on an steel mesh (70 Micron) using an optical microscope.

Determination and extraction of total flavonoid

Flavonoids of pollen grains were extracted using the maceration method of Ebrahimzadeh *et al* (Ebrahimzadeh *et al.*, 2008). Twenty grams of pollen grains were mixed with distilled water with the ratio of 1:6 and the mixture was left at room temperature for 24h. Then, flavonoid extract was separated from plant material using a Filter paper.

Total flavonoid was determined according to the Aluminium Chloride method (Ebrahimzadeh *et al.*, 2009). Briefly, 0.5 ml solution of the plant extract was separately mixed with 1.5 ml of methanol, 0.1 ml of 10٪ aluminium chloride, 0.1 ml of 1M potassium acetate and 2.8 ml of distilled water and the compounds were set aside at room temperature for 30 min. the absorption of the reaction mixture was measured at 415 nm with a double beam spectrophotometer (Perkin Elmer).

Experimental animals

45 Balb/C male mice (weighing approximately 28 gr) were used for this study. The animals were bred and maintained under standard laboratory conditions (temperature 25± 2°C and Light period of 12h) without any pathogen. The animals were divided into

nine groups as follows: group one was used as control and in groups two to nine animals were treated with different doses (1, 10, 20, 30, 50, 70 and 100 mg/Kg) of total flavonoid aqueous extract (table1).

Delayed type hypersensitivity response (DTH)

The delayed type hypersensitivity (DTH) response was determined using the method of Raisuddin *et al* (Raisuddin *et al.*, 1991). On the last day of two weeks of treatment with flavonoid extract, animals were immunized with subcutaneous injection of 1×10^9 Sheep red blood cells (SRBC). On the fifth day of immunization, all the animals were again treated with 1×10^8 cells in their left hind footpad. The right footpad was injected with the same volume of normal saline, served as the trauma control for non-specific swelling. Increase in footpad's thickness was measured 24h after the treatments using a dial caliper. All assays were performed in triplicate.

Lymphocyte proliferation assay (MTT)

The Balb/C mice spleen was removed and red blood cells were cleared by incubation in lysis buffer (0.15M NH_4Cl, 1mM Na_2EDTA, PH 7.2). Obtained mononuclear cells (2×10^5 cells/well) were grown in each well of a 96-well plate (Nunc, Denmark). The preparations were cultured with RPMI supplemented with 10٪ fetal calf serum and then different doses of flavonoid extract were added as mitogen. After 48h of incubation at 37°C in 5٪ Co_2, MTT (3-4, 5-dimethyl tetrazolyl-2) 2, 5 diphenyl) tetrazolyum bromide (Sigma chemicals) in the concentration of 5 µg/ml was added to every well and the mixture was incubated for 4h at 37°C in 5٪ Co_2. DMSO (dimethyl sulfoxide) (100µl) was added to dissolve the produced formazan crystals. Unstimulated splenocytes were used as negative control.

Plates were read at 540 nm, and the results were expressed as follows: OD values of stimulated cells minus the relative cell number of the unstimulated cells multiplied by relative OD values of unstimulated cells. All assays were performed in triplicate(Denizot and Lang, 1986).

Statistical analysis

All the values were expressed as mean± SD and data were analyzed by one-way ANOVA, followed by Duncan's test using the software SPSS version 11. Differences were considered significant when the P value was less than 0.01.

Results

Effect of total flavonoid extract on Delayed type hypersensitive (DTH) response

The total flavonoid extract at doses of 20, 30, 50 and 70 mg/Kg elicited a significant ($P<0.01$) increase in DTH response compared to control animals, which is directly correlated with cell-mediated immunity (CMI) (Fig.1). The highest DTH response was observed at doses of 50 and 70 mg/Kg. This extract at doses of 1 and 5 mg/Kg decreased the DTH response but didn't change it significantly at doses of 10 and 100 mg/Kg ($P<0.01$).

Table 1. Reviewing the effects of treatment with *T.parthenium* pollen grains' total flavonoid extract on the immune system using DTH and MTT tests.

Group	Dose (mg/Kg)	Flavonoid content (mg/ml)	DTH response(mm) Mean±SD	MTT response Mean±SD
1	–	–	0.05± 0.02	0.114± 0.09
2	1	0.15	0.008± 0.002	0.272± 0.08
3	5	0.25	0.01± 0.035	0.412± 0.05
4	10	0.3	0.07± 0.005	0.632± 0.05
5	20	0.6	0.09± 0.015	0.650± 0.02
6	30	0.9	0.092± 0.01	0.810± 0.03
7	50	1.5	0.152± 0.032	0.320± 0.07
8	70	2.1	0.160± 0.03	0.412± 0.02
9	100	3	0.066± 0.072	0.359± 0.01

Effect of total flavonoid extract on Lymphocyte proliferation assay

The lymphocyte proliferation activity of extracts was evaluated by MTT method. In this assay, the immunomodulatory effects of the total flavonoid extract was tested for its mitogenic activity. A significant increase ($P<0.01$) in the lymphocyte numbers was shown in all doses (Fig. 2) in compared to the control animals but this extract at doses of 10, 20 and 30 mg/Kg elicited the highest lymphocyte immune response. The best lymphocyte immune response was found at dose of 30 mg/Kg.

Discussion

The mechanism responsible for elevating DTH as an indicator of CMI responses could be due to the presence of sensitized T-lymphocytes. When challenged by the antigen, they convert into lymphoblasts and secrete a variety of molecules including proinflammatory lymphokines, attracting more scavenger cells to the site of reaction. The infiltrating cells are probably immobilized to promote the inflammatory reaction (Fulzele *et al.*, 2003). Increase in DTH response at doses of 20, 30, 50 and 70 mg/Kg indicates that specific doses of *T.parthenium* pollen flavonoid extract have stimulatory effects on lymphocytes at the site of injection. Low doses (1 and 5 mg/Kg) of the extract decreased the DTH response and the highest dose (100 mg/Kg) of the extract didn't have any effect on it. Total flavonoid is known by their antibacterial, antiviral, antioxidant, immunomodulatory and inhibiting pro-inflammatory cytokine production and their receptors. Flavonoids have been shown to affect many enzyme systems involved in allergic and inflammatory responses (Verma *et al.*, 1988).

In our study, The MTT assay was used to assess the mitochondrial activity in order to estimate the rate of lymphocyte proliferation (Nashikkar *et al.*, 2012). The immunomodulatory effect of pollen flavonoid extract was tested to evaluate its mitogenic activity on mice's splenocytes. Comparison of the lymphocyte proliferation in extract-treated and non-treated groups revealed that all doses of total flavonoid extract increased the lymphocyte immune response but the highest mitogenic activity was observed at the dose of 30 mg/Kg of the extract. Increased proliferation of cells in the MTT assay suggests that total flavonoid extract of *T.parthenium* pollen grains in all doses, has an stimulatory effect on the expansion of splenocytes.

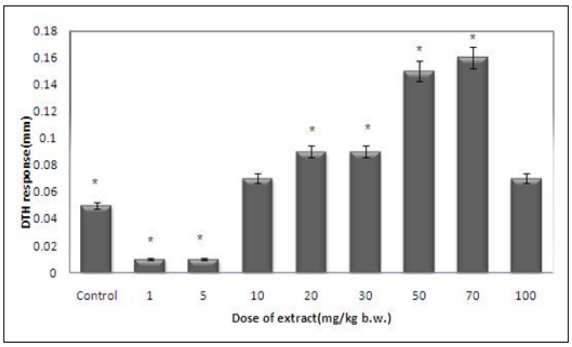

Fig. 1. Delayed type hypersensitivity (DTH) response in Balb/C mice after treatment with different doses of *T.parthenium* pollen grains' total flavonoid extract. $P<0.01$ is marked with * and is indicative of a significant difference, when compared with the control animals.

These results are in accordance with several studies performed by other researchers indicating the stimulatory properties of flavonoids on immune system and flavones on human peripheral blood leukocyte proliferation. They significantly increase the activity of helper T cells, cytokines, interleukins, gama-interferon and macrophages and so they are useful in the treatment of several diseases caused by immune dysfunction (Verma *et al.*, 1988, Kawakita *et al.*, 2005, Sharififar *et al.*, 2009). Lee *et al* (2009) (Lee *et al.*, 2009) indicated that Pine pollen extract had significant antioxidant activity and moreover, Carpes *et al* (2009) (Carpes *et al.*, 2007) reported that the high antioxidant activity of bee pollen extract is probably due to high concentrations of phenolic compounds in it. The antioxidant activity of phenolic compounds depends on their chemical structure and can be explained by the action of these compounds as a reducing agent (Rice-Evans *et al.*, 1996). In vitro experiments have demonstrated that some flavonoids have greater antioxidant activity than vitamins E and C (Almaraz-Abarca *et al.*, 2007). These antioxidant properties may induce the immunostimulant effect,

as several antioxidants have been reported to possess immunomodulatory properties(De la Fuente and Victor, 2000).

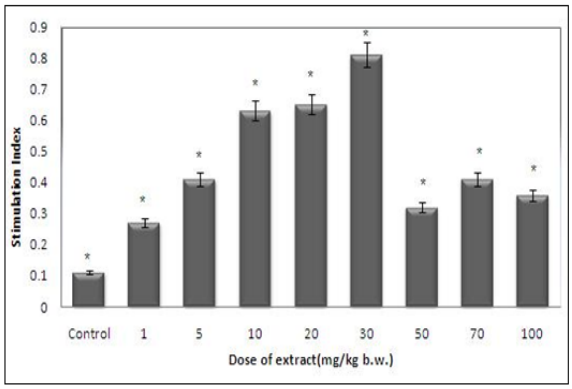

Fig. 2. Lymphocyte immune response test (MTT) in Balb/C mice after treatment with different doses of *T.parthenium* pollen grains' total flavonoid extract. P<0.01 is marked with * and is indicative of a significant difference, when compared with the control animals.

In this study we conclude that total flavonoid extract of *T.parthenium* bee pollens has appreciable immunomodulatory activity. Moreover we found the best effective doses of total flavonoid extract for DTH test at 50 and 70 mg/Kg and MTT test at 30mg/Kg. Our results showed that flavonoids of *T.parthenium* bee pollens probably can be useful in production of new drugs for prevention or cure of various diseases related to the immune system. Therefore results are very promising for further immunological and pharmacological experiments, which will focus on isolating various flavonoids from total flavonoids and investigating about their immunomodulatory effects separately.

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