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Original Article

The effects of methanolic, chloroform, and ethylacetate extracts of the *Cucurbita pepo* L. on the delay type hypersensitivity and antibody production

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Abstract

Pumpkin, as a dietary plant, has been used in traditional medicine around the world. In addition, during the last decade, antidiabetic, antihypertensive, antitumor, intestinal antiparasitic, antibacterial, anti hypercholesterolemia, anti-inflammatory, immunomodulatory and analgic effects of pumpkin has been reported. The aim of the present study was to determine the effects of different extracts of *Cucurbita pepo* L. on the immune responses. Methanolic, chloroform and ethylacetate extracts of *C. pepo* fruits was obtained using percolation method. Mice were used to study the effects of *C. pepo* extracts on the acquired immunity. Sheep red blood cell (SRBC) was injected (S.C., 1×10^8 cells/ml, 20 µl) and 5 days later, methanolic, chloroform and ethylacetate extracts of *C. pepo* at diiferent doses (10, 100 and 500 mg/kg), betamethasone and levamisol at equal doses (4 mg/kg) as positive controls and normal saline as a negative control were given i.p. After 1 h SRBC was injected to the footpad (S.C., 1×10^8 cells/ml, 20 µl) and the footpad swelling was measured up to 72 h. To investigate the effects of *C. pepo* on the innate immunity the same procedure was used, but animals received only one injection of SRBC 1 h after i.p. injection of test compounds. Our findings showed that SRBC induced an increase in the paw swelling with maximum response at 6-8 h. Betamethasone inhibited the paw swelling in both models. In both innate and acquired immunity models, methanolic, chloroform and ethylacetate extracts of *C. pepo* fruits significantly reduced the paw swelling dose dependently. The data suggest that the pumpkin extracts may have immunomodulatory effects.

Keywords: *Cucurbita pepo* L.; Pumpkin; Acquired immunity; Innate immunity

INTRODUCTION

During the last decades there has been considerable attention all over the world to the use of dietary plants and herbal preparations as alternative medicine. Several herbs have been used traditionally to prevent and treat diseases. About 12.1% of adults in the United States used herbal medicines in 1997 (1). In 2001, about 4.2 billion Dollars was spent on the herbal remedies in America (2). It should be considered that in the developing countries the use of the herbal remedies is much higher, as 70-95% of the population relies on the use of traditional medicines for primary medical problems. WHO reported an exponential increase in the rate of consumption of herbal remedies, as in 2008 the global market for traditional medicines was about 83 billion US Dollars annually (3). Recently, scientific evaluation of dietary plants and preparations of plant origin medications have received more attention. One of these plants is pumpkin which has been frequently used as functional food or medicine.

Pumpkin is a gourd-like squash of the genus Cucurbita and the family Cucurbitaceae. The family Cucurbitaceae comprises about 80 genera and over 800 species (4). It commonly refers to cultivars of anyone of the species *C. pepo*, *C. mixta*, *C. maxima* Duchesne, *C. Ficifolia*, and *C. moschata*. Among these, *C. pepo*, *C. maxima* Duchesne and *C. moschata* Duchesne have great economical and social

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value in the horticulture worldwide with high production (5-7). They have typically a thick, orange or yellow shell, creased from the stem to the bottom, containing the seeds and pulp.

Pumpkin is cultivated throughout the world for use as vegetable as well as medicine. It has been used traditionally as medicine in many countries such as China, Argentina, India, Brazil and Iran (8-10).

Although pumpkin is a well-known edible plant, it has been used in the traditional medicine worldwide. Many compounds have been isolated from the pumpkin spp, but only some of them have biological activities and medicinal properties (11).

In most countries it is commonly used as antidiabetic and internally as well as externally for management of worms and parasites. In the last few decades, researches have been focused on the antidiabetic (12,13), antihypertension, antitumor (14-19), antibacteria, antifungal (20), antihypercholesterolemia, intestinal anti-parasitia (21), immunomodulatory, anti-inflammatory (22,23) and antalgic effects of pumpkin. (24,25). The pumpkin poly-saccharides, by increasing the cell immune function, are responsible for the immuno-modulatory activity of pumpkin (26). In addition, enhancement of splenic lymphocyte proliferation, natural killer cell activity and an increase in the number of CD4+, CD8+ T cells and the CD4+/CD8+ ratio by pumpkin extracts has been reported (27). In the current study delay type hypesensitivity (DTH), a model of cell-mediated response that is well-defined *in vivo* was used. DTH reaction can be quantified by measuring the amount of the paw swelling after injection of antigen (28-30). The aim of this study was to investigate the effects of different extracts of the *C. pepo* fruit on the cellular immune system or antibody potential in mice or rats.

MATERIALS AND METHODS

Plant material

The fruits of *C. pepo* were purchased from Najafabad market of Isfahan province. The plant specimen was identified by the Department of Pharmacognosy, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran.

Preparation of the extracts

Air-dried fruits of the plant (100 g) were coarsely powdered and perculated with 400 ml of methanol:water (75:25), chloroform or ethylacetate:water (17:3) for 48 h. After filtration, the extracts were evaporated until dryness in a vacuum evaporator (31,32).

Determination of polyphenolic compounds of C. pepo

Using Folin-Ciocalteau method, total polyphenolic compounds of *C. pepo* were determined spectrophotometrically at 765 nm. Gallic acid was used as the standard (33).

Animals

Six to eight-weeks old Balb/c male mice and the Wistar rats were purchased from the Pasteure Institute (Tehran, Iran). Treatment of the animals was in accordance with the institutional guidelines. They were maintained in a tempe-rature and light-controlled environment with free access to standard rodent chow and water.

Sheep red blood cell-induced paw swelling

Nine groups of Balb/c mice each consisted of eight animals, received the methanolic, chloroform or ethyl acetate extracts of the *C. pepo* at different doses of 10, 100 and 500 mg/kg. In addition, three other groups of animals received equal doses of 4 mg/kg of betamethasone and levamisol as the positive controls and normal saline as the negative control. The sheep red blood cell (SRBC) was prepared by centrifugation which followed by 3 times washing with normal saline. To investigate the effects of the extracts of the *C. pepo* on the acquired immunity, 20 µl of SRBC containing 1×10^8 cells/ml was injected subcutaneously (S.C) to the shaved back of animals on day 0. The mice were challenged on day 5 by S.C. injection of 20 µl of 1×10^8 SRBC/ml into the right hind of the footpad. The footpad swelling was measured with an engineer's caliper (Diamond brand, China, 150 × 0.02 mm) up to 72 h after antigen challenge, and the degree of the footpad swelling was calculated as follows:

Increase (%) = (the footpad swelling after antigen challenge – the footpad swelling before antigen challenge/the footpad swelling before antigen challenge)×100

To assess the effects of the extracts of the *C. pepo* on the innate immunity, the rats received one injection of SRBC on day 0 into the footpad (S.C) 1 h after i.p. injection of the test compounds (28-30). The time course of studies was the same in all the experiments.

Measurement of the humoral immune response

Procedure for immunization was the same as for the acquired immunity. The challenge was made on the day 6. For collecting blood samples, light ether anaesthesia was used. To avoid any circadian influence, blood sampling and sacrificing were made between 8.00-9.00 a.m. The serum was separated and kept at minus 20°C until use. Direct haem-agglutination test in microtiter plate was performed to determine the circulating antibody titer (34). The plates were incubated for two h at room temperature. Antibody titer was determined as the reciprocal of the highest dilution exhibiting hemagglutination. SRBC was obtained from the same animal source for all the experiments.

Statistical analysis

SIGMASTAT^{TM} (Jandel Software, San Raphael, CA) was used to perform statistical analyses. The data are presented as means ± S.E.M. The significance of the differences between various experimental groups was determined by analysis of variance, followed by Kruskal Wallis post hoc test. Significance was assumed at 5% level.

RESULTS

Using percolation method for extraction, the extract yield of semi-solid masses after evaporation and solvent removal of methanol:water, chloroform and ethylacetate: water of *C. pepo* were 30, 25 and 10%, respectively.

Effects of methanolic, chloroform and ethylacetate extracts of C. pepo on the innate immunity

The animals were randomly divided into twelve groups, each composed of eight mice. The first nine groups of animals received a single dose of methanolic, chloroform or ethylacetate extracts of *C. pepo* (10, 100, and 500 mg/kg). The tenth and eleventh groups of mice (positive controls) received betame-thasone or levamisol (4 mg/kg), while the twelfth group was injected normal saline which served as the negative control. After 1 h SRBC was injected into the footpad and the paw swelling was measured up to 24 h. SRBC injection significantly increased the paw swelling with maximum response at 6-8 h (P<0.05, Figs. 1-3).

Injection of betamethasone (4 mg/kg) significantly inhibited the paw swelling while levamisol (4 mg/kg) significantly increased the paw swelling (Figs. 1-3). Methanolic, chloroform, and ethylacetate extracts of *C. pepo* fruits at the doses of 10, 100 and 500 mg/kg significantly in a dose-dependent fashion reduced the paw swelling (P<0.05, Figs. 1-3) .

Effects of methanolic, chloroform and ethylacetate extracts of C. pepo on the acquired immunity

Different groups of mice (similar to those of the innate immunity model) were used. They were challenged with S.C injection of SRBC on day 0 followed by day 5 one h after i.p. injection of betamethasone, levamisol or extracts the paw swelling was measured up to 72 h. SRBC injection significantly increased the paw swelling with maximum response occured at 4 h (P<0.05) (Figs. 4-6). Percent changes in the paw swelling in the control group was significantly higher than those of the innate immunity (P<0.05). Injedction of betamethasone (4 mg/kg) significantly decreased the paw swelling, while levamisol (4 mg/kg) significantly increased the paw swelling (Figs. 4-6).

Our findings showed that methanolic, chloroform, and ethylacetate extracts of *C. pepo* fruits significantly and dose dependently reduced the paw swelling (P<0.05, Figs. 4-6).

Circulating antibody titers

The anti-SRBC antibody dilution is shown in Table 1. In unchallenged rats there was no significant change in the SRBC specific circulating antibody levels. After an immune

challenge, betamethasone decreased the antibody levels while levamisol increased the antibody concentration. Methanolic, chloroform and ethyl acetate extracts of the *C. pepo* significantly increased the serum titer (P<0.05).

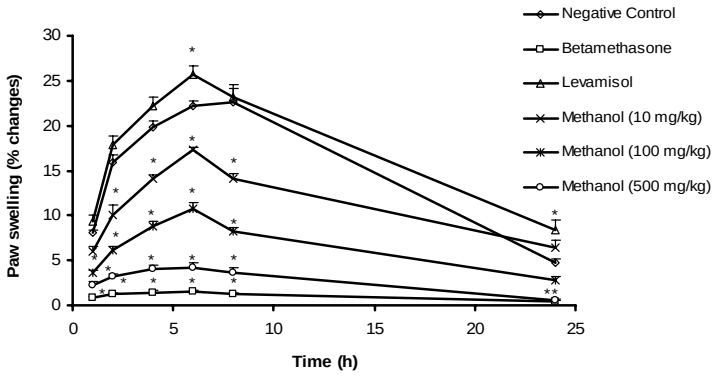

Fig. 1. Effect of the methanolic extract of the *C. pepo* on the innate immunity. Results are shown as percent increases in the paw swelling ± S.E.M. Groups of 8 mice per condition were used. * = P<0.05 compared with the negative control group.

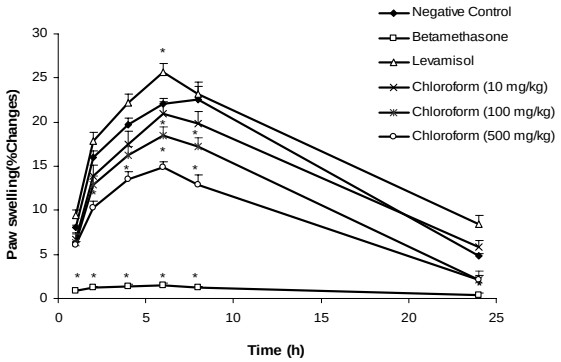

Fig. 2. Effect of the chloroform extract of the *C. pepo* on the innate immunity. Results are shown as percentage increases in the paw swelling ± S.E.M. Groups of 8 mice per condition were used. * = P<0.05 compared with the negative control group.

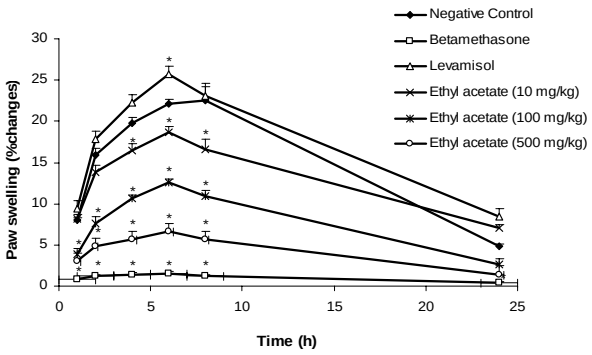

Fig. 3. Effect of the ethylacetate extract of the *C. pepo* on the innate immunity. Results are shown as percentage increases in the paw swelling ± S.E.M. Groups of 8 mice per condition were used. * = P<0.05 compared with the negative control group.

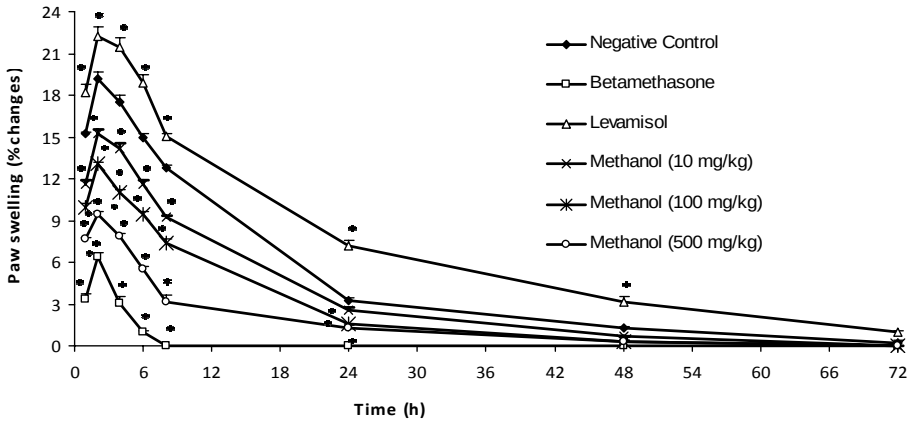

Fig. 4. Effect of the methanolic extract of the *C. pepo* on the acquired immunity. Results are shown as percent increases in the paw swelling ± S.E.M. Groups of 8 mice per condition were used. * = $P<0.05$ compared with the negative control group.

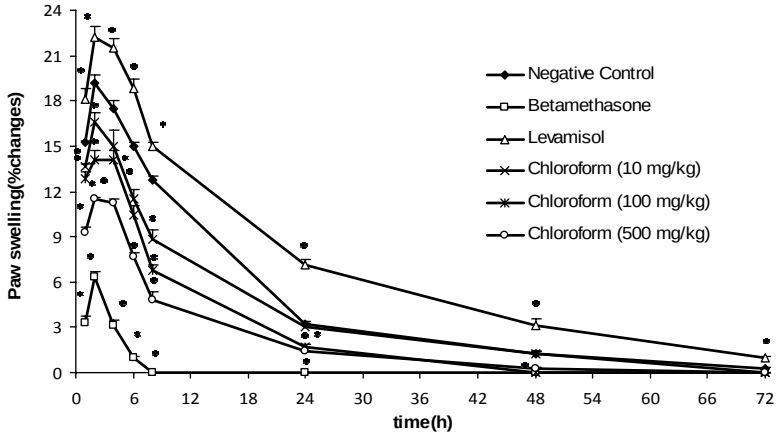

Fig. 5. Effect of the chloroform extract of the *C. pepo* on the acquired immunity. Results are shown as percent increases in the paw swelling ± S.E.M. Groups of 8 mice per condition were used. * = $P<0.05$ compared with the negative control group.

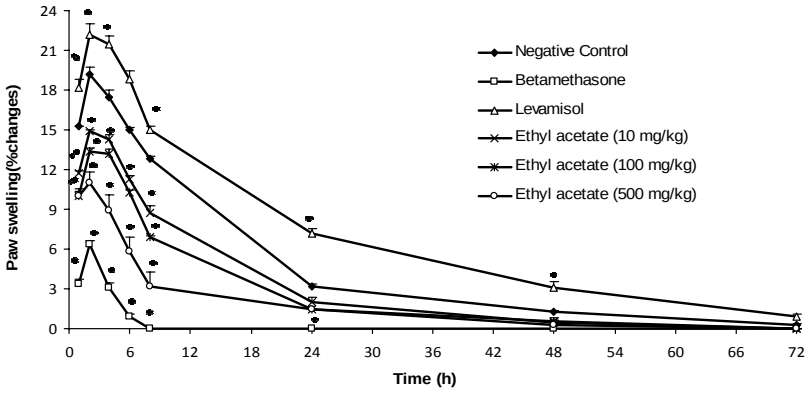

Fig. 6. Effect of the ehylacetate extract of the *C. pepo* on the acquired immunity. Results are shown as percent increases in the paw swelling ± S.E.M. Groups of 8 mice per condition were used. * = $P<0.05$ compared with the negative control group.

Table 1. Rat serum anti-SRBC antibody dilution

Groups	Rat serum anti-SRBC antibody dilution
Negative control	1/1000
Betamethasone (positive control)	1/600 *
Levamisol (positive control)	1/30000 *
Methanolic extract	1/10000 *
Chloroform axtract	1/20000 *
Ethyl acetate extract	1/10000 *

Results are shown as serum anti-SRBC antibody dilution. Groups of 8 mice per condition were used. * = $P<0.05$ compared with the negative control group.

DISCUSSION

The aim of this study was to look at the effects of different extracts of the *C. pepo* as a potential source of immunomodulatory compounds by measuring of the DTH. Disturbance in the immune system can cause many clinical disorders. In the management and treatment of inflammation and allergic diseases we need to suppress the immune system, while stimulation of the immune system is highly desirable for the treatment of HIV, immunodeficiency and infectious diseases (35). However, it has been shown that certain agents normalize or modulate pathophysiological processes and are hence called immunomodulatory agents withought being specifically stimulatory or suppressive (36). In addition, increase in antibiotic resistant strains of microorganisms is a serious problem in the treatment of infectious diseases which prompted scientists to look for the herbal immunomodulators (37). It has been suggested that most of the therapeutic effects of medicinal plants in the treatment of infectious diseases are mainly due to their effects on the immune system. This encourages scientists to identify and characteriz natural compounds with immunomodulatory activity (38). Many compounds including polysaccharides, phenolic compounds, alkaloids etc. are immunomodulator. Pumpkin contains several compounds including polysaccharides, anti-fungal proteins, such as a- and b-moschins, myeloid antimicrobial peptide (39-40). In addition, bryonolic acid, a naturally occurring triterpenoid, has been identified in multiple species of the Cucurbitaceae family. Several biological activities such as anti-allergic, cytotoxic and anti-tumor activities have been reported for bryonolic acid (41).

Betamethasone (4mg/kg), an immunosuppressive drug (41-42), inhibited the paw swelling and levamisol (4mg/kg), an immunostimulating drug (43), increased the paw swelling in both the innate and acquired immunity models indicating the accuracy of the method used in these experiments (**Figs. 1-6**). Methanolic, chloroform and ethylacetate extracts of the *C. pepo* reduced significantly the paw swelling in both models and there was a clear dose-dependent response in 100 mg/kg and 500 mg/kg doses ($p<0.05$) (**Figs. 1-6**). In the innate immunity model the methanolic and ethylacetate extracts were more potent than the chloroform extract. The immunomodulating activities of *C. pepo* may depend on various chemical compounds such as different kinds of cucurbitacins, proteins and vitamin E. The most important role may belongs to the cucurbitacins. The cucurbitacins are the most important compounds in cucurbitaceae family. They are anticancer natural triterpenoids that nowadays has been focused because of the vast spectrum of effects (44-45). In addition, they have free radical scavengering effect and antioxidant activity (46). The *C. pepo* has also a considerable amount of vitamin E that is a very important antioxidant compound (47). Pupkin fruits consist of up to 50 % fatty oil, proteins, carotenoids, tocopherols, phytosterols and phytoestrogens as well (48-51). Aminoacids, polysaccharides and polyphenols as polar constituents of plants can be extracted with polar solvents such as methanol and water. It has been shown that polysaccharides and polyphenols possess

immunomodulatory effects (26). However, further pharmacological and phytochemical studies are needed to identify the constituents of the *C. pepo* and precisely evaluate their immunomodulating activities and mechanisms.

CONCLUSION

It can be concluted from this study that different extracts of *C. pepo* fruits have immunomodulatory activity which makes it a good candidiate for further studies.

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REFERENCES

1. Ang-Lee MK, Moss J, Yuan CS. Herbal medicines and perioperative care. JAMA. 2001;286:208-216.
2. Marcus DM, Grollman AP. Botanical medicines: The need for new regulations. N Engl J Med. 2002;347:2073-2076.
3. Robinson MR, Zhang X. The World Medicines Situation, 3rd Ed. Traditional medicine: Global situation, issues and challenges, WHO, Geneva, 2011.
4. Giampan JS, Rezende JAM, Silva RF. Reaction of cucurbits species to infection with *Zucchini lethal chlorosis* virus. Scientia Horticulturae. 2007;114:129-132.
5. Whitaker TW, Davis GN. Cucurbits. New York: Interscience Publ. Inc.1962.
6. Robinson RW, Decker-Walters DS. Cucurbits. New York: CAB International.1997.
7. Taylor MJ, Brant J. Trends in world cucurbit production, 1991 to 2001. In: Maynard DN (ed), Cucurbitaceae. Alexandria, VA: ASHS Press, 2002;373-379.
8. Popovic M. On growing squash and pumpkin (Cucurbita sp.) in Yugoslavia. Savremena Poljoprivreda. 1971;11:59–71.
9. Jia W, Gao W, Tang L. Antidiabetic herbal drugs officially approved in China. Phytother Res.2007;17:1127-1134.
10. Adolfo AC,Michael H. Mexican plants with hypoglycaemic effect used in the treatment of diabetes. J Ethnopharmacol. 2007;99:325-348.
11. Caili F, Huan S, Quanhong L. A review on pharmacological activities and utilization technologies of pumpkin. Plant Foods Hum Nutr. 2006;61:73-80.
12. Xia T, Wang Q. Hypoglycaemic role of *Cucurbita ficifolia* (Cucurbitaceae) fruit extract in streptozotocin-induced diabetic rats. J Sci Food Agric. 2007;87:1753-1757.
13. Kwon YI, Apostolidis E, Kim YC, Shetty K. Health benefits of traditional corn, beans, and pumpkin: *in vitro* studies for hyperglycemia and hypertension management. J Med Food. 2007;10:266-275.
14. Cheong NE, Choi YO, Kim WY, Bae IS, Cho MJ, Hwang I, Kim JW, Lee SY, Cheong NE *et al*. Purification and characterization of an antifungal PR-5 protein from pumpkin leaves. 1997;7:214-219.
15. Ito Y, Maeda S & Sugiyama T. Suppression of 7, 12-dimethylbenz [a]anthracene-induced chromosome aberrations in rat bone marrow cells by vegetable juices. Mutat Res. 1986;172:55-60.
16. Hongjian Z, Xunyi N, Giangong D. Experimental study of the effect of *Sophora flavescens Ait* and *Cucrbita pepo* L. on benign prostatic hyperplasia. Chin J Surg Intergrated Tradit West Med. 1999;72:42-50.
17. Liu T, Zhang M, Zhang H, Sun H, Yang X, Deng Y, Ji W. Combined antitumor activity of cucurbitacin B and docetaxel in laryngeal cancer. Eur J Pharmacol. 2008;587:78-84.
18. Nakashima S., Matsuda H., Kurume A. Cucurbitacin E as a new inhibitor of cofilin phosphorilation in human leukemia u937 cells. Bioorg Med Chem Lett. 2010;20:2994-2997.
19. Escandell JM, Kaler P, Recio MC, Sasazuki T, Shirasawa S, Augenlicht L, *et al*. Activated kRas protects colon cancer cells from cucurbitacin-induced apoptosis: The role of p53 and p21. Bioch Pharmacol. 2008;76:198-207.
20. Vassiliou AG, Neumann GM, Condron R, Polya GM. Purification and mass spectrometry-assisted sequencing of basic antifungal proteins from seeds of pumpkin (*Cucurbita maxima*). Plant Sci. 1998;134:141-162.
21. Diaz-Obrego D, Lloja-Lozano L, Carbajal-Zu. Preclinical studies of *Cucurbita maxima* (pumpkin seeds) a traditional intestinal antiparasitic in rural urban areas (article in Spanish). Rev Gastroenterol Peru. 2004;24:323-327.
22. Fahim AT, Abd-el Fattah AA, Agha AM, Gad MZ. Effect of pumpkin-seed oil on the level of free radical scavengers induced during adjuvant-arthritis in rats. Pharmacol Res. 1995;31:73-79.
23. Zuhair HA, Abd El-Fattah AA, El-Sayed MI. Pumpkin-seed oil modulates the effect of felodipine and captopril in spontaneously hypertensive rats. Pharmacol Res. 2002;41:555-563.
24. Wang P. Experimental study on pharmacological actions about analgesia, anti-inflammation of *Cucurbita moschata* Duch. Shizhen Med Mteria Med Res. 1999;19:567-569.
25. Caili F, Huan S, Quanhong L. A review on pharmacological activities and utilization technologies of pumpkin. Plant food Hum Nutr. 2006;61:73-80.
26. Xu GH. A study of the possible antitumour effect and immunom petence of pumpkin polysaccharide. J Wuhan Prof Med Coll. 2002;28:1-4.

27. Xia HC, Li F, Li Z, Zhang ZC. Purification and characterization of Moschatin, a novel type I ribosome-inactivating protein from the mature seeds of pumpkin (*Cucurbita moschata*), and preparation of its immunotoxin against human melanoma cells. Cell Res. 2003;13(5):369-374.
28. Hurtrel B, Maire MA, Hurtrel M, Lagrange PH. Different time course patterns of local expression of delayed-type hypersensitivity to sheep red blood cells in mice. Cell Immunol. 1992;142:252-263.
29. Belkaid Y, Valenzuela JG, Kamhawi S, Rowton E, Sacks DL, Ribeiro JM. Delayed-type hypersensitivity to *Phlebotomous papatasi sand* fly bite: an adaptive response induced by the fly. Proc Natl Acad Sci. 2000;97:6704-6709.
30. Barrington-Leigh J. The *in vitro* induction of delayed-type hypersensitivity to allo-antigens of the mouse. J Immunol Meth. 1984;69:149-163.
31. "Osterreichisches Arzneibuch". Wien: Editio Nona Osterreichisches-taatsdruckerei; 1960.
32. Momeni T. Herbal Extracts. Tehran: Reza Press; 2000.
33. Ough CS, Amerine MA. Methods for analysis musts and wines, John Wiley & Sons, New York. 1988;204-206.
34. Basseri HR, Safari N, Mousakazemi SH, Akbarzade K, Basseri HR *et al*. Comparison of midgut hemagglutination activity in three different geographical populations of *Anopheles stephensi* Iranian J Publ Health. 2004;33:60-67.
35. Koko WS, Mesaik MA, Yousaf S, Galal M, Choudhary MI. *In vitro* immunomodulating properties of selected Sudanese medicinal plants, J Ethnopharmacol. 2008;118:26-34.
36. Bafna AR, Mishra SH. Immunomodulatory activity of methanol extract of roots of *Cissampelos pareira* Linn. Ars Pharm. 2005;46:253-262.
37. Srisilam K, Sumalatha DV, Thilagam E, Veeresham C. Immunomodulators from higher plants, Indian J Nat Prod. 2000;20:3-15.
38. Pragathi D, Vijaya T, Anitha D, Mouli KC, Sai Gopal DVR. Botanical immunomodulators-potential therapeutic agents. J Global Pharma Technol. 2011;3:11-14.
39. Du B, Song Y, Hu X, Liao X, Ni Y, Li Q. Oligosaccharides prepared by acid hydrolysis of polysaccharides from pumpkin (*Cucurbita moschata*) pulp and their prebiotic activities. Int J Food Sci Technol. 2011;46:982-987.
40. Emily C. Barker, Tonibelle N. Gatbonton-Schwager, Yong Han, Jennifer E. Clay, John J. Letterio and Gregory P. Tochtrop bryonolic acid: A large-scale isolation and evaluation of heme oxygenase 1 expression in activated macrophages J Nat Prod. 2010;73:1064-1068.
41. Elliott AB, Chakravart EF. Immunosuppressive medications during pregnancy and lactation in women with autoimmune diseases. Women's Health 2010;6:431-442.
42. van Runnard Heimel PJ, Franx A, Schobben AFAM, Huisjes AJM, Derks JB, Bruinse HW. Corticosteroids, Pregnancy, and HELLP Syndrome: A Review. Obstetrical Gynecol Survey. 2005;60:57-70.
43. Fabrizi1 F, Dixit V, Messa1 P, Martin P. Meta-analysis: levamisole improves the immune response to hepatitis B vaccine in dialysis patients. Alimentary Pharmacol Ther. 2010;32: 756-762.
44. Tannin-Spitz T, Bergman M, Grossman S. Cucurbitacin glucosides**:** Antioxidant and free-radical scavenging activities. Biochem Biophysic Res. 2007;364:181-186.
45. Chan TK, Meng FY, Li Q Ho CY, Lam TS, To Y, Lee WH, *et al*. Cucurbitacin B induces apoptosis and S phase cell cycle arrest in BEL-7402 human hepatocellular carcinoma cells and is effective via oral administration. Cancer Letters.2010;294:118-124.
46. Chan TK., Li K., Lam Liu S, Chu KH, Toh M, Xieand WD. Cucurbitacin B inhibits STAT3 and the Raf/MEK/ERK pathway in leukemia cell line K562. Cancer Lett. 2010;289:46-52.
47. Tarhan L, Kayali HA, Urek RO. In vitro antioxidant properties of *Cucurbita pepo* L. male and female flowers extracts. Plant food Hum Nutr. 2007;62:49-51.
48. Sicilia T, Niemeyer HB, Honig DM, Metzler M. Identification and stereochemical characterization of lignans in flaxseed and pumpkin seeds. J Agric Food Chem. 2003;26:1181-1188.
49. Rodriguez JB, Gros EG, Bertoni MH, Cattaneo P. The sterols of Cucurbita moschata ("calabacita") seed oil. Lipids. 1996;31:1205-1208.
50. Murkovic M, Hillebrand A, Winkler J, Leitner E, Pfannhauser W. Variability of fatty acid content in pumpkin seeds (Cucurbita pepo L.). Z. Lebensm Unters Forsch. 1996;203:216-219.
51. Matus Z, Molnar P, Szabo LG. Main carotenoids in pressed seeds (Cucurbitae semen) of oil pumpkin (Cucurbita pepo convar. pepo var. styriaca). Acta Pharm Hung. 1993;63:247-256.