Stimulatory Effects *of Euphorbia cheiradenia* on Cell Mediated Immunity and Humoral Antibody Synthesis

Zahra Amirghofran[1,2,3]*, Abbas Azadmehr[1], Masoud Bahmani[1], Katayoun Javidnia[3]

[1]Department of Immunology, [2]Autoimmune Disease Research Center, [3]Medicinal & Natural Products Chemistry Research Center, Shiraz University of Medical Sciences, Shiraz, Iran

Abstract

Background: Studies have demonstrated that plant extracts possess various biological characteristics including immunomodulatory activity. **Objective:** *Euphorbia cheiradenia Boiss* et Hohen (Euphorbiaceae), a medicinal herb native to Iran was investigated for its immunomodulatory effects. **Methods:** The methanolic extract of the plant was prepared and added to mitogen-induced human peripheral blood lymphocyte cultures at different concentrations. Effect of *E. cheiradenia* on in vivo cell-mediated immunity was measured by delayed type hypersensitivity (DTH) reaction. The effect of the extract on humoral antibody synthesis was also measured in immunized mice treated with different extract concentrations. **Results:** The stimulation index (SI) for cultures treated with 0.01 to 200 μg/ml of the extract ranged from 1.3±0.04 to 2.4±0.06, ($p<0.01$) showing a significant stimulatory effect of *E. cheiradenia* on the lymphocytes. IL-2 secreted from lymphocytes treated with the extract was significantly higher than that from the non-treated cells ($p<0.001$). Cell cycle analysis on mitogen-treated lymphocytes exposed to different concentrations of the extract showed an increase in the percentage of cells at G2M phase with increases in the concentration of the extract, but the results was not significant. In DTH skin test, the mean footpad thickness of all mice groups treated with 1, 50 and 100 mg/kg of the extract at 24 hours after immunization with antigen was 3.5±0.6 mm compared to 2.5±0.5 mm for the non-treated group ($p=0.005$). Moreover, an increase in production of specific antibody in mice immunized with different extract concentrations was also demonstrated. **Conclusion:** Results of this study showed the ability of the *E. cheiradenia* extract to induce proliferation of lymphocytes and enhance both cellular and humoral specific immune responses.

Keywords: *Euphorbia cheiradenia*, Euphorbiaceae, Delayed type hypersensitivity, Humoral immune response

*Corresponding author: Dr. Zahra Amirghofran, Department of Immunology, Medical School, Shiraz University of Medical Sciences, Shiraz, Iran. Tel/ Fax: (+) 98 711 2351575, e-mail: amirghz@sums.ac.ir

INTRODUCTION

The genus *Euphorbia* (Euphorbiaceae family) comprises more than 1000 species with a broad distribution in both temperate and tropical regions (1). In Iran over 70 species have been reported, 17 of which are endemic (2). Some of the plants of this family are used in folk medicine to cure skin diseases, gonorrhea, migraines, intestinal parasites, and warts (3). *Euphorbia* has been the source of a large number of biological active compounds. Tannins, flavonoids, unsaturated sterols/triterpenes, carbohydrates, lactones and proteins/amino acids were reported as major active constituents of some *Euphorbia* species such as *E. paralias* (4).

A variety of diterpenoids with antibacterial, anticancer, prostaglandin E2-inhibitory, antifeedant, anti-HIV, and analgesic activity have also been isolated from different Euphorbia species. They include jatrophane, ingenol, and myrsinane diterpenoids (5-7). These diterpenoids have been reported to act in diverse ways; they have been found to be skin-irritants, tumor-promoters, anti-cancer agents, and recently, agents for overcoming multidrug-resistance (6, 8-9). In addition to anti-tumor activity, several species of this genus have been investigated for their immunomodulatory activity (8) and some immunotoxic, immunosuppressive and immunostimulatory effects have been reported. These broad range and diversity of biological activities in the Euphorbia genus, perhaps due to the presence of various components with different modes of action in the plants, encouraged us to investigate the possible immunomodulatory activity of *E. cheiradenia Boiss* et Hohen, a native plant of this genus to Iran. *E. cheiradenia* is used for the treatment of infectious diseases and for scorpion bites in Iranian traditional medicine. This plant grows wild on the slopes of Dena Mountain (near Yasouj, Kohkiloyeh and Boirahmad Province, Iran). In a study by Abbas et al, (10) three new diterpene esters, cheiradone, cheiradone A, and cheiradone B, have been isolated from the aerial parts of *E. cheiradenia.* In the present study, in order to explore the possible immunomodulatory activities of *E. cheiradenia,* the effect of its extract on in vitro human lymphocyte proliferation and in vivo cell-mediated and humoral immune response in mice was investigated.

MATERIALS AND METHODS

Materials. [^{3}H]-Thymidine was purchased from Amersham, Germany. Ficoll-hypaque and phytohemagglutinin (PHA**)** were obtained from Biotest, Germany. Propidium iodide, proteinase K and 3-[4, 5-dimethyl-2-thiazolyl]-2, 5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Sigma, St. Louis, Missouri, USA. The following items were also used. Tissue culture plates from Nunclone, Denmark. RNase A from Boehringer Mannheim, Germany. Interleukin 2 (IL-2) commercial enzyme immunoassay (ELISA) kit from Roche, Germany. RPMI 1640 and fetal calf serum (FCS) from Gibco-BRL, Germany. Penicillin and streptomycin as antibiotics from Iran Daru Co, Tehran, Iran.

Animals. Male BALB/c mice (20–22 g) were obtained from Pasteur Institute, Tehran, Iran. Animal experiments were conducted in accordance with current ethical regulations on animal research. Mice were randomized and housed five to a cage in plastic cages. The animals were maintained under standard laboratory conditions of a temperature of 25±2°C and a photoperiod of 12 h and received standard mouse chow and water ad libitum.

Plant Material and Preparation of the Extract. Aerial parts of *E. cheiradenia* were collected from Dena Mountain, at the time of flowering in May. The plant was identified by Mr. Azizolah Jafari from the Department of Botany, Research Center for Natural Resources and Animal Husbandry, Yasuj, Iran. The specimen (herbarium No.3212) was deposited in the herbarium of the above mentioned center.

The aerial parts were air dried in a shaded area, powdered and defatted with petroleum ether for 4 hours. A methanol extract was obtained by maceration of the plant in 3x1500 ml methanol at room temperature for 48 hours. The methanol extract was filtered and concentrated under reduced pressure and yielded 5.3% (w/w). Dried extract were later dissolved in DMSO followed by RPMI culture medium to obtain 20 mg/ml and mixed at 37ºC for 20 min. This solution was passed through 0.22 µm filters for sterilization and then diluted with the medium and prepared at different concentrations.

Lymphocyte Activation Assay. Peripheral blood lymphocytes (PBLs) from at least three healthy adult individuals, who provided informed consent, were separated using Ficoll-hypaque gradient centrifugation.

100 µl PBL (10^6 cells/ml) was added to each of the wells of a 96-well flat-bottomed tissue culture plate. Then the same volume of the media containing two-fold concentrations of 0.1 to 200 µg/ml of the extract was added. Triplicate wells containing the extract-untreated cells and 20 µg/ml of PHA as the mitogen, and those containing the extract-untreated cells and the solvent in a final concentration equal to the test wells, were considered as controls.

The effect of the extract on lymphocyte proliferation in the presence of a sub-optimal dose of PHA was also assessed by adding PHA (10 µg/ml) into the wells. After 3 days of incubation at 37ºC in an atmosphere of humidified air containing 5% CO_2, cultures were pulsed with 0.5 µCi/well of [^{3}H]-Thymidine prior to harvesting. Radioactivity was measured using a beta-counter (Pharmacia, Sweden). The mean CPM value of each of the three independent experiments performed in triplicate was determined and then the stimulation index (SI) calculated.

Assay of IL-2 Secretion. PBL from normal donors, suspended at 10^5 cells per well, were cultured with PHA alone or in combination with varying concentrations of the extract for 3 days as mentioned above. Culture supernatants were then collected and assayed for IL-2 concentration by ELISA method. The IL-2 assay had a lower sensitivity limit of 5 pg/ml.

Analysis of Cell Cycle and DNA Synthesis. $2x10^6$ cells/ml were seeded into a 24 well plate and treated with PHA (10 µg/ml) and the appropriate concentrations of the extract for 72 hours. Cells were then collected by centrifugation at 1000 rpm for 15 min and thoroughly rinsed with PBS. The pellets were fixed overnight in ice cold 70% ethanol at -20 °C. After being washed twice with PBS, the cells were resuspended in phosphate buffered citrate and then centrifuged (1000 rpm for 15 min). After washing, the cells were resuspended in 1 ml of PBS containing 10 mg/ml RNase and 1mg/ml propidium iodide and incubated for 1 hour at 37ºC in the dark. The DNA content of the cells was then analyzed with a FACScalibur flow cytometer (Becton-Dickinson, USA). The distribution of DNA content was expressed as G1, S, G2 and M.

Delayed Type Hypersensitivity (DTH) Skin Test. Sheep red blood cells (SRBC), collected in Alsever's solution, were washed three times in large volumes of pyrogen free 0.9% normal saline and adjusted to a concentration of 5×10^9 cells/ml for immunization and challenge. DTH test was performed as described previously with some modifications (11). Mice were divided into four groups, each group containing 5 mice. Different

concentrations of the extract (1, 50 and 100 mg/kg) were injected (ip) to three groups at days -2, -1, 0, 1 and 2. The solvent was injected to group 4 on the same days as in the control. The animals were immunized subcutaneously by injection of 10^8 SRBC/100 µl on day 0. The mice were then challenged by injection of SRBC suspension in the right hind foot pad at day 7. The thickness of the right hind foot pad was measured using vernier caliper (Mitutoyo, Japan) after 24 hours.

Humoral Antibody Synthesis. The method used was that of Marake et al. with some modifications (12). Five groups of 5 mice were immunized intraperitoneally (ip) with $5x10^9$ SRBC on days 0 and +7. In three groups, different doses of extract (1, 50 and 100 mg/kg) were administrated on days -2, -1, 1 and 2 of immunization (ip). The mice in the fourth group were injected with levamisol on the same days as an immunopotentiating agent (2 mg/kg, ip). The fifth group was considered as non-treated control and were only injected with equal amounts of the solvent. Blood samples were obtained from each mouse on day +7 for primary and on day +14 for secondary responses. Antibody titer was determined by hemagglutination assay. 25µl of 0.1% SRBC suspension was added to 25 µl of two-fold diluted serum samples in V-shaped microtitration plates. After 1 hour of incubation, the highest dilution of serum samples producing hemagglutination was considered as antibody titer. To compare results, mean Log2 of the titers was determined.

Statistical Analysis. Data were presented as mean±SD and the differences between groups were assessed using SPSS software (SPSS Inc, Chicago, IL, USA) and the Mann-Whitney U test. P values less than 0.05 were considered significant.

RESULTS

Effect of *E. cheiradenia* on Lymphocyte Activation. The immunomodulatory effects of the methanolic extract of *E. cheiradenia* were tested for its mitogenic activity in human lymphocytes. Comparison of the lymphocyte proliferation in extract-treated and non-treated cultures revealed a mild mitogenic activity in extract concentrations above 10µg/ml (Figure 1A). Effect of the extract on lymphocyte proliferation in the presence of a suboptimal dose of PHA was determined. A marked increase in mitogen-induced proliferation was observed at all concentrations of the extract. The SI in cultures treated with 0.01 to 200 µg/ml of the extract was from 1.3±0.04 to 2.4±0.06, $p<0.01$ showing a significant dose dependent stimulatory effect of E. cheiradenia on the PHA-treated cells (Figure 1B).

Effect of E. cheiradenia on the Production of IL-2. The mean IL-2 concentration at 1 to 100 µg/ml of the extract in lymphocyte proliferation assay was from 325±13 to 2256±60 pg/ml, demonstrating a strong increase compared to that of the non-treated culture (32±2.9 pg/ml) ($p< 0.001$).The concentration of IL-2 increased dose dependently with an increase in the concentration of the extract (Figure 2).

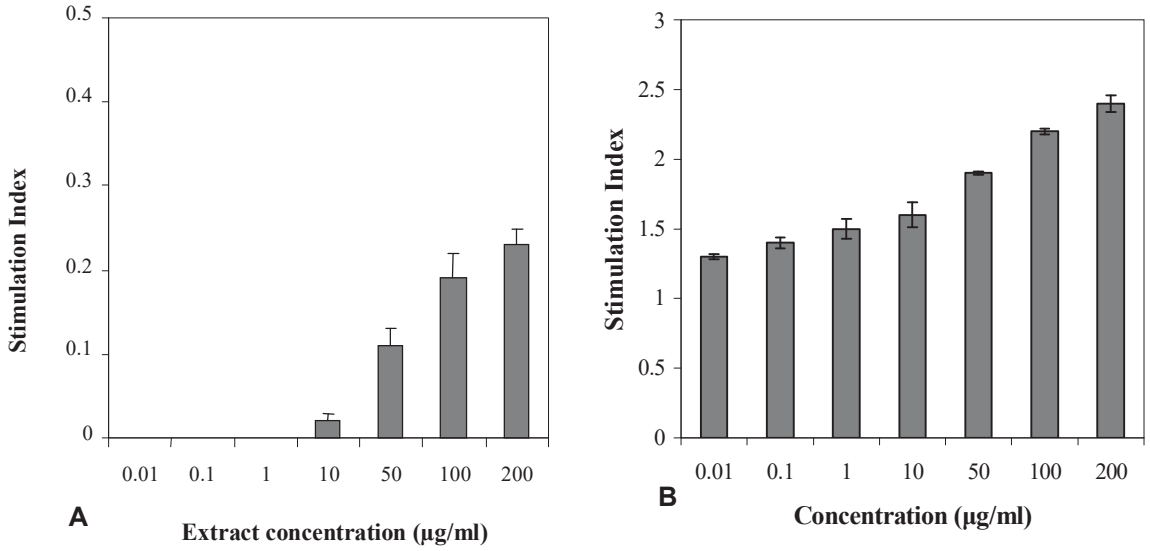

Figure 1. The Effect of different concentrations of *E. cheiradenia* on the human peripheral blood lymphocytes. A. Direct effect of the extract on the cells, B. Effect of the extract on cells treated with a suboptimal dose of phytohemagglutinin as mitogen. Values represent the mean of three independent experiments. A significant dose dependent stimulatory effect of the extract on the cells is observed ($p<0.01$).

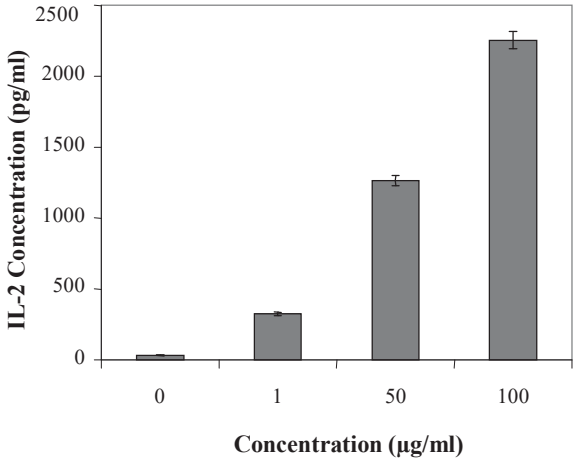

Figure 2. Effect of different concentrations of the *E. cheiradenia* on the secretion of IL-2 by lymphocytes treated with phytohemagglutinin. A marked increase in IL-2 production in the extract treated cultures compared to the non-treated culture is observed ($p< 0.001$).

Effect of *E. cheiradenia* on Cell Cycle. Cell cycle analysis on PHA-treated lymphocytes exposed to 1, 100 and 200 µg/ml of the extract was performed by flow cytometer. The corresponding percentages of cells at G2M phase were 10±0.21, 13.2±1.6 and 14.01±0.85, respectively. The percentage for PHA-treated cells was 14.7± 0.05 (Table 1) indicating that with increasing concentrations of the extract, the number of proliferative cells was weakly and non-significantly augmented.

Effect of *E. cheiradenia* on Delayed Hypersensitivity Reaction. Footpad thickness was measured after immunization of extract-treated mice with SRBC. The mean footpad thickness of all mice groups treated with 1, 50 and 100 mg/kg of the extract at 24 hours after immunization was 3.5±0.6 mm compared to 2.5±0.5 mm for the non-treated group (p=0.005). The mean footpad thicknesses of the mice groups treated with 1, 50 and 100 mg/kg of the extract were 2.8±0.5, 3.7±0.4 and 4.1±0.1 mm, respectively, indicating that the exposure to higher concentrations of the extract increased hypersensitivity reaction (Figure 3).

Table 1. Flowcytometric analysis of the cell cycle of mitogen-treated lymphocytes

Extract concentration (µg/ml)	Percentage of cells at G2M	
1	10±0.21	
100	13.2±1.6	p= 0.1
200	14.01±0.85	
Negative control*	14.7± 0.05	

*Cells that were stimulated with a suboptimal dose of phytohemagglutinin without adding the extract were considered as the negative control

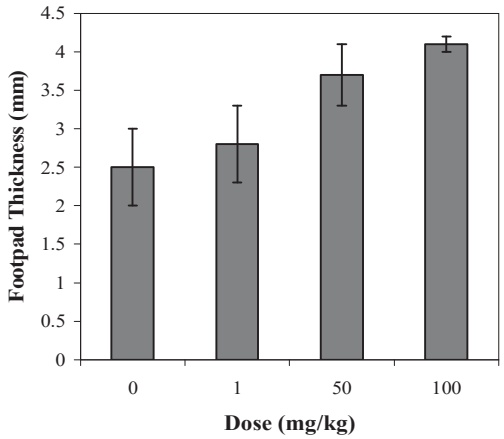

Figure 3. Effects of *E. cheiradenia* extract on delayed type hypersensitivity (DTH) reaction in mice immunized with sheep red blood cells. Values represent mean±SD of footpad thicknesses measured 24 hours after immunization. The mean footpad thickness of mice groups treated with the extract was more than that of untreated group (p=0.005).

Effect of *E. cheiradenia* on Humoral Antibody Response. Figure 4 shows the effect of the extract on humoral antibody synthesis. Log2 of the mean antibody titer for 100 mg/kg of the extract was 10.5±0.6 compared to 8.3±0.5 for the non-treated mice after the primary response, and 11.6±0.5 versus 9±0.4 at the secondary response (p<0.007). The stimulatory effect of the extract on antibody production at this concentration was comparable to the effect of levamisol as a positive control at both primary (10.5±0.6) and secondary (11.2±0.4) responses.

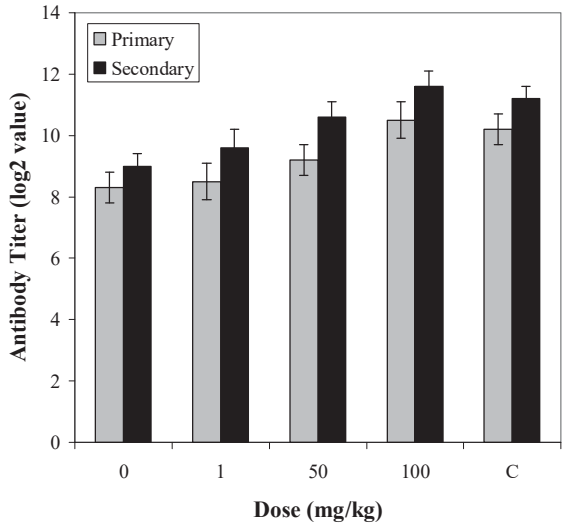

Figure 4. Effects of different doses of *E. cheiradenia* extract on antibody synthesis in mice. Values represent mean±SD of Log2 of antibody titer at primary and secondary response to sheep red blood cells. C: Levamisol treated mice. A significant increase in antibody titer in both primary and secondary immune responses is observed ($p<0.007$).

DISCUSSION

In previous studies on *Euphorbia* species, some immunomodulatory effects have been reported. Xu et al (13) have shown the immunomodulatory activity of the ethanolic extract of *E. ebracteolata*. In a study by Valente et al (14), three new diterpene polyesters were isolated and characterized from the whole dried plant of *E. pubescens* and their in vitro effect on the proliferation of human peripheral blood lymphocytes were evaluated. These diterpene compounds exhibited no effect on the mitogenic response of human lymphocytes to PHA. Mitogenic activity on the murine spleen lymphocytes and/or human T-lymphocytes in three other studies on the latex of *E. neriifolia* (15), *E. marginata* (16) and *E. characias L.* (17) have shown to be due to lectins present in the plant extracts. In contrary to these reports, immunosuppressive effects of an ethyl acetate fraction from *E. Royleana* has also been reported (18) which may indicate the presence of different compounds in *Euphorbia* species with a variety of modes of action on the immune system. In the present study we demonstrated that the methanolic extract of *E. cheiradenia* significantly stimulated the proliferation of human lymphocytes in vitro, triggered by a suboptimal concentration of a mitogen. This is the first report showing the immunomodulatory properties of *E. cheiradenia* extract on peripheral blood lymphocytes. We also showed that *E. cheiradenia* weakly stimulated proliferation of lymphocytes in the absence of PHA.

The results of our study showing a significant increase in the production of IL-2 in cultures treated with the extract, demonstrates the ability of *E. cheiradenia* to enhance activation of T cells and release of IL-2 cytokine.

In analysis of the effect of *E. cheiradenia* on the lymphocyte cell cycle, we found a mild increase in the percentage of cells in G2M phase. This increase did not exceed the value obtained for the control, perhaps due to the presence of other compounds in the extract with different modes of action.

The present study showed that cell-mediated immunity, as evaluated by DTH to SRBC, can be potentiated by the injection of the extract. The immunopotentiating action of the extract on the DTH could be due to its effect on the number of specifically committed lymphocytes and the availability of blood monocytes that could be recruited locally at the site of interaction (19). The mechanism (s) whereby *E. cheiradenia* can enhance the activity of these components is unclear, but it may be due to the increased secretion of cytokines or the increased chemotactic response of the immune cells.

The extract also showed stimulatory effects on the humoral immune response. Injection of *E. cheiradenia* into immunized mice showed a significant increase in anti-SRBC hemagglutinine titer in both primary and secondary immune responses. The antibody production, directed at T dependent antigen SRBC, requires the cooperation of T and B lymphocytes (20). The stimulation of humoral response to SRBC could be due to different cytokines produced at the site of T cell and B cell stimulation (21). Taken together the result of this study indicates the immunostimulatory effects of *E. cheiradenia* that may justify the traditional use of this plant in infectious diseases, however, the possible direct effect on the microbial agents should also be considered. The direct antimicrobial effects of a few species of Euphorbia have also been reported (22-24), but there is no study on the antimicrobial property of *E. cheiradenia*.

In our previous study, *E. cheiradenia* showed some cytotoxic activity on different tumor cells which was in line with the anti cancer activity reported for some other species of Euphorbia genus (24). Several medicinal herbs have been reported to possess immunostimulatory effects with antitumor activity. These dual effects have mostly been attributed to different compounds particularly certain polysaccharides present in plants that can directly inhibit the proliferation of mammalian tumor cells and stimulate the immune system upon acting as a mitogen (25,26).

In conclusion, the enhancement of antibody synthesis together with DTH response indicates that the extract contains a bioreactive components that stimulate both humoral and cellular immune responses.

ACKNOWLEDGEMENT

This work was supported by grant number 3311 from Shiraz University of Medical Sciences.

REFERENCES

1 Ravikanth V, Lakshmi Niranjan Reddy V, Vijender Reddy A, Ravinder K, Prabhakar Rao T, Siva Ram T et al. Three new ingol diterpenes from Euphorbia nivulia: evaluation of cytotoxic activity. Biol Pharm Bull (Tokyo). 2003; 51: 431-4.
2 Mozaffarian V. A Dictionary of Iranian Plant Names. Farhang Mo'aser, Tehran, 1996; p. 219.
3 Singla AK, Pathak K. Phytoconstituents of Euphorbia species. Fitoterapia. 1990; 61: 483-516.
4 Atta AH, Mouneir SM. Evaluation of some medicinal plant extracts for antidiarrhoeal activity. Phytother Res. 2005; 19: 481-5.
5 Abdelgaleil SAM, Kassem SMI, Doe M, Baba M, Nakatani M. Diterpenoids from Euphorbia paralias. Phytochem. 2001; 58: 1135-9.
6 Hohmann J, Re´dei D, Forgo P, Molna´r J, Dombi G, Zorig T. Jatrophane diterpenoids from Euphorbia mongolica as modulators of the multidrug resistance of L5128 mouse lymphoma cells. J Nat Prod. 2003; 6 : 976-9.
7 Ravikanth V, Reddy VLN, Rao TP, Diwan PV, Ramakrishna S, Venkateswarlu Y. Macrocyclic diterpenes from Euphorbia nivaline. Phytochem. 2002; 59: 331-5.
8 Valente C, Pedro M, Duarte A, Nascimento MS, Abreu PM, Ferreira MJ. Bioactive diterpenoids, a new jatrophane and two ent-abietanes, and other constituents from Euphorbia pubescens. J Nat Prod. 2004; 67: 902-4
9 Corea G, Fattorusso C, Fattorusso E, Lanzotti V. Amygdaloidins A-L, twelve new 13 a-OH jatrophane diterpenes from Euphorbia amygdaloides L. Tetrahedron. 2005; 61: 4485-94.

10 Abbas M, Jassbi AR, Zahid M, Ali Z, Alam N, Akhtar F et al. Three new diterpenoids from Euphorbia cheiradenia. Helvetica Chimica Acta. 2000; 83: 2751–5.
11 Sharma ML, Rao CS, Duda PL. Immunostimulatory activity of Picrorhiza kurroa leaf extract. J Ethnopharmacol 1994; 41:185-92.
12 Makare N, Bodhankar S, Rangari V. Immunomodulatory activity of alcoholic extract of Mangifera indica L. in mice. J Ethnopharmacol. 2001; 78:133-7.
13 Xu ZH, Qin GW, Xu RS. An isopimarane diterpene from Euphorbia ebracteolata Hayata. J Asian Nat Prod Res 2000; 2: 257-261.
14 Valente C, Ferreira MJ, Abreu PM, Pedro M, Cerqueira F, Nascimento MS. Three new jatrophane-type diterpenes from Euphorbia pubescens. Planta Med. 2003; 69: 361-6.
15 Seshagirirao K, Prasad MN. Purification and partial characterization of a lectin from Euphorbia neriifolia Latex. Biochem Mol Biol Int. 1995; 35: 1199-204.
16 Stirpe F, Licastro F, Morini MC, Parente A, Savino G, Abbondanza A et al. Purification and partial characterization of a mitogenic lectin from the latex of Euphorbia marginata. Biochim Biophys Acta. 1993; 1158: 33-9.
17 Barbieri L, Falasca A, Franceschi C, Licastro F, Rossi CA, Stirpe F. Purification and properties of two lectins from the latex of the euphorbiaceous plants Hura crepitans L. (sand-box tree) and Euphorbia characias L. (Mediterranean spurge). Biochem J. 1983; 215: 433-9.
18 Bani S, Kaul A, Khan B, Ahmad SF, Suri KA, Satti NK et al. Immunosuppressive properties of an ethyl acetate fraction from Euphorbia royleana. J Ethnopharmacol. 2005; 3: 185-92.
19 Fuster MJ, Lagrange PH. Immunomodulation with P40, an insoluble delipidated fraction of Corynebacterium granulosum. Immune response to sheep red blood cells. Ann Immunol (Paris). 1982; 133: 253-67.
20 Eldridge JH, Kimura S, Morisaki I, Michalek SM, Hamaoka T, Hamada S et al. Immunoregulation in the rat: cellular and molecular requirements for B cell responses to types 1, 2 and T-dependent antigens. J Immunol. 1985; 134: 2236-46.
21 Luzzati AL, Giordani L, Giacomini E. Interleukin-12 up-regulates the induction of an antigen-specific antibody response in cultures of human lymphocytes. Eur J Immunol. 1997; 27: 2696-701.
22 Sudhakar M, Rao ChV, Rao PM, Raju DB, Venkateswarlu Y. Antimicrobial activity of Caesalpinia pulcherrima, Euphorbia hirta and Asystasia gangeticum. Fitoterapia. 2006; 77: 378-80.
23 Natarajan D, Britto SJ, Srinivasan K, Nagamurugan N, Mohanasundari C, Perumal G. Anti-bacterial activity of Euphorbia fusiformis--a rare medicinal herb. J Ethnopharmacol. 2005; 31: 123-6.
24 Amirghofran Z, Bahmani M, Azadmehr A, Javidnia K. Induction of apoptosis in leukemia cell lines by Linum persicum and Euphorbia cheiradenia. J Cancer Res Clin Oncol. 2006; 132: 427-32.
25 Gamal-Eldeen AM, Djemgou PC, Tchuendem M, Ngadjui BT, Tane P, Toshifumi H. Anti-cancer and immunostimulatory activity of chromones and other constituents from Cassia petersiana. Z Naturforsch [C]. 2007; 62: 331-8.
26 Brown AC, Reitzenstein JE, Liu J, Jadus MR. The anti-cancer effects of poi (Colocasia esculenta) on colonic adenocarcinoma cells In vitro. Phytother Res. 2005; 19: 767-71.