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Screening of fourteen Moroccan medicinal plants for immunomodulating activities.

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Abstract

The aim of this study is to determine the immunomodulating ability of total extract of fourteen plants used in traditional Moroccan medicine, *Citrullus colocynthis* L., *Elletaria cardamomum* L., *Piper cubeba* L., *Ammi Visnaga* L., *Urtica dioïca* L., *Juncus acutus* L; *Capparis spinosa* L., *Aristolochia longa* L., *Marrubium vulgare* L., *Delphinium staphysagria* L., *Sinapis nigra* L., *Tetraclinis articulata* L., *Lepidium sativum* L. and *Datura stramonium* L. The immunomodulatory activity was tested using primary culture of rabbit splenocytes. Cell proliferation in presence or absence of plant extracts and with or without a mitogen (Concanavalin-A, Con-A) was assayed by MTT assay. We observed firstly that (i) the addition of plant extract to splenocytes didn't show significant cytotoxicity; (ii) except for *Citrullus colocynthis* L., *Elletaria cardamom* L., *and Piper cubeba* L., other plant extracts stimulate splenocyte proliferation with high effect for *Ammi Visnaga* L., (iii) *Citrullus colocynthis* impaired partially the mitogenic action of Con-A. In the second time, we investigate the hemaglutination properties of plant extracts. Results obtained showed that the plant extracts which stimulate splenocyte proliferation bind to blood cells inducing hemagglutination except for *Ammi visnaga* L., and *Urtica dioïca* L. This study revealed interesting immunomodulating actions of plants which could explain their traditional use.

Key words: *immunomodulatory activity, mitogenic, hemagglutination, Citrullus colocynthis* L.

Introduction

For long time, the natural products have been used as the main source of therapies, about 25% of the drugs prescribed world wide came from plants and it is estimated that 60% of anti-tumour and anti-infectious drugs already on the market or under clinical trial are of natural origin (Rates, 2001).

Many medicinal plants have been used for treatment of several diseases by widely Moroccan population; many studies have show that therapeutic activity of various extracts of plants may be mediated by interaction with the host's immune system (Wagner & Proksch 1984, Franz 1989, Wagner 1990).

In traditional Moroccan medicine, plants studied were used to treat several disorders as asthma, allergy, pulmonary infection, rheumatic pains (Bellakdar 1997, Hmamouchi 1999). Scientific reports have showed several actions for plants selected but few of them reported indication of immunomodulating activity as for *Citrullus colocynthis* L.,(Benjeddou *et al*., 2003), *Datura stramonium* L., (Katsuko *et al*. 1987, Mc Currach & Kilpatrick 1988). *Urtica dioïca* L., *Ammi visnaga* L., (Galleli & Truffa, 1993).

Controlled proliferation is a universal method of screening plants. Used in this study, we have undertaken to examine the effect of the aqueous extracts of plants on cultured rabbit splenocytes, part of the immune system, in order to clarify their immunomodulating activity.

In this paper, fourteen plants has been tested, results indicated that eight plants extracts stimulated splenocyte proliferation when *Citrullus colocynthis* L., inhibited this proliferation suggesting an immunosuppressive action against immunity cells.

Experimental

Material

Except where otherwise specified, the compounds were purchased from Sigma (St Louis, MO, USA). Con-A was dissolved in PBS (Phosphate buffered saline) (1 mg/ml) and used at a final concentration of 7µg/ml. MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide) was dissolved in PBS at 5 mg/ml.

Plant material and extraction

All plants were collected from Fès Boulmane region except for *Piper cubeba* L., which is imported by herborists. The plants studied are usually used by Moroccan people as traditional remedies. The parts of plants used were according to their medicinal traditional use by Moroccans.

First, the parts of plant were twice washed by distilled water, air dried, and ground to fine powder. Then 10 g was dissolved in 100 ml of PBS and stirring during 2 hours. The suspension was centrifuged (15 min at 4500 rpm, 4°C) and the supernatant sterilised by filtration through 0.45 µm nitrocellulose filters. In this study, we have used 100 µg/ml of crude extract as final concentration for all plants.

The aqueous extracts obtained from different plants were tested against rabbit splenocytes cultivated in RPMI medium during 72 hours at 37°C.

Cell culture

Cell suspension used in this study was obtained from rabbits. Briefly, spleens were removed aseptically from animals and then suspension prepared by pressing the organs trough a fine wire mesh. Cell suspension was washed by centrifugation repeated in RPMI and the red blood cells lysed by 154 mM of Ammonium Chloride. The number of viable cells was determined microscopically by trypan blue exclusion test.

The culture was performed in RPMI medium (without glucose) supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 10% FCS (fetal calf serum), and two antibiotics (ampicillin 100 U/ml and streptomycin 100µg/ml).

Cell proliferation assay

Cell proliferation was measured by the MTT assay as described before (Mossman 1983). Briefly, cells were plated at 5000 cells/100µl/well in 96 well plates and incubated at 37°C in humidified chamber under an atmosphere of 95% air and 5% CO2 for 72 hours. Plant extracts and/or Con-A were added to cells before incubation period in a final volume of 10µl.

Thereafter, 10 µl of MTT solution (5 mg/ml in PBS) was added and plate incubated 3 hours at 37°C. At the end, the supernatant was removed and replaced by 100 µl of DMSO. The coloration obtained after 1 hour was measured using a wavelength at 570 nm.

Hemaglutination assay

Assay for hemagglutination activity was performed using modified method of Kabat & Mayer (1961). The reaction is realised in a final volume of 100 µl: 50 µl of 0.15 M NaCl in 0.05 M Tris/HCl buffer, pH 7.4, 25 µl of rabbit erythrocytes suspension (3 times washed) and 25 µl of the total plant extract. After gentle mixing, the aliquots were incubated for 30 min at 37°C. Assays were performed in 96 wells plate. Hemagglutination was monitored macroscopically.

Statistical analysis

In each experiment, conditions were realised at least in duplicate (n=2). General means presented were calculated from those obtained in "N" experiments. Student's *t*

test for unpaired data was used to compare general means of two groups.

For simultaneous comparisons of means from more than two groups, one-way ANOVA was used. Differences were considered statistically significant for *P* values <0.05.

Results

Plants were selected according to their popular use in Morocco. As shown by table 1, all plants are used to treat, among other diseases, some disorders of immunity system like asthma, splenic complaints and inflammation. Use of plants, by population in major cases, is to stimulate host defences. In same situations like asthma or eczema, plants were used to inhibit the action of the immunity system for example use of *Urtica dioïca* L., to treat eczema (Table 1).

Table 1. Ethno botanical data of medicinal plants used.

Family Plant name	Vernacular name	Part used	Use in Moroccan traditional medicine
Apiaceae *Ammi visnaga* L	Bachnikha	Seed	Asthma, toothpicks, fumigated, diabetes, skin disorders
Aristolochiaceae Aristolochia longa L	Baraztam	Root	dermatitis, anti-septic
Brassicaceae *Lepidium sativum* L	Habb archad	Seed	Stimulant of host defences, skin diseases
Brassicaceae *Sinapis nigra* L	Khardal	Seed	Rheumatic inflammation, bronchitis, constipation, , and stimulant
Capparidaceae *Capparis spinosa* L	kabbar	Fruit	Splenic complaint, cholagogue
Cucurbitaceae *Citrullus colocynthis* L	Handal Hdaj	Seed	rheumatic diseases, tuberculosis, and spasmolytic
Cupressaceae *Tetraclinis articulata* L	Al' Aaraâr	Seed	Asthma, digestive and hair tonic
Juncaceae *Juncus acutus* L	Assmar	Seed	Inflammation, Kidney disorders
Lamiaceae *Marrubium vulgare* L	Marriwa	Flower	Asthma, tonic, bilious, stimulation, and stomachic
Piperaceae *Piper cubeba* L	kbaba	Seed	Stimulant of host defences, Rheumatic diseases, antiseptic and anti diarrhoeic
Ranunculaceae *Delphinium staphysagria* L	Habb'arrass	Seed	Prostatic inflammation, hair tonic, and for toothache.
Solanaceae *Datura stramonium* L	Chdak-jmel	Seed	Asthma, narcotic, sedative
Urticaceae *Urtica dioïca* L	Hourrayga	Seed	Spleen disorder, eczema, skin and kidney affections
Zingiberaceae *Elletaria cardamomum* L	Qaâqolla	Seed	Inflammation, digestive stimulant and aphrodisiac

1-Cytotoxicity effect

In the first part, we tested the effect of plant extracts on cell proliferation. From the fourteen plants tested, thirteen didn't show any inhibition of cell proliferation except for *Citrullus colocynthis* L. which induces a slight not statistically significant inhibition (83.1% of response; N=5).

These thirteen plants can be divided in three groups according to their effect against cells (Figure 1). First group, without action on cell proliferation, *Elletaria cardamomum* L., (102%, N=5) and *Piper cubeba* L., (114%, N=12). Second group including *Urtica dioïca* L., and *Juncus acutus* L., induces a moderate increase in

cell proliferation, *Urtica* =136%, N=10 and *Juncus* =144.2% , N=5 as compared to control. The latest group shows a high mitogenic activity against splenocytes, this group is formed by *Capparis spinosa* L.,, *Aristolochia longa* L.,, *Marrubium vulgare* L.,, *Delphinium Staphysagria* L.,, *Ammi visnaga* L.,, *Sinapis nigra* L.,, *Tetraclinis articulata* L.,, *Lepidium sativum* L., *and Datura stramonium* L. The high mitogenic activity was recorded for *Ammi visnaga* L.,: 417.2%, N=10.

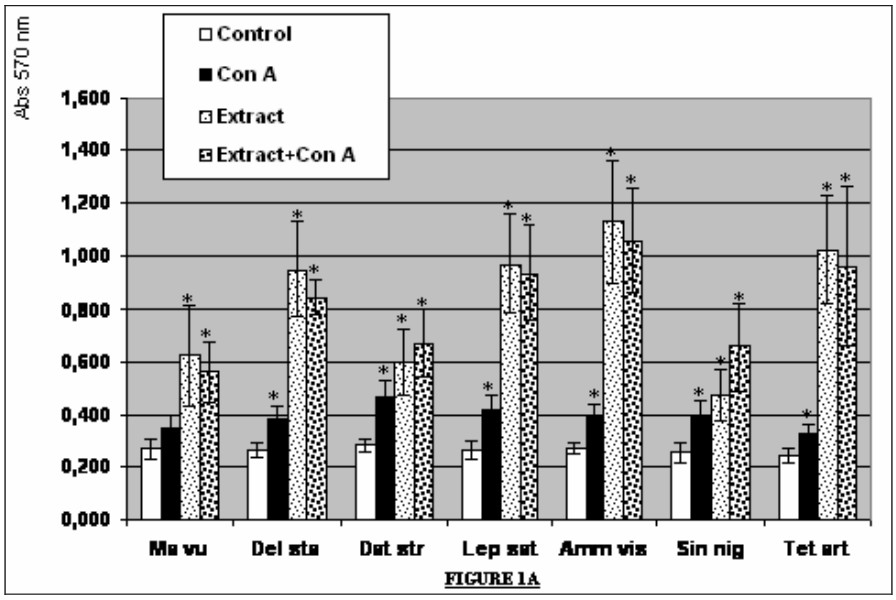

FIGURE 1A

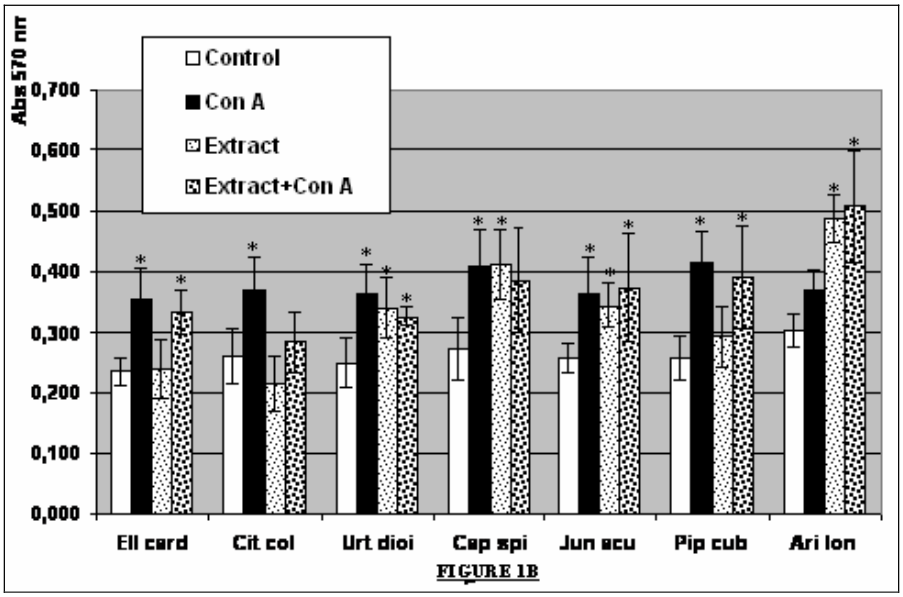

FIGURE 1B

Figure 1. Effect of plant extracts on the proliferation of rabbit splenocytes. Cell proliferation was measured by absorbance at 570nm. Plant extracts and /or Con-A were added before incubation of cells at 37°C during 72h. Results were compared to the response of cells alone (control). Results are expressed as Means ± SEM from 10 independent experiments. * : at least $p<0.05$

2-Addition of Con-A

We used Con-A to stimulate splenocyte proliferation. Then we tested plant extracts on Con-A proliferation to characterize their possible immunosuppressive effects. Plant extracts and Con-A were added to cells before incubation period.

In the experimental series, Con-A at 7 µg/ml induced an increase in cell proliferation varying from 123.6 % to 166.9% compared to control response.

When cells were treated by plant extracts and Con-A, we observed that extract of *E. cardamomum* L., *C. spinosa* L., *P. cubeba* L., *U. dioïca* L., *and J. acutus* L., didn't modify the splenocyte proliferation induced by Con-A (figure 1). In contrast, we noted an increase in cell proliferation more than Con-A alone with *A. longa* L., (136.6% of Con-A response, N=3), *D. stramonium* L., (142.6% ; N=6), *M. vulgare* L., (162.2% ; N=9), *S. nigra* L., (168.5% ; N=4), *D. staphysagria* L., (218.2% ; N=8), *L. sativum* L., (222.9% ; N=9), *A. visnaga* L., (270.4% ; N=10) and *T. articulata* L., (292.4% ; N=5).

An interesting result was observed with *C. colocynthis* L., since the response observed with the extract combined to Con-A was lower than that observed with Con-A alone. It seems that *C. colocynthis* L., extract reduced the splenocyte proliferation induced by Con-A since we obtained 76.1% (N=5) of response compared to Con-A.

3-Hemagglutination test

The hemagglutination test was performed with plant extracts using Con-A as positive control. Results obtained from 10 experiments are represented in table 2. In this table we compared agglutination test to mitogenic effect observed before in figure 1. We observed that positive agglutination was correlated to mitogenic action for *A. longa* L., *L. sativum* L., *S. nigra* L., *C. spinosa* L., *T. articulata* L., *J. acutus* L., *M. vulgare* L., and *D. stramonium* L.,. The absence of agglutination is also correlated to the absence of mitogenic effect for extracts of *E. cardamomum* L., *P. cubeba* L., and *C. colocynthis* L., (Table 2). In contrast, *D. staphysagria* L., (N=5) and *A. visnaga* L., (N=8) had a high mitogenic activity but didn't induce agglutination of blood cells in any of experiments performed.

Table 2. Hemagglutination test of plant extracts.

Plant	Hemagglutination	Mitogenic effect
Ammi visnaga L	-	+++
Aristolochia longa L	+	+
Lepidium sativum L	+	+++
Sinapis nigra L	+	+
Capparis spinosa L	+	+
Citrullus colocynthis L	-	-
Tetraclinis articulata L	+	+++
Juncus acutus L	+	+
Marrubium vulgare L	+	++
Piper cubeba L	-	-
Delphinium staphysagria L	-	+++
Datura stramonium L	+	++
Urtica dioïca L	-	+
Elletaria cardamomum L	-	-

Discussion

The aim of this study is to explore the immunomodulating activity of fourteen plants used in traditional Moroccan medication. Rabbit splenocytes had been used to assess this objective by measuring their proliferation in presence of the plant extracts.

In this study, we observed that *C. colocynthis* L., didn't alter significantly viability of splenocytes at the concentration of 100 µg/ml. Same conclusion was made before on hepatic cells where *C. colocynthis* L., extract in the range of 25–100 µg/ml didn't alter the measured viability parameters in liver slices (Barth *et al*., 2002).

In this study, in presence of *C. colocynthis* L., we noted a reduction in cell proliferation induced by Con-A. This result indicates an immunosuppressive effect for *C. colocynthis* L. Bendjeddou *et al*. (2003) have obtained equivalent indication since they observed an immunosuppression in mice by *C. colocynthis* L., after oral administration (from 25 to 100 mg/Kg/day). These conclusions can be a strong suggestion for an immunosuppressive action of *C. colocynthis* L.

Results indicated also an absence of cytotoxicity for other thirteen plants studied at the concentration of 100µg/ml. This data confirms results obtained before for *U. dioïca* L. on human cells (Konrad *et al*., 2000) for *L. sativum* L., on rats (Adam 1999) and the cytoprotective effect of *M. vulgare* L., (Martin-Nizard *et al*., 2003). In contrast, it has been described a cytotoxicity by *T. articulata* L. essential oil on human lymphocytes with an IC_{50} of 160µg/ml (Buhagiar *et al*., 1999). This concentration of essential oil, higher than that in total extract of *T. articulata* L, can explain absence of cytotoxicity observed in this work. For *J. acutus* L., it has been observed toxicity only on alga cells (DellaGreca *et al*., 2004). This study indicates absence of toxicity on animal cells at least at the concentration used.

Mitogenic activity was described for *C. spinosa,* L., *A. longa* L.*., M. vulgare* L., *D. staphysagria* L., *A. visnaga* L., *S. nigra* L., *T. articulata* L., *L. sativum* L., *U. dioïca* L., *J. acutus* L., *and D. stramonium* L. This proliferative property has been observed before for *U. dioïca* L., which contained a lectin with specific T cell mitogenic action (Galleli & Truffa, 1993); and for *D. stramonium* L., which a lectin is responsible of human lymphocyte proliferation (Katsuko *et al*. 1987, Mc Currach & Kilpatrick 1988). For other plants, we described for the first time their stimulation of splenocytes indicating an immunostimulating profile. These plants with a mitogenic activity agglutinated blood cells except for *A. visnaga* L., and *U. dioïca* L. which didn't induced hemagglutination instead of their highly mitogenic effect. It has been reported that *U. dioïca* L., contains a lectin with low agglutination activity (Galelli & Truffa, 1993). It is possible for these two plants that the concentration of agglutinin in total extract is very low since we observed agglutination with concentrated protein plant's extract (data not shown).

In conclusion, by studying rabbit splenocytes proliferation under various plant extracts, we described an immunostimulating activity for eight new plants correlated to an hemagglutination action. This funding indicated probably the presence of a lectin responsible of splenocyte stimulation. In contrast, we observed that *Citrullus colocynthis* L., has an immunosuppressive action which suggests a possible application as anti-proliferative substance.

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