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Immunostimulant effect of *Brassica rapa* and *Raphanus sativus* seeds on thymic cells and their cytotoxicity

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Abstract

Immunomodulators from natural products are widely used for the treatment of infectious diseases, allergies, and cancer in traditional Moroccan medicine. The purpose of this study was the study of two plant seeds of *Brassica rapa* (Turnip) and *Raphanus sativus* (Radish) used by Moroccans in traditional medicine to enhance immunity. We have prepared three different extracts from seeds using ethanol, Ethyl Acetate, or water. Immunomodulatory effects of these two plant seeds were tested on rabbit immunity cell proliferation (splenocytes, thymocytes, and macrophages) and their functions (IgG production, cytotoxicity, and phagocytosis). The results obtained indicated that only aqueous extract of *B. rapa* seeds revealed an immunostimulant effect on both splenocyte and thymocyte proliferation with an increase in cytotoxicity of thymocytes (MLR assay). With *R. sativus* seeds, we observed an important stimulation of thymocyte proliferation and their cytotoxicity under aqueous extract without effect on splenocyte or macrophages. We concluded that aqueous extract of both seeds (*B. rapa* and *R. sativus*) possessed immunostimulant properties leading to stimulation of cellular immunity responsible for defense against viruses.

Keywords: Brassica Rapa, cell proliferation, IgG production, Immunostimulation, phagocytosis, Raphanus Sativus, splenocytes, thymocytes

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Introduction

For centuries, Morocco has been known for its herbal medicine traditions. Moroccan culture shows many use cases of pharmacological natural products[1, 2] with plants used as immunomodulators. Immunomodulators are divided into two categories: immunostimulatory substances that enhance immune defense against infectious agents and immuno-suppressive substances that inhibit it in case of allergies and transplants.

Brassica rapa var. Rapa L. (B. rapa), known as turnip and belonging to the Brassicaceae family, is known to contain numerous nutrients including vitamins, minerals, phytochemicals, and fibers.[4] *B. rapa* also contains bioactive metabolites described as potent antimicrobial,[3] antioxidant and anticancer,[17] and immunomodulatory.[6] *Raphanus sativus (R.*

sativus), commonly known as radish and part of the same Brassicaceae family, is widely consumed as a vegetable or condiment in human diets around the world. Radish possesses different pharmacological properties as an antioxidant[29] anti-inflammatory, antitumor,[20, 7] antimicrobial, antiviral, and antimutagenic.[13] However, the immunostimulant activities of these Moroccan seeds were rarely reported.

Most research so far was carried out on other parts (leaves, roots… etc) and only a few scientific studies on the effects of the seeds of these plants on immunity were carried out.[28,30,31]

In Moroccan culture, seeds of both plants (Brassica and Raphanus) are indicated by traditional herbalists to treat infections but also included in formulas to treat cancers. In this study, our goal was to verify the traditional herbalist use of these seeds in formulas by exploring their immunostimulant impact. In particular, the immunomodulatory effects of three extracts of *B. rapa* and *R. sativus* seeds on rabbit lymphocyte cell culture were assessed.

Materials and Methods

Animal materials

We used bred Zemmouri male rabbits weighing 1.5 to 2.5 Kg for all in-vitro investigations. Rabbits were distributed in cages under a 12h light/dark cycle in a temperature-controlled room (22 to 24°C). Rabbits had free access to standard feed and water. The study protocol was reviewed and approved by the Local Ethical Committee of Sidi Mohamed Ben Abdellah University (Ref, AL13-10-2021). All studies were carried out according to guidelines of animal care as prescribed by national ethical standards.

Preparation of extracts

Seeds of *B. rapa* (turnip) and *R. sativus* (radish) were purchased from the local market and fully identified by botanists, then washed twice using distilled water and desiccated at 40 °C until stabilization of weight. Afterward, they were grounded to a fine powder for extraction. A portion (100 g) of fine powder was then defatted using petroleum-ether and the mixture was filtered, defatted powder was dried from solvent at 40 °C. Then, 100 g from defatted powder was extracted by maceration for 3 hours with 200 ml of water, ethanol (EtOH), or ethyl acetate (EtOAc). The mixture was then filtered, and the solvent evaporated from the filtrate. The dried extracts were stored at -20°C until used.

Cell culture

Cell suspensions used in this study were obtained from the rabbits sacrificed above. Rabbits were sacrificed after anesthesia with petroleum-ether. Then organs were rapidly removed. Spleen and Thymus were removed aseptically from animals and cell suspensions were prepared by pressing the organs through a fine wire mesh as described in previous studies.[14,8,9] These cells were washed by RPMI (Sigma-Aldrich, USA) and the red blood cells were lysed by 154 mM Ammonium Chloride. The number of viable cells was determined microscopically by trypan blue 0.1% exclusion test.

The culture used RPMI medium supplemented with 2 mM glutamine, 10 % of serum, antibiotics (ampicillin 100 U/mL and streptomycin 100 mg/mL), and antifungal (Fluconazole 2mg/ml).

Cell proliferation assay

Cell proliferation was measured by the MTT assay according to Mosmann et al., 1983[23] and as described previously.[8,9,14] In short, cells were plated at 150,000 cells/well in 96 well plates (Citotest Labware Manufacturing CO., LTD, Jiangsu, China), then incubated at 37 °C in a humidified chamber under an atmosphere of 95% air and 5 % CO2 for 72 hours. The extracts diluted in RPMI were added to cells at 1mg/ml and 2mg/ml before their incubation. After 72 hours of incubation, 10 µL of MTT solution (Sigma-Aldrich, USA; 5 mg/mL in PBS) was added. After three hours of incubation, dimethyl sulfoxide (DMSO) was added to all wells to dissolve the formazan formed. Finally, the optical density was measured through wavelength at 570 nm using the spectrophotometer (Bio Tek L800, Bio Tek Instruments, USA).

Isolation of macrophages and evaluation of their proliferation and activity

In summary, macrophages were separated from spleen cells by their adherence capacity to plate wells. For this, 100 µL of spleen cells suspension at 2.106 cells/mL was added in 96 well plates that were incubated at 37 °C for 2 hours for adherence of macrophages. Afterward, non-adherent cells were removed and every well washed twice with sterile RPMI.

To evaluate macrophage proliferation, adherent macrophages were incubated in RPMI with different extracts. Then proliferation was evaluated using MTT assay as described above (see cell proliferation essay).

The phagocyte test was performed as described by El Youbi et al., 2010[14] using neutral red. In every well, adherent macrophages were incubated in 100 µL of RPMI supplemented with 0.075% of neutral red. Then, 10 µL of plant extracts at 2mg/ml were added and plates were incubated for 2 hours. Finally, after removing the supernatant and washing cells, the reaction was stopped with a solution containing acetic acid (1M) /ethanol (1:1 v/v) which dissolves phagocyted neutral red. The extent of the phagocytic activity was evaluated by measuring absorbance at 540 nm which is the maximum of absorption of neutral red used in the assay.

Allogeneic mixed lymphocyte reaction (MLR)

Thymocytes were freshly isolated from the thymus as described above (see Cell culture).[8,9,14] Chicken red blood cells (CRBC) were obtained from sacrificed white chicken (*Gallus Gallus domesticus*). They were then washed twice using NaCl 0.9 % and diluted at a cell density of 106 cells/mL in NaCl 0.9 %. Thereafter, 105 CRBC was added to 106 of thymocytes in RPMI supplemented with serum. The cell mixture was then incubated in the presence or absence of extracts, for 24 h at 37°C in a humidified atmosphere. Cytotoxicity of thymocytes against CRBC was assessed by detecting CRBC lysis in the medium and measuring absorbance at 540nm, as described by El Youbi et al., 2012.[15]

Complement test

The complement test was realized by evaluation of mouse red blood cells lysis (MRBC) by the complement pathway in the presence of anti-MRBC antibodies prepared in immunized rabbits using Freund adjuvant and mouse RBC as antigens. MRBC was washed twice with NaCl 0.9 %, and 1 % MRBC cell suspension was prepared. Then, MRBC were incubated in RPMI supplemented with serum containing anti-MRBC specific antibodies for 4h in presence of 2mg/ml extracts at 37 °C. After incubation, samples were centrifuged, and absorbance of supernatants was determined at 540 nm.

Evaluation of total IgG production by ELISA assay

Isolated Splenocytes were incubated with or without 2 mg/ml extracts in RPMI for 72 H at 37°C. Then, 100 µL of supernatant of cell culture was deposited on a microtiter plate for IgG evaluation. The plate's wells were then washed twice overnight at 4 °C, with BBS (100mM boric acid, 24.3 mM borate sodium, 147.5mM NaCl, pH 8.4), containing 1 mL/L of Tween-20 and wells saturated with Tween 20 at 2.5 mg/mL for one hour at 37 °C (200 L per well). After elimination of Tween 20 and washing, the anti-IgG rabbit antibody peroxidase-labeled was added (100 µL per well) and incubated for two hours at 37 °C. The revelation of the immune complex was carried out by the addition of the chromogen, orthophenylenediamine (OPD), at a concentration of 0.5 mg/m. The reaction was stopped by adding HCl 3M and the absorbance was then measured at 490 nm.

For specific IgG assay, splenocyte culture was carried out by adding ovalbumin in RPMI with or without extracts. The assay began firstly by coating wells with 100 µL of ovalbumin and then adding 100 µL of cell culture supernatant to carry out assay as indicated for total IgG above.

Statistical analysis

Each experimental condition was realized at least in triplicate (n=3). Values presented are expressed as the means ± SEM. Statistical analyses were carried out using the ANOVA test. Differences were considered statistically significant at $p<0.05$.

Results

Effect of B. rapa and R. sativus seeds extracts on humoral immunity

Figures 1 and 2 describe the effect of *B. rapa* and *R. sativus* seed extracts obtained with ethanol (EtOH), ethyl acetate (EtOAc), and water on splenocyte proliferation. In case of *B. rapa,* we observed that EtOAc extract did not affect splenocyte proliferation. In contrast, EtOH and aqueous extracts stimulated cell proliferation with a higher effect for the aqueous extract (162 % vs control [(samples/control) *100] compared with EtOH (127 % vs control). In case of *R. sativus* extracts, splenocyte proliferation was not statistically modified but we remarked a slight inhibition of cell proliferation which did not exceed 25% [(Control-Sample)/Control)*100 of inhibition].

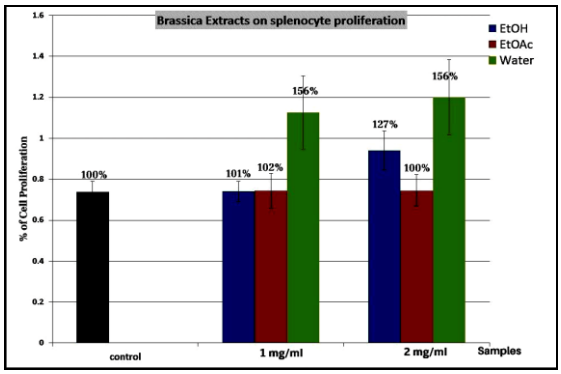

Figure 1. Effect of Brassica seeds extracts, Ethanol extract, Ethyl Acetate extract and Aqueous extract on splenocyte proliferation. EtOH: Ethanol extract; EtOAc: Ethyl Acetate extract; Water: Aqueous extract (N=7).

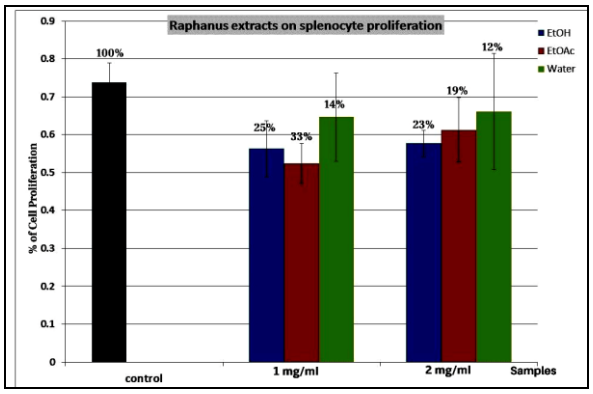

Figure 2. Effect of Raphanus seeds extracts, Ethanol extract, Ethyl Acetate extract and Aqueous extract on splenocyte proliferation. EtOH: Ethanol extract; EtOAc; Ethyl Acetate extract; Water: Aqueous extract (N=7).

The level of total and specific IgG produced by splenocytes under different extracts of *B. rapa* or *R. sativus* seeds was evaluated. Results shown in Table 1 indicated that EtOH and EtOAc extracts of *B. rapa* inhibited the production of total IgG (Table 1; EtOH: 43.1%; EtOAc: 33.1% of inhibition; $p<0.005$; N=5) and specific IgG (EtOH: 11.4%; EtOAc: 41.5% of inhibition, $p<0.005$; N=5). Whereas the aqueous extract did not significantly affect the production of IgG. With *R. sativus*, we observed a slight inhibition IgG production (EtOH: 28.9%; EtOAc: 20.2% inhibition; $p>0.05$ N=5; Table 1).

Table 1. Effect of *B.rapa* and *R. sativus* extracts (at 2 mg/ml) on total IgG production.

Plant name	Plant extract		
	EtOH (N=5)	EtOAc (N=5)	Water (N=5)
B. rapa	56.5 ± 17.4 %	66.9 ± 7.1 %	97.2 ± 8.3 %
R. sativus	71.17 ± 12.8 %	79.8 ± 16.4 %	100.2 ± 12.1 %

Results are represented as % of control obtained in same experiments

EtOH: Ethanol extract; EAC: Ethyl Acetate extract; Water: Aqueous extract

We examined the complement activity under these extracts; we observed that EtOH and EtOAc extracts of *B. rapa* or *R. sativus* did not modify the complement activity (Table 2). But aqueous extracts of both seeds seem to exert an important stimulation of complement activity ($p<0.005$; N=10; Table 2).

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Table 2. Effect of *B. rapa* and *R. sativus* extracts on Complement activity.

Plant name	Plant extract		
	EtOH (N=10)	EtOAc (N=10)	Water (N=10)
B. rapa	92.6 ± 6.8	90.1 ± 9.5	228.9 ± 40.2
R. sativus	96.9 ± 8.2	113.1 ± 16.8	185.3 ± 72.2

EtOH: Ethanol extract; EtOAc: Ethyl Acetate extract; Water: Aqueous extract.

Effect of B. rapa and R. sativus seeds extracts on cellular immunity

In this section, we evaluated the effects of the extracts on thymocyte proliferation. Figures 3 and 4, showed that for both seeds of *B. rapa* and *R. sativus*, neither EtOH nor EtOAc extracts modified the thymic cell proliferation except for EtOH extract of *B. rapa* which at high concentration stimulated proliferation by 132.1 % vs control. However, aqueous extractions of both seeds induced a higher stimulation of thymic cell proliferation, *B. rapa*: 301.8 %; *R. sativus*: 255.3% of response; $P<0.001$ N=5.

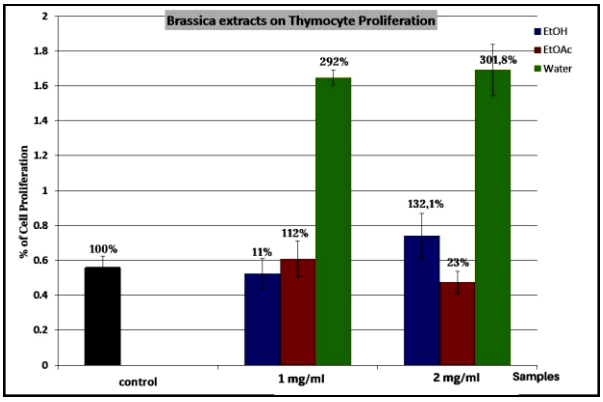

Figure 3. Effect of Brassica seeds extracts, Ethanol extract, Ethyl Acetate extract and Aqueous extract on Thymocyte proliferation. EtOH: Ethanol extract; EtOAc: Ethyl Acetate extract; Water: Aqueous extract (N=7).

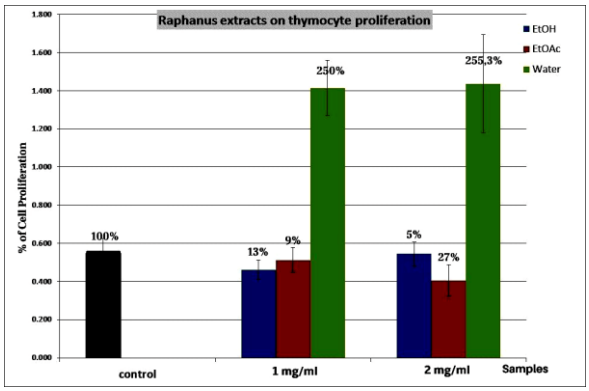

Figure 4. Effect of Raphanus seeds extracts, Ethanol extract, Ethyl Acetate extract and Aqueous extract on Thymocyte proliferation. EtOH: Ethanol extract; EtOAc: Ethyl Acetate extract; Water: Aqueous extract (N=7).

By evaluating the cytotoxicity of thymic cells against foreign cells, we remarked that like thymic proliferation results, EtOH and EtOAc extracts of Raphanus did not change MLR (Table 3). The *B. rapa*, EtOAc extract did not modify MLR, but EtOH indicated a stimulation of this MLR activity. For aqueous extracts, we found that both extracts of *B. rapa* and *R. sativus* stimulated MLR activity ($p<0.005$; N=7; Table 3).

Table 3. Effect of *B. rapa* and *R. sativus* extracts (at 2 mg/ml) on MLR activity.

Plant name	Plant Extract		
	EtOH (N=7)	EtOAc (N=7)	Water (N=7)
B. rapa	143.4 ± 44.5	91.1 ± 13.5	171.5 ± 24.5
R. sativus	102 ± 22	91.2 ± 14.8	173.7 ± 21.9

Results are represented as % of control obtained in same experiments.
EtOH: Ethanol extract; EtOAc: Ethyl Acetate extract; Water: Aqueous extract.

Effect of B. rapa and R. sativus on Macrophage proliferation and Phagocytosis Activity

Table 4 shows the effect of *B. rapa* and *R. sativus* extracts on macrophage proliferation. We observed that aqueous extracts of both seeds did not significantly change the macrophage proliferation, but other extracts induced an inhibition that was more pronounced with EtOAc extract of *B. rapa* with 44.6% inhibition.

For phagocytosis activity, all extracts of both seeds did not statistically change phagocytosis except for aqueous extracts which induced an inhibition that reached 48% of inhibition for *B. rapa* and 24% for *R. sativus*.

Table 4. Effect of *B. rapa* and *R. sativus* extracts on Macrophage proliferation and Phagocytosis activity.

	Plant name	Plant Extract			
		Control (N=5)	EtOH (N=5)	EtOAc (N=5)	Water (N=5)
Macrophage proliferation	*B. rapa*	1.12 ± 0.07	0.80 ± 0.15	0.62 ± 0.1	1.20 ± 0.05
	R. sativus		0.75 ± 0.03	1.06 ± 0.16	1.25 ± 0.06
Phagocytosis	*B. rapa*	0.25 ± 0.02	0.21 ± 0.03	0.21 ± 0.03	0.13 ± 0.01
	R. sativus		0.21 ± 0.06	0.19 ± 0.01	0.19 ± 0.04

EtOH: Ethanol extract; EtOAc: Ethyl Acetate extract; Water: Aqueous extract .

Discussion

In the present study, we investigated the Immunomodulators from natural products are commonly used for the treatment of infectious diseases, allergies, and cancer. For this reason, this study aimed at the assessment of in-vitro and ex-vivo of the immunomodulatory effect of seeds of *B. rapa* and *R. sativus* traditionally used to treat diseases in Moroccan traditional medicine. Extraction of *B. rapa* and *R. sativus* seeds was conducted by three different solvents (water, ethanol, and Ethyl acetate).

For *B. rapa* seeds, we remarked by evaluating the effect of the extracts on humoral immunity that aqueous extract induced a mitogenic effect on splenocyte proliferation. This mitogenic effect was also observed on thymic cells where the aqueous extract highly stimulated their proliferation leading to the presence of a mitogenic compound in this extract. This stimulation of immunity cell proliferation was probably mediated by a lectin (glycoprotein) which is present in vegetal seeds and particularly in *B. rapa* as indicated by Raval et al., 2004[24] and for Lepidium sativum (Brassicaceae specie).[10]

Similar immunostimulation of cells was also reported by Wang et al., 2021[12] by measuring the weight of the spleen and thymus under polysaccharides from *B. rapa*. Another study[32]

with petroleum extract from *B. rapa* showed an immunostimulatory effect by increasing the proportions of B cells, CD4+ T cells, CD8+ T cells, and activated CD8+ T cells in the spleens of tumor-bearing mice.

On the thymic cells, we observed an increase in the function of lymphocyte cytotoxicity as measured by MLR. This finding is in accordance with data obtained by Yamamoto et al., 2018[19] who observed an increase by *B. rapa* extract of cytotoxicity of NK lymphocytes.

In contrast, Ethanolic and Et-Acetate extracts inhibited the production of IgG by the splenocytes, indicating the presence of immunosuppressive metabolites in these extracts. This immunosuppression of *B. rapa* was suggested before by Jafarian-Dehkordi et al., 2013,[16] where for other *Brassicaceae* species, an increase in antibody production was observed.[26]

On isolated macrophages, ethanolic and ethyl acetate extracts of *B. rapa* seeds both inhibited macrophage proliferation. In contrast, other authors observed stimulation of macrophages under *Brassica* roots extract[12, 28] or by aqueous extract of total plant from *Brassica campestris* with the experiments conducted on macrophage cell line RAW 264.7 and not on isolated macrophages[11] We indicated, for the first time, an inhibition of freshly isolated macrophage proliferation under *B. rapa* seeds extracts leading to an anti-inflammatory effect of compounds present in *B. rapa* extracts. This conclusion is confirmed by previous publications indicating an anti-inflammatory effect of *B. rapa* root compounds on cultured macrophage cell lines.[18, 22]

As for *R. sativus* seeds extracts, we observed that, the three extracts did not significantly modify splenocyte proliferation or IgG production. In contrast, we reported a high stimulation of cellular immunity by aqueous extract which stimulates proliferation of thymic cells and their cytotoxicity against foreign cells with no effect on macrophage proliferation. It was shown before that radish roots[21] and radish leaves[25] extracts contained compounds that enhanced cytotoxicity of NK cells without modifying splenocyte or macrophage viability. These reported results are in accordance with our findings leading to the conclusion that aqueous extract of radish seeds have an immunostimulant action on thymic cells and their cytotoxicity.

Moroccan traditional medicine use *B. rapa* and *R. sativus* seeds for improving the body immune system.[34] In addition, these seeds are rich with several bioactive compounds such as glucosinolates, isothiocyanate, phenolic compounds, flavonoids, polysaccharides, and organic acids, which provide a wide range of pharmacological activities for example anticancer, immunomodulatory, anti-hypoxia and anti-oxidation activities.

Our study showed that aqueous extract of *B. rapa* and *R. sativus* seeds increased humoral immunity by immunostimulation of spleen cell, complement activity. Further, cellular immunity was also stimulated.

In conclusion, our research data showed that aqueous extract of Radish seeds exerted an important immunostimulation of thymic cells and their function. However, it has no effect on spleen cells and macrophage proliferation. In addition, aqueous extract of Turnip seems to improve immunity by stimulation the proliferation of spleen cells, complement activity, thymic cells, and their cytotoxicity. These findings indicated a possible use of aqueous extract of Radish and Turnip to enhance humoral, cellular immunity and lymphocyte toxicity in different diseases, which give support to the traditional use of these plants in Moroccan traditional medicine.

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Author Contributions

SL and LA; contributed to the study design and conception. SL; wrote the first draft of manuscript. LA; supervision. IO; Contributed to the methodology and revised the manuscript. All authors read and approved the final manuscript.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical approval

The study protocol was reviewed and approved by the Local Ethical Committee of Sidi Mohamed Ben Abdellah University (Ref, AL13-10-2021). All studies were carried out according to guidelines of animal care as prescribed by national ethical standards.

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