MORICANDIA ARVENSIS CHLOROFORM EXTRACT INCREASES T-LYMPHOCYTE PROLIFERATION, SUPPRESSES NO PRODUCTION, AND AFFECTS CELL ANTIOXIDANT ACTIVITY *IN VITRO*

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ABSTRACT

Moricandia arvensis (Brassicaceae/crucifer) is a medicinal plant widespread in southern Tunisia. Despite its many uses in traditional medicine, there is no report addressing the effects of *M.arvensis* extracts on mice immune cell function. In this study, the immunomodulatory and anti-inflammatory activities of *M. arvensis* Chloroform root (ChlR) extract were investigated. The extract might act as modulator of cellular immune responses *in situ* by stimulating the proliferation of T-lymphocytes. On the contrary, ChlR extract inhibits LPS stimulated splenocytes proliferation, implying a potential inhibition of B-cells and thus the humoral immune responses. Without mitogens stimulation, ChlR extract enhances significantly cell proliferation and affects macrophage functions by modulating their lysosomal enzyme activity and nitric oxide release. Considerable antioxidant effects were also observed on the cellular antioxidant activity. Overall ChlR extract from *M. arvensis* enhanced cellular immune responses and exerted an anti-inflammatory effect fortifying the importance of the use of its products as anti-inflammatory agents.

KEYWORDS: *Moricandia arvensis*, proliferation, splenocytes, immunomodulation, cellular antioxidant activity.

1. INTRODUCTION

Moricandia arvensis has been used in Tunisia in traditional medecine in the treatment of sexually transmitted diseases and scorbut.[1,2] This specie also used in traditional food[1], belongs to the *Brassicaceae* (*Cruciferae*) family; which is characterized by its high content in phenolic compounds, glucosinolates, and indole derivatives. Some glucosinolates possessing an aromatic or indolic side chain, have been reported to be linked to the anticarcinogenic activity[3] and have shown antioxidant effects.[4] Though this plant and its products are frequently used in traditional medicine and cooking, the literature on effects on the immune system is lacking. This fact prompted us to study the potential immunomodulatory activity and antioxidant potential of *M. arvensis*, more precisely its chloroform root (ChlR) extract, *in vitro*. To our knowledge, this appears to be the first report of immunomodulatory and cellular antioxidant activities associated with this plant.

The importance of lymphocyte functions in host defense against infection, in self-tolerance, and in tumor surveillance, as well as in inflammation is well established. Inflammation is a defense mechanism comprised of many complex events; mediators involved in the inflammatory reaction can induce, maintain and aggravate many disorders. Nitric oxide (NO) is a critical mediator produced by inducible NO synthase (iNOS) in macrophages stimulated with bacterial membrane components (like lipopolysaccharide [LPS]) and/or cytokines. Over-formation of agents like NO can somehow contribute to the rheumatoid arthritis, systemic lupus erythematosus and inflammatory myositis.[5] Recently, the intentional modulation of macrophage and lymphocyte activities has been seen as an important pharmacologic/therapeutic approach to potentially treat many disease conditions[6] with chronic inflammatory etiology and pathology, such as atherosclerosis, rheumatoid arthritis and Alzheimer's disease.[7] Hence, the employment of anti-inflammatory agents might be helpful in the therapeutic treatment of those pathologies.[8] In fact, products issued from medicinal plants have been used as an additional and alternative therapy.[9] The study reported here, contributes to finding new interesting species of medicinal plants indigenous to Tunisia.

2. MATERIAL AND METHODS
2.1. Plant material
M. arvensis subsp. *eu-arvensis* was collected from Oued Ghezran at Gafsa, southern Tunisia and was identified by

Dr. Mohamed Cheieb (Department of Botany, Faculty of Sciences, University of Sfax, Tunisia).

2.2. Preparation of plant extracts
To prepare the extract for use in this present study, collected roots were powdered at room temperature (RT). The powders were then macerated in methanol (MeOH) over a 24 hr period at RT with continuous stirring. Thereafter, the material was filtered, and the solvent then air-evaporated from the extract. The dried extract was re-constituted in 100% distilled water, and then underwent repeated extractions with petroleum ether and chloroform (liquid/liquid) (v/v). The final extract was air-dried and weighed; the chloroform root extract (ChlR) concentrate yield (per gram dried plant material) was determined as 100 x weight (g) dried extract/dry weight (g) plant material. The percentage yield ChlR extract was routinely 0.1%.

From this material, extract solutions containing concentrations of 12.5; 25, 50, 100, or 200 µg extract/ml were then prepared for use in the evaluation of their effects on immune cells *in vitro*.

2.3. Cell preparations from mice
Naive BALB/c mice (6-8-wk-old, male, 18-22 g) obtained from the Pasteur Institute (Tunis, Tunisia) were housed under standard conditions of temperature (22-28°C), humidity (30-70%), and light (12-hr light/dark) in an accredited pathogen-free facility. All mice were provided *ad libitum* access to standard rodent chow and filtered water. We evoke that all experiments were performed in accordance with the guidelines for the care and use of laboratory animals as published by the US National Institute of Health; in addition the experiments received the explicit approval of the Ethics Animal Committee in Tunisia.

Splenocytes were obtained as previously reported.[10] After cervical dislocation of mice, each spleen was isolated aseptically and then minced with a sterile forceps. Splenocytes were then obtained by centrifugation (1500 rpm, 10 min, 4°C); any red blood cells were lysed by re-suspending the pellet in lysing buffer (144 mM NH_4Cl, 1.7 mM Tris-Base) and placing the cells on ice for 10 min. Cells were then washed twice with phosphate-buffered saline (PBS, pH 7.4) and re-suspended in complete RPMI medium. Other mice were used solely to provide peritoneal macrophages. After intraperitoneal injection of 4 ml sterile PBS, massaging of the peritoneum, and drawing back of the fluid ($\approx$ 4 ml) into the syringe, we obtained the peritoneal cells which were washed and re-suspended in complete RPMI medium. The trypan blue exclusion technique was used to assessing cell viability.

2.4. Cell proliferation assay
Assays of lymphocyte proliferation were performed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method.[11] In brief, splenocyte suspensions in RPMI 1640 medium (5×10^6 cells/ml; 100 µl aliquot/well) were pre-incubated in 96-well plates for 24 hr before addition of mitogens (lectin or LPS, each at 5 µg/ml) alone or in combination with increasing concentrations of ChlR extract (0, 12.5, 25, 50, 100, or 200 µg/ml).

Lectin was used to trigger T-lymphocyte cell division, whereas LPS-stimulated splenocyte proliferation implying thus the activation of B-cells proliferation and humoral immune responses. Cells were then incubated (37°C, 5% CO_2 atmosphere) for an additional 48 hr. Thereafter, the plates were centrifuged (1500 rpm, 10 min) and then the cell pellet in each well was re-suspended in 50 µl MTT (5 mg/ml in RPMI) solution and incubated a further 4 hr at 37°C. After this period, the plate was centrifuged again, the MTT in each well was removed, and 100 µl dimethyl sulfoxide (98% DMSO) was added. The absorbance was then measured at 570 nm using microplate reader (Thermo Scientific, Vantaa, Finland). The percentage of proliferation was calculated by using the following equation:
Proliferation (%) = 100 x (OD sample-OD control)/OD control.

For cells treated with extract without mitogen:
OD sample= OD of treated cells with ChlR extract alone
OD control: OD of untreated cells

For cells treated with extract and Lectin:
OD sample: OD of treated cells with ChlR extract+Lectin
OD control: OD of treated cells with Lectin.

For cells treated with extract and LPS:
OD sample: OD of treated cells with ChlR extract +LPS
OD control: OD of treated cells with LPS.

2.5. Assessment of lysosomal enzyme activity
Lysosomal enzyme activity was measured as described in Manosroi *et al*.[12] Macrophage suspensions (100 µl aliquot of 6×10^6 cells/ml stock preparation) were seeded into flat-bottom 96-well plates, treated with different concentrations of extract and then incubated at 37°C in a 5% CO_2 humidified atmosphere for 48 hr. After that period, the medium was removed and 20 µl of 0.1% Triton X100, 100 µl of 100 mM *p*-nitrophenyl phosphate solution and 50 µl of 0.1 M citrate buffer (pH 5.0) were added to each well. The plate was then incubated for 30 min at 37°C before 150 µl 0.2 M borate buffer (pH 9.8) was added to each well and the absorbance was then measured at 405 nm. The percentage of lysosomal enzyme activity in treated cultures relative to that in control cells was calculated as:
Lysosomal enzyme activity (%) = 100 x (OD sample-OD control)/OD control.
Where OD sample= OD of treated cells with ChlR extract
OD control= OD of untreated cells

2.6. Nitrite determination

Using a Griess reaction, the amount of NO released by the peritoneal macrophages was measured by determining the amounts of accumulated nitrite (NO_2^-) in cell free supernatants.[13] Peritoneal macrophages (100 µl aliquot of 6×10^6 cells/ml) were seeded into flat-bottom 96-well plates and incubated for 48 hr in the presence of increasing doses of ChlR extract. Cells treated with LPS (5 µg/ml) alone were used as a positive control. NO_2^- was then measured by adding of 100 µl Griess reagent (1% sulfanilamide and 0.1% naphthylenediamine in 5% phosphoric acid) to 100 µl harvested supernatant. The optical density was then measured at 570 nm using the microplate reader and NO concentrations were calculated by comparison with a standard curve of $NaNO_2$ (diluted in culture mediums).

2.7. Cellular antioxidant activity (CAA) assay

A CAA assay was employed to measure effects on cell antioxidant potentials[14] by the extract. In brief, splenocytes were seeded at 6×10^4 cells (in 100 µl RPMI) in 96-well microplates and incubated for 24 hr at 37°C. The medium was then removed and the wells were washed with PBS. Triplicate wells were then treated for 1 hr with 95 µl extract along with 5 µl of a 25 µM solution of 2',7'-dichlorofluorescin (DCFH) in medium. After incubation, all wells were washed with 100 µl PBS and a 100 µl aliquot of 600 µM ABAP in RPMI was applied to the cells. ABAP is an exogenous source of peroxyl radicals used to oxidize DCFH-DA to fluorescent DCF product. Accordingly, cells treated with ChlR extract that have antioxidant activity should have lower fluorescence relative to that with untreated cells. Fluorescence was read every 5 min for a total of 1 hr using a fluorescence microplate reader (Biotek, Winooski, VT) (538 nm emission and 485 nm excitation). Each plate included triplicate control and blank wells: control wells contained cells treated with DCFH-DA and ABAP; blank wells contained cells with DCFH-DA and RPMI without ABAP. Fluorescence values for the blank samples and initial fluorescence values were subtracted from the sample values (ChlR extract). The area under the fluorescence vs. time-curve was integrated at each timepoint to calculate the CAA units using the following equation: CAA (U) = 100 - (∫SA / ∫CA) x 100,

Where ∫SA and ∫CA= the integrated areas under the fluorescence vs. time-curves for sample and control curves, respectively.

2.8. Statistical analysis

All data were expressed as mean (± SD) and compared using a Student's *t*-test followed by the Mann-Whitney U-test for tests that we have used more than one mice (SPSS 11.0 software (SPSS INC; Chicago, IL)). Statistical significance was assigned at p values < 0.05.

3. RESULTS

3.1. Splenocyte proliferation

To test whether ChlR extract promoted or inhibited cell proliferation, splenocytes were isolated and cultured with extract alone, extract + lectin, or extract + LPS. In the absence of mitogen, extract at test concentrations > 12.5 µg/ml was able to induce significant splenocyte proliferation (Figure 1A) albeit that the effect was not dose-dependent below 100 µg/ml. In the studies with lectin, only the highest concentrations of ChlR extract (100 and 200 µg/ml) up-regulated lectin-induced proliferation (Figure 1B) above the levels from the lectin alone. Lower doses appeared to be inhibitory, with the effect from 12.5 µg/ml of extract causing a significant reduction relative to that of the lectin itself. In contrast, all doses of ChlR extract significantly inhibited LPS-induced proliferation of the cells (Figure 1C). Again, the inhibitory effects were worse as the dose of extract tested was lowered.

3.2. Peritoneal macrophage lysosomal activity

The *in vitro* effects of the ChlR extract on lysosomal enzyme activity in macrophages are demonstrated in Figure 2. The ChlR extract modulated phagocytic activity in a biphasic dose-related manner. Concentrations of 12.5 and 25 µg/ml of ChlR extract appeared to induce measurable levels of AP activity in the cells. At 12.5 µg/ml, the AP activity compared to values in control cells was increased by 38 [± 0.2]%. However, as the dose level increased to 25 µg/ml (relative AP activity = 22.27 [± 0.10]%), a trend toward a lessening of the extent of induction was noted. By 50 µg/ml (0.66 [± 0.2]%), any stimulatory effect was no longer evident. At doses above 50 µg/ml, inhibition of phagocytic activity was then documented, with activities at 100 and 200 µg/ml extract being, respectively, -53.57 [± 0.19] and -90.35 [± 0.02]% compared to control cell values.

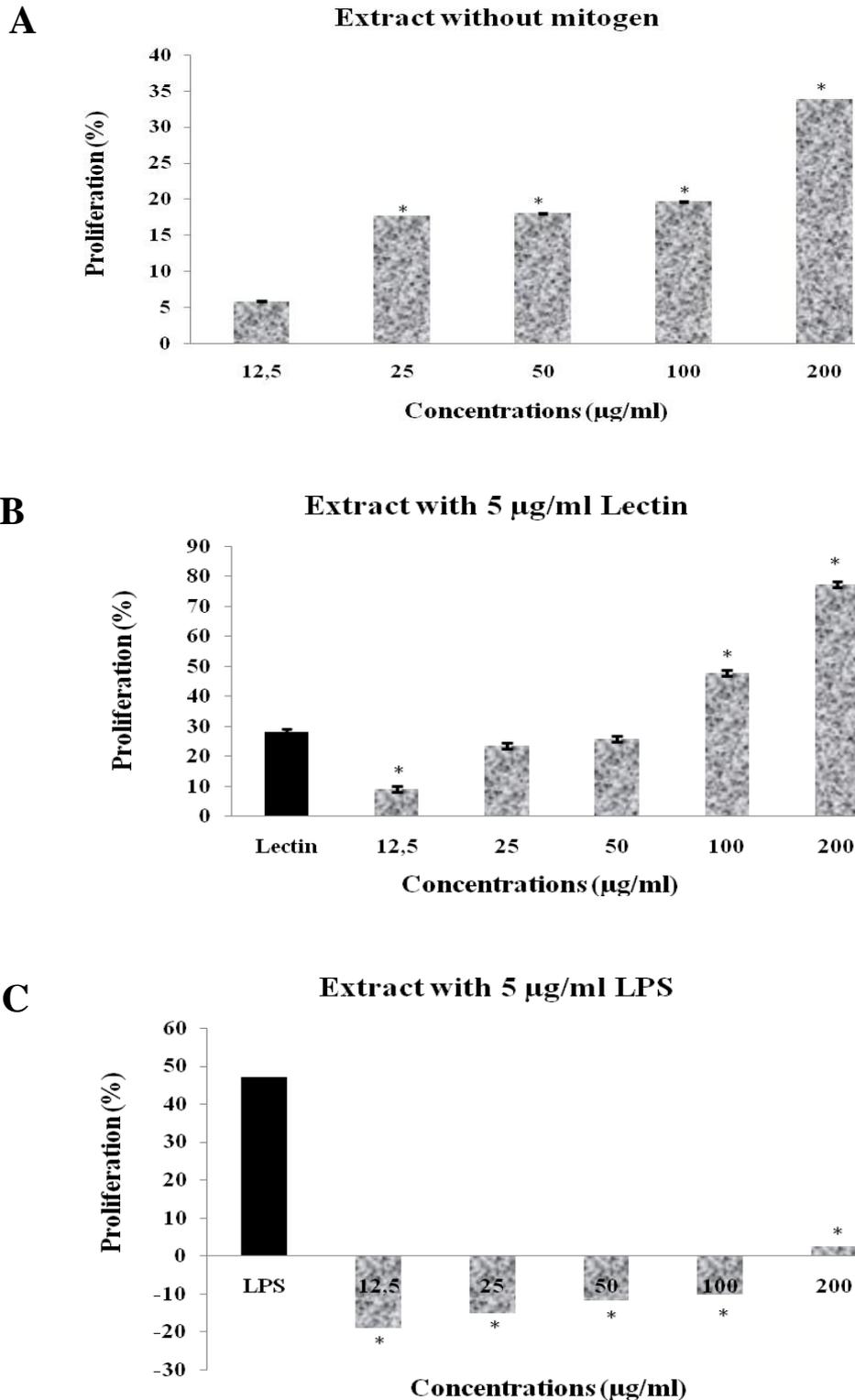### 3.3. Peritoneal macrophage NO production

Effects of the ChlR extract on peritoneal macrophage NO production are shown in Figure 3. Macrophages treated with various concentrations of extract (12.5-100 µg/mL) for 48 hr appeared to display a significant dose-independent decrease in NO production in comparison to that induced by LPS alone. Release of NO by macrophages increased from 6.04 to 41.0 µM as extract concentration decreased from 200 to 12.5 µg/mL.

3.4. Cellular antioxidant activity (CAA)

The CAA assay used to quantitatively evaluate antioxidant activity of the ChlR extract in splenocytes indicated that the extract could be absorbed into the cells and exert antioxidant activity. Production of fluorescence by ABAP-mediated oxidation of DCFH to DCF was inversely dose-relatedly reduced by the extract over the range of the tested concentrations (Figure 4). The maximum antioxidant capacity (56.8 [± 1.1]%) was attained with 12.5 µg/mL of tested extract (Figure 5) and this then progressively (non-dose-dependent) decreased

all the way down to 10.87 [± 0.54]% (a value now non-significantly different from activity in control cells)

when the cells had been treated with 200 µg/ml of extract.

A   **Extract without mitogen**

B   **Extract with 5 µg/ml Lectin**

C   **Extract with 5 µg/ml LPS**

Fig. 1: *In vitro* **effects of ChlR extract on proliferation of splenocytes. Cells incubated with: (A) increasing doses of extract only; (B) lipopolysaccharide (LPS, 5 µg/ml) in absence and presence of extract; and, (C) lectin (5 µg/ml) in the absence and the presence of ChlR extract. All treatments were for 48 hr prior to addition of MTT reagent. Data shown are mean (± SD) percentage proliferation from three independent experiments. (A) *Value significantly different from RPMI-only negative control ($p < 0.05$). (B) and (C) *Value significantly different from mitogen (LPS or lectin) only-treated cells ($p < 0.05$).**
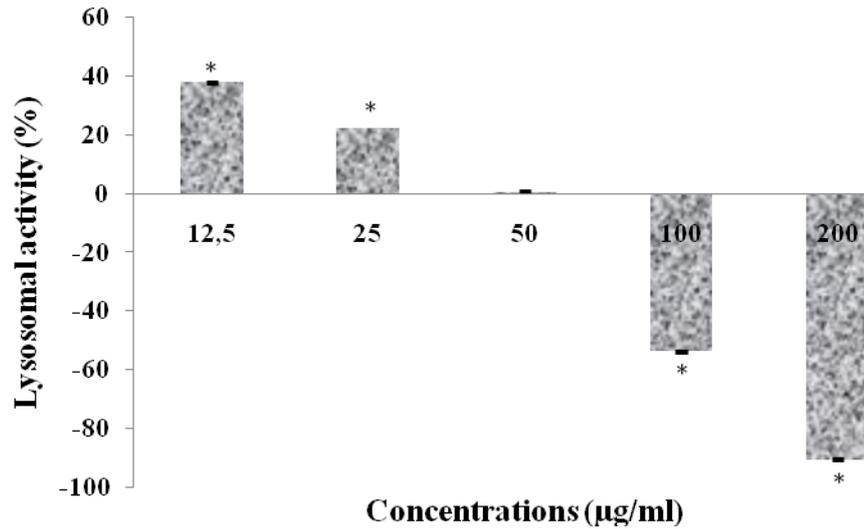
Fig. 2: Stimulation of mouse peritoneal macrophage lysosomal enzyme activity by ChlR extract. Macrophages were incubated in the presence of increasing concentrations of extract for 48 hr. Controls cells were incubated with medium only. Lysosomal enzyme activity was then assessed as indicated in Materials and Methods. Data shown are mean (± SD) percentage increases in lysosomal enzyme activity (relative to values seen with untreated control cells) from three independent experiments. *Value significantly different from untreated control cells (p < 0.05).
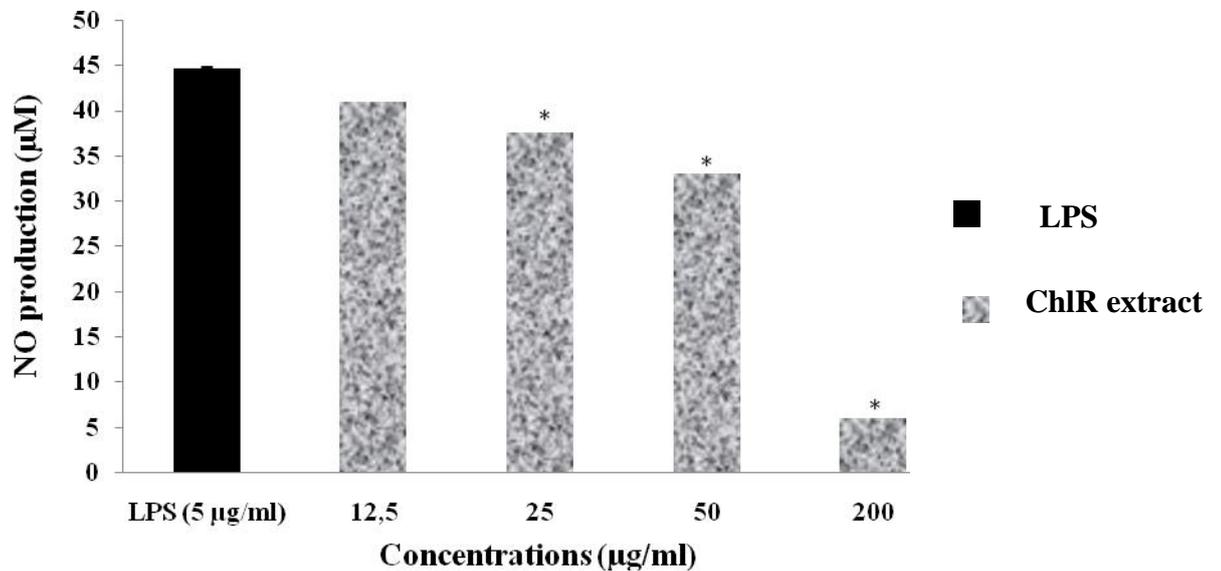
Fig. 3: Production of nitrite by mouse peritoneal macrophages in response to tested extracts from *M. arvensis*. Macrophages (6×10^6 cells/well) were incubated in microtiter plates in RPMI 1640 medium in the presence of increasing concentrations of ChlR extract for 48h. Cells treated with LPS (5 µg/ml) alone were used as a positive control. Data shown are mean (±SD) of NO production from three independent experiments. *Value significantly different compared with that of LPS stimulated [untreated] control cells control ($p < 0.05$).
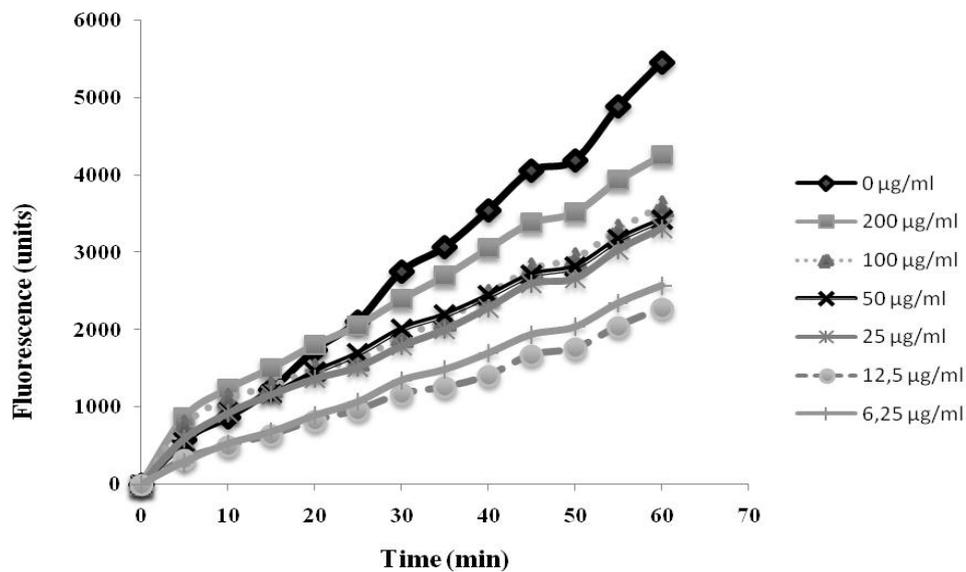
Fig. 4: Kinetics of inhibited oxidation by the ChlR extract. The kinetics of the inhibition of oxidation of DCFH in splenocytes using a cellular antioxidant activity assay in the presence of ChlR extract. Values shown are means (n = 5/dose tested).
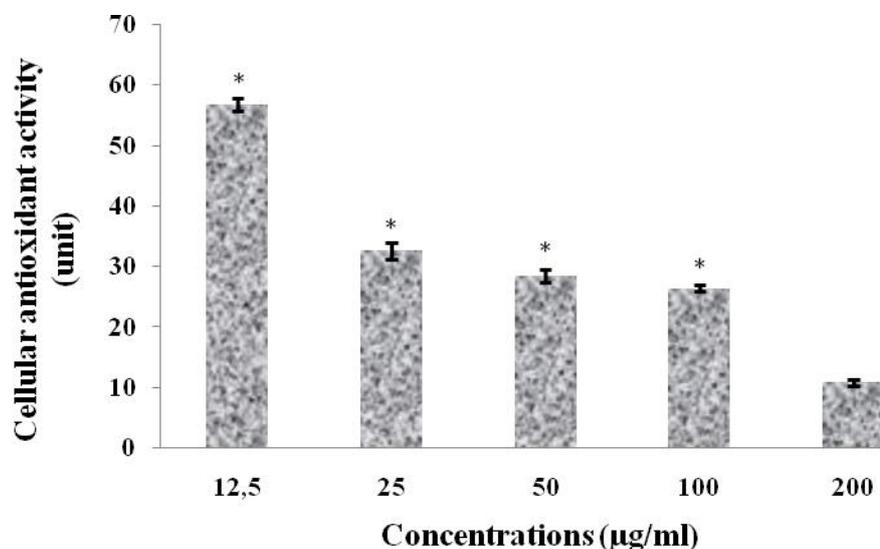

Fig. 5: Effect of ChlR extract on cellular antioxidant activity in splenocytes. Cells were incubated in PBS in the absence or presence of DCFH-DA and increasing concentrations of extract for 1 hr. Data shown are mean (± SD) percentage changes in cellular antioxidant activity (relative to values in untreated control cells) from three independent experiments. *Value significantly different vs. untreated control cells ($p < 0.05$).

4. DISCUSSION

Several medicinal plants are considered immunomodulatory as they impart a variety of anti-inflammatory, anti-microbial, and anti-tumoral effects.[15] Investigating the effects of substances that promote or inhibit macrophage and lymphocyte inflammatory response represents a potent mean to study immunomodulation and drug discovery. This study focused on how extract from a medicinal plant, *i.e.*, *Moricandia arvensis*, could modulate immune cell function.

Here, the effects of ChlR extract on both LPS and lectin stimulated proliferation of B- and T-lymphocytes, respectively, were assessed. In the presence of LPS and ChlR extract, stimulation of cell proliferation was decreased at all tested doses. These results suggested to us potential antagonistic or competitor effects from the extract against the LPS. How this occurred is not clear; it is possible that constituents in the extract may down-regulate the pro-inflammatory interleukin (IL)-1 and tumor necrosis factor (TNF)- production by mouse peritoneal macrophages. Further studies will help to clarify more precisely mechanisms underlying the effects of the extract on B-lymphocyte function. At the highest

concentrations tested here (100 and 200 μg/ml), ChlR extract up-regulated lectin-induced cell proliferation suggesting that ChlR extract in the presence of lectin seems to be more sensible than B cells response. The types of results seen in the current study with regard to the lectin-induced effects on the splenocytes suggested potential synergistic effects of the extract, i.e., the extract may contain some factors that themselves are acting as mitogens. The presence of lectin and other mitogens in plant extracts argues in favor of this explanation. Several published data indicate that numerous secondary metabolites isolated from plants have been proven to possess an immunomodulatory, properties.[16,17]

The proliferation of the splenocytes in response to *M. arvensis* ChlR extract alone could be ascribed to several compounds. A presence of polyphenols, and tannins, was clearly seen in our previous preliminary results of phytochemical screening[18] a class of agents already known to be active immunomodulatory substances.[19] The stimulation of T-lymphocytes could also be attributed to flavonoids in the extract[18] as several flavonoid compounds have been previously shown to modulate the immune system.[20]

The modulation of anti-tumor activities of macrophages by various biological response modifiers is an area of active interest in cancer chemotherapy and is often closely associated with immunomodulatory activity of test drugs.[21] An important non-specific immune activity *in situ* is phagocytosis by macrophages accompanied by release of lysosomal enzymes for killing\digesting microbial pathogens. The results of the analyses here indicated that macro-phage lysosomal enzyme activity appeared to be inversely affected by the dose of ChlR extract. Macrophage phagocytosis is also accompanied by release of free radicals including NO involved in pathogen killing. Compared with LPS, a potent inducer of macrophage NO production[22], the studies here showed that low concentrations of ChlR extract were able to induce NO synthesis by peritoneal macrophages, even without addition of any other exogenous stimuli. Previous studies by other investigators noted that inducing NO production inhibited tumor cell survival *in situ*.[23] As was observed for macrophage lysosomal enzyme activity, the production of NO appeared to be inversely related to extract dose used. Taken together; the results here showed that ChlR extract potentially could affect macro-phage inflammatory functions. An elevated release of NO can reflect phagocytic stimulation of the cells; this would then imply that low doses of the extract could potentially act as immunostimulatory factors of innate immunity. In contrast, at high doses, ChlR extract appeared to act as a potent inhibitor of lysosomal macrophage activity (accompanied by a low release of NO). These outcomes would imply that high doses of the extract could potentially induce an anti-inflammatory effect. Our results were in accordance with other components present in cruciferous vegetable like an indole-3-carbinol (I3C) and -phenylethyl

isothiocyanate (PEITC) which inhibited NO production.[24]

Many plants have been shown to influence lysosomal activity[25] and some plant derived products have been shown to act as NO inducers or inhibitors via different mechanisms such as Concanavalin A which induces the NO production *in vitro*, but not *in vivo*, by stimulating cytokines.[26] We also mentioned as examples of plants, aqueous extract of *Limoniastrum guyonianum* gall[16] and Babassu mesocarp flour aqueous extract were described to inducing the production of NO. In the context of the current study, the results here might be explained by the presence of tannins and flavonoids in the ChlR extract. Tannins are known to enhance macrophage nitroblue tetrazolium (NBT) reducing activity[27], activate morphological changes (spreading) in macrophages, and induce various formation of cytokines including tumor necrosis factor-□ and interleukine-1.[28] Flavonoids such as kempferol also induce NO synthase by splenocytes.[20]

Lastly, it is well known that changes in cell oxidant status can impart a stress that, in turn, impacts on function; in regard to immune system cells, this could give rise to changes reflected as immunomodulation. The CAA assay used here to quantitatively evaluate potential relations between anti-inflammatory and cellular antioxidant activity by the ChlR extract showed that the extract could cause a reduction in intracellular oxidation of DCFH. This implied that either agents in the extract were acting directly as antioxidants and/or they could trigger processes in the cell that augmented antioxidant monitoring processes. This latter activity of the ChlR extract may be a result of its action through other mechanisms and radicals than NO scavenging effect.

As was observed for the production of NO, CAA activity appeared to be inversely related to extract dose used. This effect may be explained by a biphasic reaction between radicals and some polyphenols described by many authors.[29] This reaction consists of initial fast scavenging activity where the more active compounds react immediately with ROS and a second reaction where products are formed and together with the less reactive molecules. *M. arvensis* ChlR extract is a complex mixture of several compounds, in particular phenolic compounds with a diversity of chemical structures that give them particular properties of reaction and solubility.

5. CONCLUSIONS

In summary, the present work illustrated the effects of *M. arvensis* ChlR extract on murine immune cell function *in vitro*. This is the first report describing the anti-inflammatory properties, as well as immunomodulating effects of *M. arvensis* extract in a mouse immune cell model. Clearly, further detailed studies in animal models are necessary to clarify how/to what extent immune cell modulation might occur *in vivo*. Fractionation/full characterization of the immunomodulatory compounds in the ChlR extract is also essential and should prove

beneficial in ongoing effort to isolate and identify active anti-inflammatory and antioxidant molecules.

REFERENCES
1. Skandrani, I., Limem, I., Neffati, A., Boubaker, J., Ben Sghaier, M., Bhouri, W., Bouhlel, I., Kilani, S., Ghedira, K., Chekir-Ghedira. (2010). Assessment of phenolic content, free-radical-scavenging capacity genotoxic and anti-genotoxic effect of aqueous extractprepared from *Moricandia arvensis* leaves. *Food and Chemical Toxicology*, 48(2): 710-715.
2. Le Floch, E. (1983). Contribution à une Etude Ethnobotanique de la Flore Tunisienne. Tunis: Ministère de L'Enseignement Supérieur et de la Recherche Scientifique.
3. Belkhiri, A., Lockwood, G.B. (1990). An indole derivative and glucosinolates from *Moricandia arvensis*. *Phytochemistry*, 29(4): 1315-1316.
4. Braham, H., Mighri, Z., Jannet, H.B., Matthew, S., Abreu, P.M. (2005). Antioxidant phenolic glycosides from *Moricandia arvensis*. *Journal of Natural Product*, 68(4): 517-522.
5. Harput, U.S., Saracoglu, I., Ogihara, Y. (2005). Stimulation of lymphocyte proliferation and inhibition of nitric oxide production by aqueous *Urtica dioica* extract. *Phytotherapy Research*, 19(4): 346-348.
6. Nworu, C.S., Temchura, V., Okoye, F.B., Akah, P.A., Esimone, C.O., Uberla, K. (2010). Activation of murine lymphocytes and modulation of macrophage functions by fractions of *Alchornea cordifolia* (Euphorbiaceae) leaf extract. *Immunopharmacology*, 32(1): 28-36.
7. Szabo, A. (2015). Psychedelics and immunomodulation Nnovel approaches and therapeutic opportunities. *Frontiers in Immunology*, 8: 356.
8. Sosa, S., Balick, M.J., Arvigo, R., Esposito, R.G., Pizza, C., Altinier, G., Tubaro, A. Screening of the topical anti-inflammatory activity of some Central American plants. (2002). *Journal of Ethnopharmacology*, 81(2): 211-215.
9. Vickers, A. (1999). Reflections on complementary medicine research in the UK. *Complementary Therapies in Medicine*, 7(4): 199-200.
10. Limem, I., Harizi, H., Ghedira, K., Chekir-Ghedira, L. (2010). Leaf extracts from *Phlomis crinita Cav. subs. mauritanica Munby* affect immune cell functions *in vitro*. *Immunopharmacology*, 33(2): 309-314.
11. Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, 65: 55-63.
12. Manosroi, A., Saraphanchotiwitthaya, A., Manosroi, J. (2003). Immunomodulatory activities of *Clausena excavata Burm*. f. wood extracts. *Journal of Ethnopharmacology*, 89(1): 155-160.
13. Green, L.C., Wagner, D.A., Glogowski, J., Skipper, P.L., Wishnok, J.S., Tannenbaum, S.R. (1982). Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. *Analytical Biochemistry*, 126(1): 131-138.
14. Wolfe, K.L., Liu, R.H. (2007). Cellular antioxidant activity (CAA) assay for assessing antioxidants, foods, and dietary supplements. *Journal of Agricultural and Food Chemistry*, 55(22): 8896-8907.
15. Davicino, R., Mattar, A., Casali, Y., Porporatto, C., Correa, S.G., Micalizzi, B. (2007). *In vivo* immunomodulatory effects of aqueous extracts of *Larrea divaricata Cav*. *Immunopharmacology and Immunotoxicology*, 29: 351-366.
16. Krifa, M., Bouhlel, I., Ghedira-Chekir, L., Ghedira, K. (2013). Immunomodulatory and cellular anti-oxidant activities of an aqueous extract of *Limoniastrum guyonianum* gall. *Journal of Ethnopharmacology*, 146(1): 243-9.
17. Krifa, M., El Mekdad, H., Bentouati, N., Pizzi, A., Ghedira, K., Hammami, M., El Meshri, S.E., Chekir-Ghedira, L. (2015). Immunomodulatory and anticancer effects of *Pituranthos tortuosus* essential oil. *Tumour Biology*, 36(7): 5165-70.
18. Skandrani, I., Boubaker, J., Bouhlel, I., Limem, I., Ghedira, K., Chekir-Ghedira, L. (2010). Leaf and root extracts of *Moricandia arvensis* protect against DNA damage in human lymphoblast cell K562 and enhance antioxidant activity. *Environmental Toxicology and Pharmacology*, 30(1): 61-7.
19. Feldman, K.S., Sahasrabudhe, K., Smith, R.S., Scheuchenzuber, W.J. (1999). Immunostimulation by plant polyphenols: a relationship between tumor necrosis factor-alpha production and tannin structure. *Bioorganic and Medicinal Chemistry Letters*, 9(7): 985-990.
20. Lopez-Posadas, R., Ballester, I., Abadia-Molina, A.C., Suarez, M.D., Zarzuelo, A., Martinez-Augustin, O., Sanchez de Medina, F. (2008). Effect of flavonoids on rat splenocytes, a structure-activity relationship study. *Biochemical Pharmacology*, 76(4): 495-506.
21. Kang, N.S., Park, S.Y., Lee, K.R., Lee, S.M., Lee, B.G., Shin, D.H., Pyo, S. Modulation of macrophage function activity by ethanolic extract of larvae of *Holotrichia diomphalia*. J*ournal of Ethnopharmacology*, 79(1): 89-94.

22. MacMicking, J., Xie, Q.W., Nathan, C. (1997). Nitric oxide and macrophage function. *Annual Review of Immunology*, 15: 323-350.

23. Kozlowska, K., Cichorek, M., Zarzeczna, M. (2002). Estimation of macrophage IL-10 and NO secretion in the cytotoxicity against transplantable melanomas in relation to the progression of these tumours. *Folia Morphologica*, 61(3): 127-131.

24. Tsai, J.T., Liu, H.C., Chen, Y.H. (2010). Suppression of inflammatory mediators by cruciferous vegetable-derived indole-3-carbinol and phenylethyl isothiocyanate in lipopolysaccharide-activated macrophages. SO Mediators Inflamm, *doi: 101155/2010/293642*.

25. Sun, Y., Wang, S., Li, T., Li, X., Jiao, L., Zhang, L. (2008). Purification, structure and immunobiological activity of a new water-soluble polysaccharide from the mycelium of *Polyporus albicans* (Imaz.) Teng. *Bioresource Technology*, 99(4): 900-904.

26. Nascimento, F.R.F., Barroqueiro, E.S.B., Azevedo, A.P.S., Lopes, A.S., Ferreira, S.C.P., Silva, L.A., Maciel, M.C.G., Rodriguez, D., Guerra, R.N.M. (2006). Macrophage activation induced by *Orbignya phalerata Mart*. *Journal of Ethnopharmacology*, 103: 53–58.

27. Sakagami, H., Kawazoe, Y., Oh-hara, T., Kitajima, K., Inoue, Y., Tanuma, S., Ichikawa, S., Konno, K. (1991). Stimulation of human peripheral blood polymorphonuclear cell iodination by lignin-related substances. *Journal of Leukocyte Biology*, 49(3): 277-282.

28. El Abbouyi, A., Toumi, M., El Hachimi, Y., Jossang, A. (2004). *In vitro* effects of aqueous seeds extract of Acacia cyanophylla on the opsonized zymosan-induced superoxide anions production by rat polymorphonuclear leukocytes. *Journal of Ethnopharmacology*, 91(1): 159-165.

29. Villano, D., Fernandez-Pachon, M.S., Troncoso, A.M., Garcia-Parrilla, M.C. (2004). The antioxidant activity of wines determined by the ABTS[(+)] method: influence of sample dilution and time. *Talanta*, 64(2): 501-509.