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Study of the effects of *Lemna minor* extracts on human immune cell populations

C. CATELANI CARDOSO[1], E. MIRALDI[2], M.R. CECCARINI[3,4], Z. NAUREEN[5,6], G. BAINI[2], E. MANARA[6], K. ANPILOGOV[6], G. CAMILLERI[6], K. DHULI[6], S. PAOLACCI[7], F. RIA[8], G. DI SANTE[8], C. CAMPONESCHI[8], M. TREDICINE[8], A. ZANLARI[1,9], P. CHIURAZZI[10,11], T. BECCARI[3,4], M. BERTELLI[6,7]

[1]Stazione Sperimentale per l'Industria delle Conserve Alimentari (SSICA), Fondazione di Ricerca, Parma, Italy
[2]Department of Physical, Earth and Environmental Sciences, University of Siena, Siena, Italy
[3]Department of Pharmaceutical Science, University of Perugia, Perugia, Italy
[4]C.I.B., Consorzio Interuniversitario per le Biotecnologie, Trieste, Italy
[5]Department of Biological Sciences and Chemistry, College of Arts and Sciences, University of Nizwa, Nizwa, Oman
[6]MAGI Euregio, Bolzano, Italy
[7]MAGI'S LAB, Rovereto (TN), Italy
[8]Department of Translational Medicine and Surgery, Università Cattolica del Sacro Cuore, Rome, Italy
[9]Università Mercatorum, Università Telematica delle Camere di Commercio Italiane, Parma, Italy
[10]Sezione di Medicina Genomica, Dipartimento Scienze della Vita e Sanità Pubblica, Fondazione Policlinico Universitario A. Gemelli IRCCS, Università Cattolica del Sacro Cuore, Rome, Italy
[11]Fondazione Policlinico Universitario A. Gemelli IRCCS, UOC Genetica Medica, Rome, Italy

Abstract. – OBJECTIVE: *Lemna minor* is a plant with a huge repertoire of secondary metabolites. The literature indicates that extracts of *Lemna minor* have antioxidant, antiradical, immunomodulatory and anti-inflammatory properties. The objective of the present study was to find a suitable technique to extract active compounds from this plant and verify whether these extracts have immunomodulatory activity.

MATERIALS AND METHODS: We grew *L. minor* on a standard medium with Gamborg B5 and vitamins. We extracted compounds from the plant by maceration and decoction. The phytochemical profile of the extracts was characterized by chromatography, spectrophotometry, and spectroscopy. The extracts were tested on cultures of mononuclear cells from four human subjects. These cells were pulsed with carboxyfluorescein succinimidyl ester, grown in triplicate in standard culture medium without (control) and with increasing concentrations of Lemna extracts. Flow cytometry was used to evaluate cell death and proliferation of the total mononuclear cell population and of CD4+, CD8+, B cell and monocyte populations.

RESULTS: The Lemna extracts were not cytotoxic and did not cause cell necrosis or apoptosis in immune cells. At low concentrations, they induced very limited proliferation of CD4+ cells within 48 hours. At high concentrations, they induced proliferation of CD8+ cells and B lymphocytes within 48 hours.

CONCLUSIONS: Unfortunately, we failed to confirm any immunomodulatory activity of Lemna extracts. Growth and death rates of human immune cells were not significantly affected by adding Lemna extracts to the culture medium.

Key Words:
Lemna minor, Phytocomplex, Polyphenols, Immunomodulation, Immune cells.

Introduction

Lemna minor is a monocotyledonous herbaceous plant of the *Lemnaceae* (duckweed) family. *Lemna* is a Greek word meaning "water plant" and *minor* is Latin word meaning "small". *L. minor* is the common duckweed that grows in still or slow-moving water bodies, covering the surface[1]. Under ideal conditions of pH, water temperature, incident light and nutrient concentrations, biomass production by *Lemna minor* is similar to that of the most vigorous photosynthetic terrestrial plants. *L. minor* biomass may double in 16-48 hours. The plant has naturalized in all countries

Corresponding Author: Stefano Paolacci, Ph.D; e-mail: stefano.paolacci@assomagi.org

with moderate climate and is a valuable forage, food and medicinal plant[1,2]. *Lemna minor* has a huge repertoire of secondary metabolites[3-8] and is reported to have antimicrobial[4,9], antiparasitic[10] and immunomodulatory properties[6,7,11,12]. Recent studies have also highlighted its nutritional and therapeutic properties. Consumer health concerns, rising healthcare costs and food security issues have prompted research into the nutritional value, traditional consumption as a food source, and commercialization possibilities of duckweed[13-15]. The aim of the present pilot pre-clinical study is to explore the immunomodulatory properties of *Lemna minor*.

Materials and Methods

Cultivation Conditions

The plants were grown on liquid medium based on 1:5 dilution of Gamborg B5 complete medium with addition of vitamins (Figure 1A, B). The growth chamber was kept at a temperature of 20-25°C and illuminated with the full visible spectrum and UV from 60 30-W LEDs, reproducing the natural light-dark circadian rhythm (with a photoperiod of 12 hours). The level of the medium was checked every 2-3 days and replenished as needed. Plants were moved to a larger container when they reached confluence.

DNA Extraction

Lemna minor was dried at 50°C for 24 hours (Figure 1C). DNA from *Lemna minor* leaves was extracted using a Quick DNA Plant/seed Miniprep kit (Zymo Research) according to the manufacturer's guidelines.

Barcoding PCR

DNA was amplified with AmpliTaq Gold™ 360 PCR Master Mix (Applied Biosystem, Vilnius, Lithuania) according to the manufacturer's specifications, using the primers atpF-atpH R GCTTTTATGGAAGCTTTAACAAT and atpF-atpH F ACTCGCACACACTCCCTTTCC (melting temperature 55°C)[16]. The PCR products were purified with ExoSap-IT (Applied Biosystem, Vilnius, Lithuania) and sequenced on a CEQ8800 Sequencer (Beckman Coulter, Fullerton, CA, USA).

Extraction and Phyto-Chemical Analysis of Lemna Minor

Preparation study

In order to determine the best extraction method, various extraction techniques were tried (maceration, digestion, infusion, decoction, percolation and solid/liquid extraction). The best extraction of the active ingredients was obtained by maceration and decoction.

Solvent choice

Maceration in H_2O, decoction in H_2O and extraction in H_2O/EtOH at different concentrations (30-60-96%) were carried out to obtain four different extracts. The phytochemical profile of each of them was characterized by chromatography, spectrophotometry and spectroscopy. These anal-

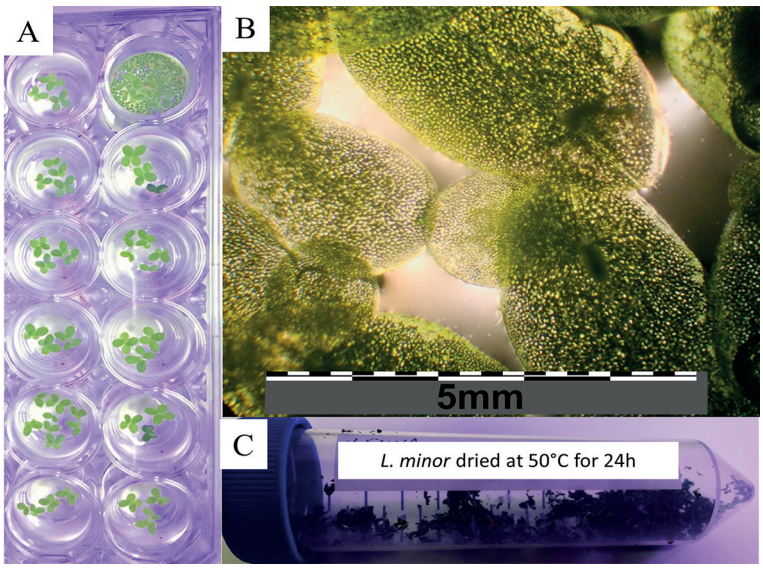

Figure 1. A, *Lemna minor* was grown on liquid medium based on Gamborg B5 complete medium diluted 1:5, with vitamins; **B,** Magnification of *Lemna minor* by stereomicroscope (X20); **C,** Lemna minor was dried at 50°C for 24 hours.

yses were necessary to determine which extraction solvent gave the best yield of the phytocomplex.

Maceration

The minced plant (500 mg) was soaked in sufficient solvent (10 times its weight) for the necessary time, shaking from time to time and replenishing the solvent in case of evaporation. Macerations lasted for 24, 48 and 72 hours, and for 2 and 4 weeks. The best results were obtained with maceration for 2 and 4 weeks. The complete phytochemical analysis was carried out on 2-week macerates. After the maceration period, the extract was filtered and the drug : extract ratio was adjusted to 1:10.

Decoction

The minced plant (500 mg) was extracted by simmering for 20 minutes. The solution was filtered hot, the residue was washed with water, and the drug : extract ratio was adjusted to 1:10.

Chemical Analysis

Quantification of total polyphenols

Total polyphenols were quantified by the Folin-Ciocalteu colorimetric method. Quantification was based on two protocols to find the more efficient extraction method: 10 µl and 20 µl of each extract were diluted in water to volumes of 3 and 1.6 ml; 500 and 100 µl of Folin-Ciocalteu reagent (Sigma-Aldrich and CARLO ERBA Reagents, respectively), diluted 1:10 in water, was added. After stirring, 1 ml and 300 µl of 30% and 20% (m/V) aqueous Na_2CO_3 solution was added.

The absorbance was read at 700 nm by spectrophotometer (SAFAS UVmc2) after 2 hours of incubation and at 765 nm after 30 minutes of incubation of the reaction mixture at room temperature and 40°C, against blanks (1 and 2 ml water). The calibration line for the quantification of polyphenols was based on eight and five increasing concentrations of standard gallic acid (Sigma-Aldrich, Milan) (100-6400 mg/l and 50-500 mg/l, R2 = 0.99). All experiments were conducted in triplicate.

Determination of total flavonoids

Total flavonoids in the extracts were quantified by direct reading of absorbance at 340 nm (maximum absorption of quercetin) of the samples diluted 1:100 in the extraction solvent. The quantity of flavonoids was obtained by interpolating the absorbance values on the calibration line constructed with standard quercetin (Sigma-Aldrich) (7.8-500 mg/l, R2 = 0.987). All experiments were conducted in triplicate.

Determination of total flavan-3-ols

Flavan-3-ols (monomeric and oligomeric catechins) were quantified by the vanillin colorimetric method. A 100 µl aliquot of aqueous solution (10 mg/ml) of the extract was diluted in double-distilled water to a volume of 0.5 ml; 500 µl vanillin (1% m/V) in ethanol 96% V/V was added, stirred and the mixture spiked with 1 ml concentrated HCl. Absorbance at 500 nm was read against a blank (10 µl double-distilled water) by spectrophotometry (Perkin Elmer Victor Nivo3s) after incubating the reaction mixture at room temperature for 15 minutes. The calibration line was based on eight increasing concentrations of (-)-epicatechin (Sigma-Aldrich, Milan) (100-6400 mg/l, R2 = 0.99). All experiments were conducted in triplicate.

Determination of total triterpenes

Total triterpenes were determined by adding 190 µl glacial acetic acid and 300 µl glacial acetic acid solution, spiked with 5% m/V vanillin, to 10 µl of the sample solution, mixing for 30 seconds, and adding 1 ml perchloric acid. The mixture was then heated to 60°C for 45 minutes, cooled, and the volume made up to 5 ml with glacial acetic acid. Absorbance was read at 548 nm and total triterpenes in the extract were quantified using a calibration curve, constructed using ursolic acid (Sigma-Aldrich). The test was performed in triplicate.

Immunomodulation assay

We isolated mononuclear cells from four subjects. We pulsed them with carboxyfluorescein succinimidyl ester (CFSE) and cultured them in triplicate in RPMI medium + human serum + gentamicin, without (control) and with increasing concentrations of *Lemna* extracts (2.4 ng/ml to 2 µg/ml). After 24, 48 and 72 hours we counted the total number of mononuclear cells and the $CD4^+$, $CD8^+$, B cell populations by flow cytometry. For each population, we evaluated cell death (necrosis, early apoptosis and late apoptosis) and proliferation (number of CFSE-low cells).

Results

Plant DNA was sequenced using the atpF-atpH noncoding spacer sequence as previously described[16], confirming species-rank determination of *Lemna minor*.

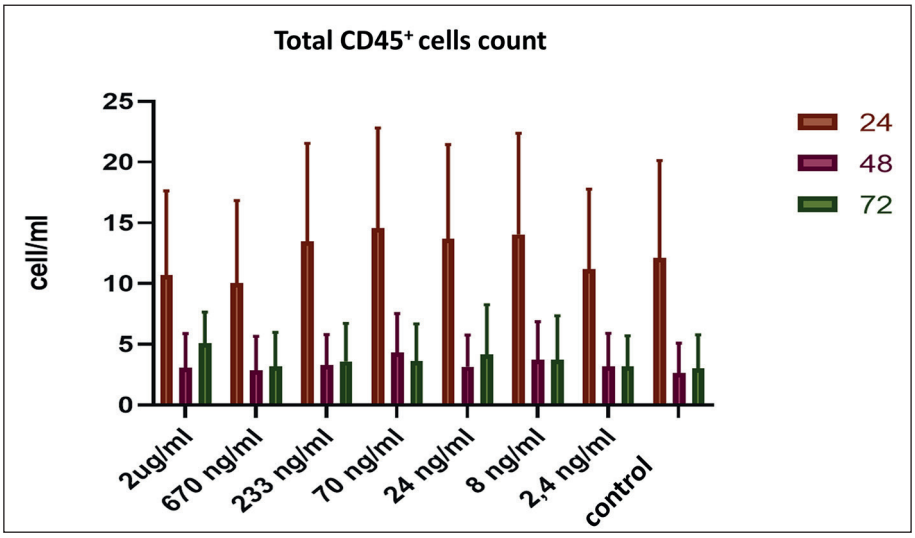

Figure 2. Total number of $CD45^+$ cells counted by flow cytometry after 24, 48 and 72 hours of growth with different concentrations of *Lemna* extracts. No significant difference was detected between experimental and control cell lines.

Maceration of *L. minor* and extraction with 96% of ethanol gave a high yield of triterpenes, whereas higher yields of polyphenols and flavan-3-ols, a subclass of flavonoids, were obtained by decoction. Total flavonoids were not extracted in detectable amounts by decoction or maceration (Table I).

The effects of *Lemna* extracts obtained by decoction were tested on immune cells. The extracts did not seem to cause cell necrosis. $CD4^+$ cells spontaneously underwent a degree of apoptosis between 24 and 48 hours. Apoptosis was unaffected or partially prevented by very high concentrations (>233 ng/ml) of *Lemna* extracts which had no substantial cytotoxic effects on other cell populations. At low concentrations (8-24 ng/ml), *Lemna* extracts induced very limited proliferation of $CD4^+$ cells within 48 hours. At high concentrations (>233 ng/ml), they induced proliferation of $CD8^+$ cells and B lymphocytes within 48 hours (Figures 2-4).

Discussion

L. minor has recently been studied for pharmacological activity, especially immunomodulatory effects. For instance, variable doses of flavonoids (1-30 mg/ml) have been shown to decrease free hemoglobin content and antibody production against specific protein antigens in virus-infected human blood samples, possibly indicating an immunosuppressive property[17]; anthocyanins have been reported to show antioxidant and immunosuppressive properties[18], while polyphenols have anti-inflammatory and immunomodulatory effects[19]. Although this literature points to a possible immunomodulatory effect of *Lemna* extracts, we were unable to replicate these findings. In fact, at the present level of analysis, none of the human immune cell populations tested seemed affected by *Lemna* extracts. Further exploration of the effects of *L. minor* extracts may be required to confirm their immunomodulatory properties.

Table I. Quantity of polyphenols, flavonoids, flavan-3-ols and triterpenes extracted from samples of *L. minor* after decoction and maceration in H_2O, and extraction. LLOD = lower limit of detection.

Method of extraction	Polyphenols (µg/mg)	Flavonoids (µg/ml)	Flavan-3-ols (µg/ml)	Triterpenes (µg/ml)
Decoction	42.30	<1 (below LLOD)	3.15	9.20
Maceration in H_2O	11.08	<1 (below LLOD)	2.70	9.10
Maceration in EtOH 30%	7.99	<1 (below LLOD)	2.15	15.14
Maceration in EtOH 60%	6.82	<1 (below LLOD)	2.8	18.23
Maceration in EtOH 96%	5.79	<1 (below LLOD)	<1 (below LLOD)	42.43

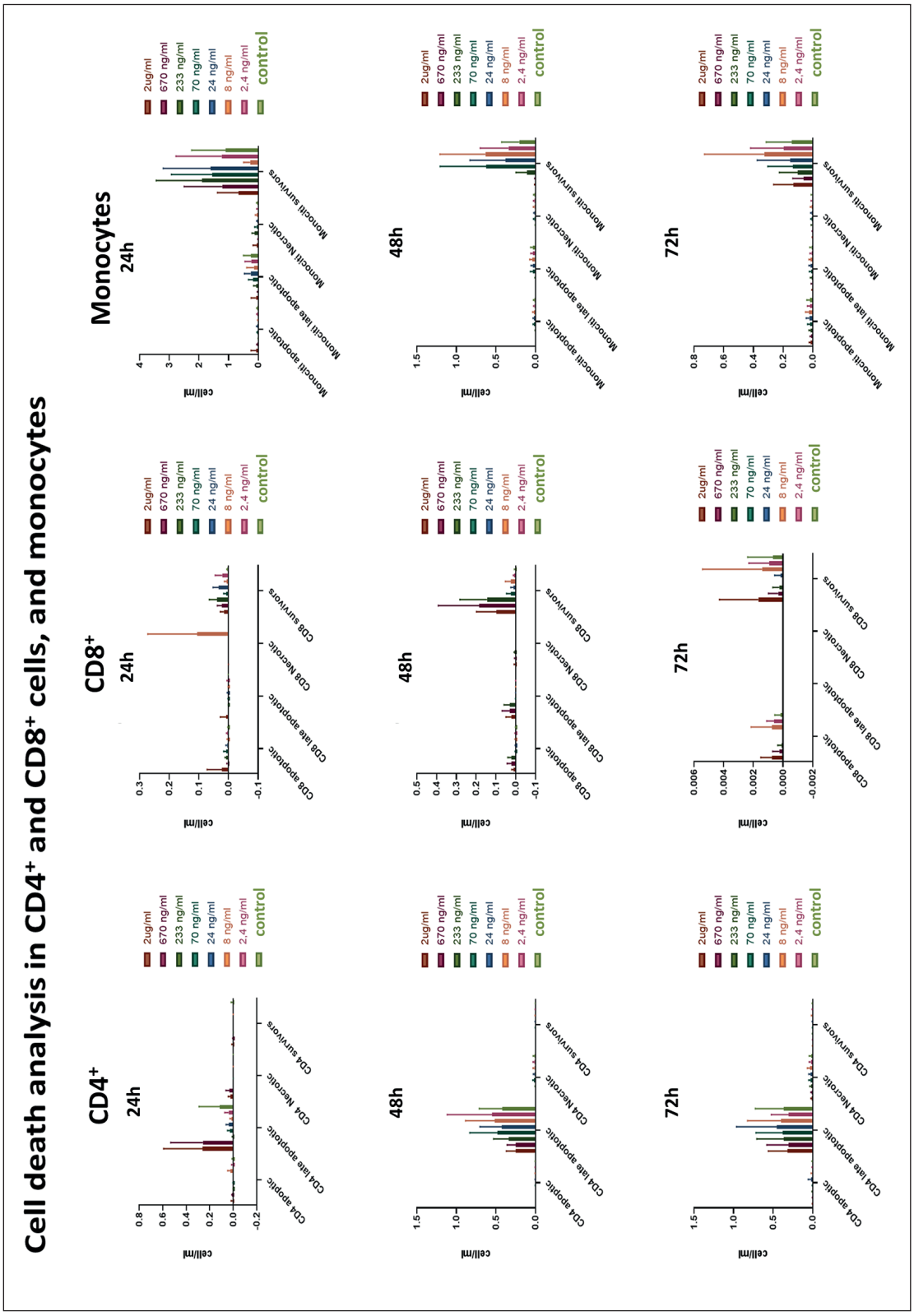

Figure 3. Number of apoptotic $CD4^+$ cells, $CD8^+$ cells and monocytes counted by flow cytometry after 24, 48 and 72 hours of growth with different concentrations of *Lemma* extracts. No significant difference was detected between experimental and control cell lines.

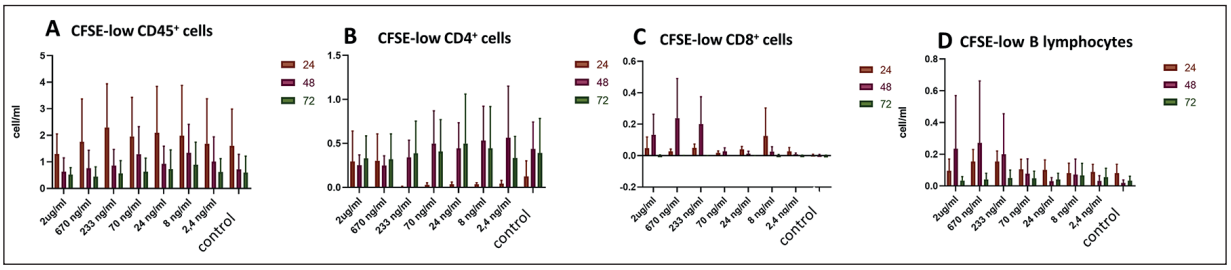

Figure 4. Number of $CD45^+$ (A), $CD4^+$ (B), $CD8^+$ (C) and B (D) cells in culture after 24, 48 and 72 hours of treatment with different concentrations of *Lemna* extracts. The number of cells containing low levels of carboxyfluorescein succinimidyl ester (CFSE) was evaluated.

Conclusions

L. minor has traditionally been consumed by humans and animals. It is currently accepted to contain compounds that have beneficial effects on human health. The present study was designed to demonstrate the alleged immunomodulatory properties of *Lemna* extracts by testing them on human immune cells. Unfortunately, we failed to confirm any immunomodulatory activity as the cells were not significantly affected when grown in medium containing *Lemna* extracts.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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