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ANTI-INFLAMMATORY EFFECTS OF EXTRACTS FROM SOME TRADITIONAL MEDITERRANEAN DIET PLANTS

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It is believed that bioactive compounds from plant foods may have health beneficial effects and reduce the risk of chronic inflammatory diseases. In this study extracts of 121 plants typical for the traditional Mediterranean diet have been screened for their potential anti-inflammatory activities. The ability of the extracts to inhibit cytokine-stimulated, iNOS-dependent synthesis of nitric oxide in murine endothelial cells, without affecting cell viability, was the primary indicator of their anti-inflammatory properties. Based on these experiments we selected eight plant extracts for further analysis: *Chrysanthemum coronarium* L., *Scandix pecten-veneris* L., *Urospermum picroides* (L.) Scop. Ex F. W. Smith, *Amaranthus cf. graecizans* L., *Onopordum macracanthum* Schousboe, *Eryngium campestre* L., *Artemisia alba* Turra and *Merendera pyrenaica* (Pourret) Fourn. Only the effects of *Onopordum macracanthum* could be non-specific since the extract strongly inhibited total protein synthesis. All remaining 7 extracts decreased nitric oxide and TNF α synthesis in the cells of monocyte origin activated with LPS, and 4 of them significantly reduced surface expression of VCAM1 on TNF α -stimulated endothelial cells. All seven plant extracts decreased cytokine or LPS-stimulated iNOS mRNA levels in both cell types. Further research to identify bioactive compounds influencing intracellular signaling pathways activated by cytokines and LPS will consequently be needed in order to better understand these *in vitro* effects.

Key words: *Mediterranean diet, chronic inflammatory diseases, nitric oxide, TNF α , VCAM1, bioactive compounds*

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INTRODUCTION

Chronic inflammation of unknown origin is a common trait of several diseases such as rheumatoid arthritis, atherosclerosis, inflammatory bowel disease or allergic disorders. Most of them are listed as accompanying rapid senescence of human population. However, there are regions of the world, such as Japan or the countries of the Mediterranean basin, which are characterized by relatively low frequency of cardiovascular disorders if compared to population of Central and Northern Europe (1, 2). The concept of correlation between the status of public health and the regional diet may lend itself to the possible explanation of that disparity. The significance of certain diet elements for preventing inflammatory-based chronic diseases was originally proposed and explored by Ancel Keys and described in his classical works known as Seven Country Studies. This concept is currently commonly accepted, although similarly to the etiology of many diseases, bioactivity of dietary elements remains mostly obscure. There is a plethora of bioactive substances in plants used as a part of everyday diet, a great number of them already identified (3). The compounds identified so far have been classified with respect to their chemical structure into several families, for instance: flavonoids and polymeric flavonoids, carotenoids, monophenolic alcohols, monoterpenes, phenolic acids, tannins and others (3). It is believed that an appropriate diet enriched in bioactive compounds with anti-inflammatory properties might prevent or mitigate some chronic disorders. Therefore, much effort is being made in order to identify and comprehensively analyze dietary plants that contain such bioactive constituents.

Preliminary results regarding the anti-inflammatory potential of plant extracts and plant-derived compounds are usually acquired from *in vitro* studies utilizing cultures of cells involved in inflammatory processes such as cells of monocyte origin, models of endothelial cells and hepatocytes. This kind of research comprises experiments that determine the abilities of the compounds to limit the synthesis of common mediators of inflammation exemplified by pro-inflammatory cytokines, chemokines, cell adhesion molecules or nitric oxide (3). Additionally, cell culture-based experiments allow to detect possible undesirable side effects of the studied compounds.

Several dietary plants-derived compounds showing anti-inflammatory activities have already been identified. Among the best characterized are flavonoids, quercetin and apigenin, belonging to the large family of polyphenols. Quercetin was shown to significantly inhibit $\text{TNF}\alpha$ and nitric oxide synthesis in LPS-activated macrophages and Kupffer cells (4, 5) as well as suppress induced expression of IL-8 and MCP-1 in human synovial cells (6). Apigenin inhibited $\text{TNF}\alpha$ -induced synthesis of IL-6 and IL-8 in human endothelial cells (7) whereas both quercetin and apigenin decreased induced expression of cell adhesion molecules in endothelial cells (7-9).

The search for bioactive dietary plants with bioactive constituents and the subsequent identification of the compounds responsible for their effects and

unraveling the mechanisms of their action may result in changing of recommended dietary patterns and introduction of new health-beneficial food supplements and natural therapeutics. In the contemporary world, often described as a “melting pot”, changing dietary habits might be easier than anytime in the past.

Here we present the results of our study identifying plants, used commonly in the traditional Mediterranean diet, which may exert anti-inflammatory activities.

MATERIALS AND METHODS

Plant Extracts

Lyophilized ethanol extracts of 121 plants characteristic for Mediterranean diet were provided by the consortium “Local Food-Nutraceuticals” (for details see the paper by Loboda et al. in this volume). After preliminary experiments eight plant were selected for extracts for further analysis: *Chrysanthemum coronarium* L. (Temenos, Greece), *Scandix pecten-veneris* L. (Monopathis, Greece), *Urospermum picroides* (L.) Scop. Ex F. W. Smith (Monopathis, Greece), *Amaranthus cf. graecizans* L. (Heraklion, Greece), *Onopordum macracanthum* Schousboe (El Jardín, Spain), *Eryngium campestre* L. (Masegoso, Spain), *Artemisia alba* Turra (Serranía de Cuenca, Spain) and *Merendera pyrenaica* (Pourret) Fourn. (Serranía de Cuenca, Spain).

The extracts were dissolved in DMSO to the concentration of 50 mg/ml and subsequent dilutions were also prepared in DMSO. The final concentration of DMSO in cell culture media during experiments did not exceed 0.2%. Fractions of the selected plant extracts obtained by means of HPLC, were provided by Dr. Antoine de Saizieu (DSM plc, Kaiseraugust, Switzerland).

Cell Lines

MBE, murine brain microvascular endothelial cells were a gift from Dr. R. Auerbach (Madison, WI, USA); MBE-SV, MBE cells immortalized with SV40 large T antigen; P388D1, murine monocyte/macrophage-like cell line was from American Type Culture Collection (ATCC #CCL-46).

Cytokines

Recombinant human tumor necrosis factor alpha (TNF α) was from Suntory Pharmaceuticals (Osaka, Japan) and from R&D Systems Inc., (Minneapolis, MN, USA) Recombinant human interleukin 1 β (IL-1 β) was from PeproTech INC, (Rocky Hill, NJ, USA).

Antibodies

Murine TNF α ELISA set was from Bender MedSystems GmbH, (Vienna, Austria); the medium from cultures of hybridoma M/K 2.7 (ATCC #CRL-1909) was used as a source of rat anti-murine VCAM1 mAb; FITC-conjugated goat anti-rat IgG was from Sigma Chemicals Co. (St. Louise, MO, USA).

Other materials

Murine iNOS cDNA was a gift from Drs. Q.-W. Xie and C. Nathan (New York, NY, USA); murine VCAM1 cDNA was a gift from L. Osborn (Cambridge, MA, USA).

All tissue culture reagents, including DMEM/Glutamax-1, fetal bovine serum (FBS), and trypsin/EDTA were purchased from Gibco BRL/Life Technologies (Paisley, UK). Radioisotopes, ^{32}P -dCTP and ^{35}S -Trans-label, were from ICN (Costa Mesa, CA, USA). Unless stated differently all remaining reagents were purchased from Sigma.

Cell cultures

Both cell lines were cultured in DMEM/Glutamax-1 supplemented with 10% FBS (complete medium) at 37°C in 5% CO₂ and passaged by trypsinization after reaching 80-90% confluence.

Nitrite assay

MBE or P388D1 were cultured in 96-well plate in complete medium. Medium was replaced with 100 μl of DMEM supplemented with 2% FCS, containing various concentrations of the plant extracts. In some experimental groups MBE cells were stimulated with TNF α (10 ng/ml) and IL-1 β (10 ng/ml), and P388D1 cells with LPS (100 ng/ml). After 24-hour incubation, nitrite concentration in the media was determined by a microplate assay. Briefly, 100 μl aliquots of the culture media were incubated with equal volumes of Griess reagent (1% sulfanilic acid/0.1% N-(1-naphthyl)ethylenediamine dihydrochloride in 2.5% H₃PO₄) at room temperature for 10 min. The absorbance at 545 nm was measured with a microplate reader (SpectraMax, Molecular Devices, Sunnyvale, CA, USA). Nitrite concentration was determined by using dilutions of sodium nitrite in medium as a standard.

Cytotoxicity assay

The cytotoxic activity of the plant extracts was determined by the MTT test (10). After incubation of the cells with the plant extracts (as described for nitrite assay), the media were replaced with the fresh DMEM containing 2% FCS and MTT (0.5 mg/ml) and the cells were incubated for additional 4 hours. Formazan crystals were solubilized in 100 μl of isopropanol containing 5 mM HCl. The absorbance was measured with a plate reader at 562 nm. The absorbance of control cells (incubated with DMSO instead of plant extract) was taken as 100% viability.

Flow cytometric analysis

MBE-SV cells were incubated for 6 h with TNF α (10 ng/ml) in the presence or absence of the plant extracts. The cells were released from plates with trypsin, washed twice with PBS and incubated on ice for 45 min in complete medium containing rat anti-murine VCAM1 moAb and then, after washing, for 30 min on ice in complete medium containing FITC-conjugated anti-rat IgG. The cells incubated only with FITC-conjugated anti-rat IgG served as a control. The staining of the cells was analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA) using Cell-Quest software.

Enzyme-linked immunosorbent assay

P388D1 cells were incubated in 96-well plate for 24 h with the plant extracts in the presence or absence of LPS (100 ng/ml). The media were collected and the measurement of TNF α levels was performed using commercial ELISA test. The assay was carried out according to manufacturer's instruction and the detection was performed using a microplate reader.

Analysis of total protein synthesis

MBE-SV cells were incubated in 6-well plates for 18 h in serum-free, methionine and cysteine-deficient DMEM with plant extracts in the presence or absence of TNF α . After addition of 50 μCi

of ^{35}S -methionine/cysteine (^{35}S -Trans-label), incubation was continued for additional 6 hours. Media were collected and cells washed thoroughly and lysed with a lysis buffer (50 mM Tris/HCl, pH 7.5, containing 0.1% (v/v) Triton X-100). The total protein in samples of lysates (20 μl) was precipitated with 10% TCA, washed 3 times with PBS containing 10% TCA and an excess of unlabelled methionine and cysteine, and dissolved in Protosol. Radioactivity was determined by liquid scintillation counting in a Wallac β -counter (Perkin Elmer). Protein concentration in the samples was determined by means of colorimetric test with bicinchoninic acid.

Northern blot analysis

Cells cultured in 6 cm diameter plates were incubated overnight in DMEM supplemented with 2% FBS and containing LPS (100 ng/ml) or appropriate cytokines (TNF α , 10 ng/ml and IL-1 β , 10 ng/ml) and the tested plant extracts. Total RNA was extracted from cells by Chomczynski and Sacchi method (11). Equal amounts of RNA (10 μg) were fractionated by electrophoresis under denaturing conditions in a 1% agarose-formaldehyde gel (12) and transferred to Hybond-NX membrane (Amersham Biosciences, Little Chalfont, UK). The membranes were hybridized overnight at 65 $^{\circ}\text{C}$ with the relevant probe labeled with [$\alpha^{32}\text{P}$]-dCTP by random priming using High Prime DNA Labeling system (Roche, Basel, Switzerland). After extensive washing, blots were exposed to a phosphorimager screen and the autoradiograms were analyzed by means of Personal Molecular Imager FX (BioRad, Hercules, CA, USA) and Quantity One software (BioRad). Autoradiographic signals were normalized to the intensity of ethidium bromide stained 28S rRNA after background subtraction.

RESULTS

Screening of plant extracts for their anti-inflammatory activity

Screening of plant extracts for their anti-inflammatory activity was performed in the biological assay using murine brain endothelial cells (MBE). MBE cells produce large quantities of iNOS-generated nitric oxide upon stimulation with proinflammatory cytokines such as TNF α + IL-1 β or TNF α + IFN γ (13). Inhibition of nitrite accumulation in media of cells incubated with the cytokines in the presence of the particular plant extract may indicate its ability to limit cell activation. Since it cannot be excluded that lower nitrite levels result from decreased cell viability or disturbed metabolism, simultaneously with the nitrite assay, the MTT assay that indirectly indicates cell viability, was performed. As shown in *Table 1*, among 121 plant extracts tested, 44 moderately or significantly affected cell viability. Among remaining extracts, 27 did not influence nitrite production, 38 showed a moderate effect and 12 strongly inhibited the cytokine-induced accumulation of nitrite in media. More detailed information will be published by The Local Food Nutraceutical Consortium in Pharmacological Research (14).

On the basis of the magnitude of the effect observed and the performance in various biological tests made by other researchers, we chose 8 plant extracts for further analysis. The selected plants included: Compositae (*Artemisia alba*, *Chrysanthemum coronarium*, *Onopordum macracanthum*, *Urospermum picroides*),

Table 1. Summary of a preliminary screen for plant extracts with anti-inflammatory properties

Cytotoxicity				
strong 24	moderate 20	none 77		
		Inhibition of nitrite synthesis		
		strong 12	moderate 38	none 27

Umbelliferae (*Eryngium campestre*, *Scandix pecten-veneris*), Amaranthaceae (*Amaranthus cf. graecizans*), and Liliaceae (*Merendera pyrenaica*).

Inhibition of nitric oxide synthesis in cytokine-activated murine endothelial cells

As a next step, we investigated the correlation between the concentration of selected plant extracts and the level of inhibition of nitric oxide synthesis in the cultures of cytokine-activated MBE cells. As shown in *Fig. 1*, the majority of selected plant extracts exerted the maximum effect at the concentrations between 50 and 100 $\mu\text{g/ml}$. The extracts of *Scandix pecten-veneris* and *Urospermum picroides* slightly affected cell viability at the concentrations of 150 and 200 $\mu\text{g/ml}$, but they significantly inhibited nitrite accumulation already at the concentration of 20 $\mu\text{g/ml}$. The extract of *Onopordum macracanthum* almost completely inhibited nitric oxide synthesis already at the concentration of 5 $\mu\text{g/ml}$. The extract did not affect cell viability up to the concentration of 40 $\mu\text{g/ml}$, however at higher doses was strongly cytotoxic. The extract of *Eryngium campestre* also showed the ability to almost completely inhibit nitrite accumulation in the medium. However, in distinction to *Onopordum macracanthum*, it did not decrease cell viability but rather stimulated cell proliferation or metabolism intensity as demonstrated by the MTT assay (*Fig. 1*).

Inhibition of nitric oxide and TNF α synthesis in LPS-activated cells of monocyte origin (P388D1)

We examined whether the selected plant extracts exert similar anti-inflammatory effect on other cells. Since monocytes and macrophages play a crucial role in inflammation, we chose murine monocyte/macrophage-like cell line P388D1 as a model. As shown in *Fig. 2A* all plant extracts significantly inhibited nitrite accumulation in LPS-stimulated P388D1 cells. Furthermore, all studied plant extracts showed very strong inhibition of the synthesis of TNF α , one of the most important proinflammatory cytokines (*Fig. 2B*). It is worth noting that the extracts not only inhibited LPS-stimulated TNF α -synthesis but also influenced the basal levels of its production. Since macrophages produce autocrine factors that may stimulate TNF α -synthesis, it is possible that the extracts affected intracellular pathways involved in the autocrine signaling.

However, it cannot be excluded that certain extracts had some deleterious effects on P388D1 that might have been undetected by means of the MTT assay.

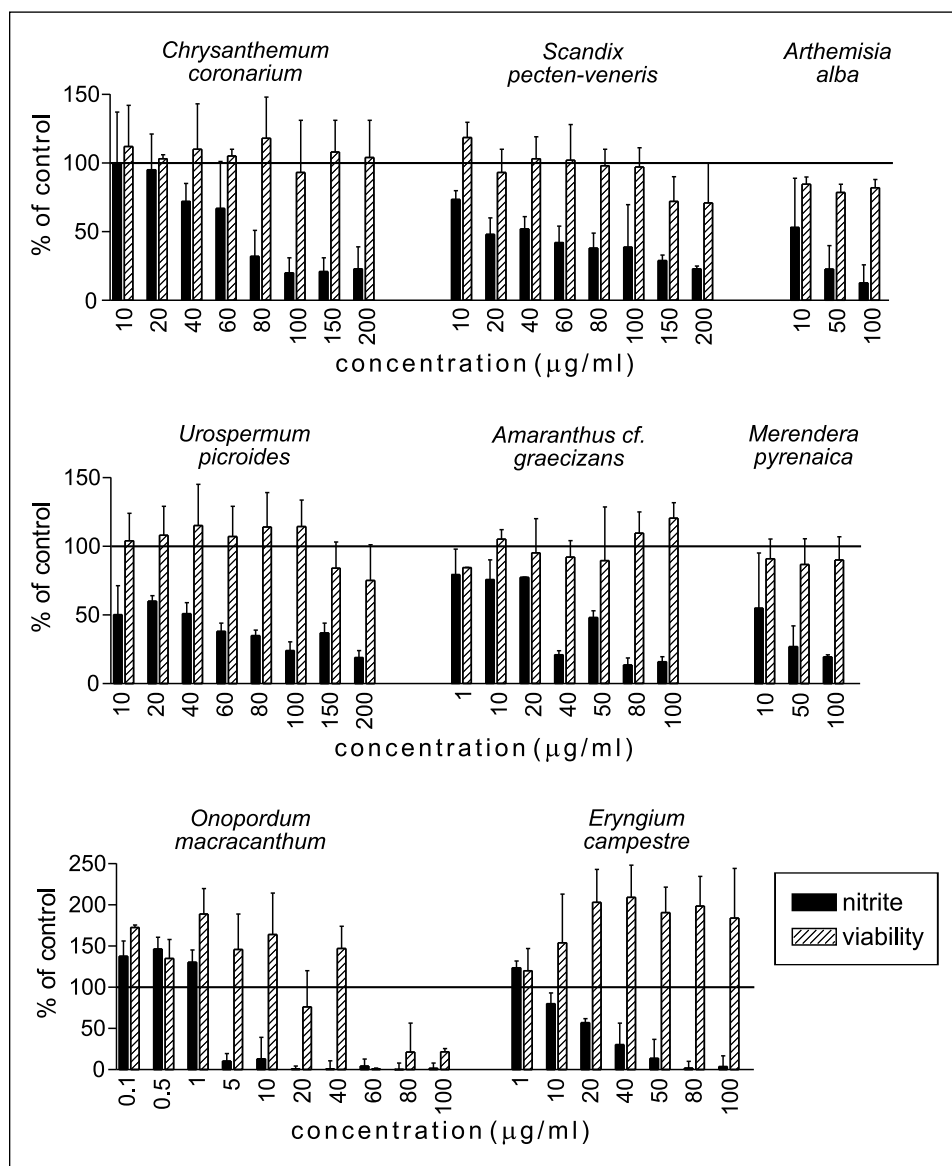


Fig. 1. Analysis of the influence of plant extracts on cell viability and cytokine-stimulated synthesis of nitric oxide in MBE cells. The nitrite concentration in media of cells exposed to the cytokines (TNF α + IL-1 β) in the absence of plant extracts was taken as 100%. Cell viability expressed as the value of A_{562} of the cells exposed to the cytokines in the absence of plant extracts was regarded as 100%. Data are shown as average \pm SD of three to four independent experiments (each in triplicates).

Inhibition of VCAM1 expression on the surface of endothelial cells

We also investigated whether the extracts were able to inhibit proinflammatory phenotypic changes in endothelial cells activated by TNF α . We examined the influence of the extracts on the surface expression of VCAM1, one

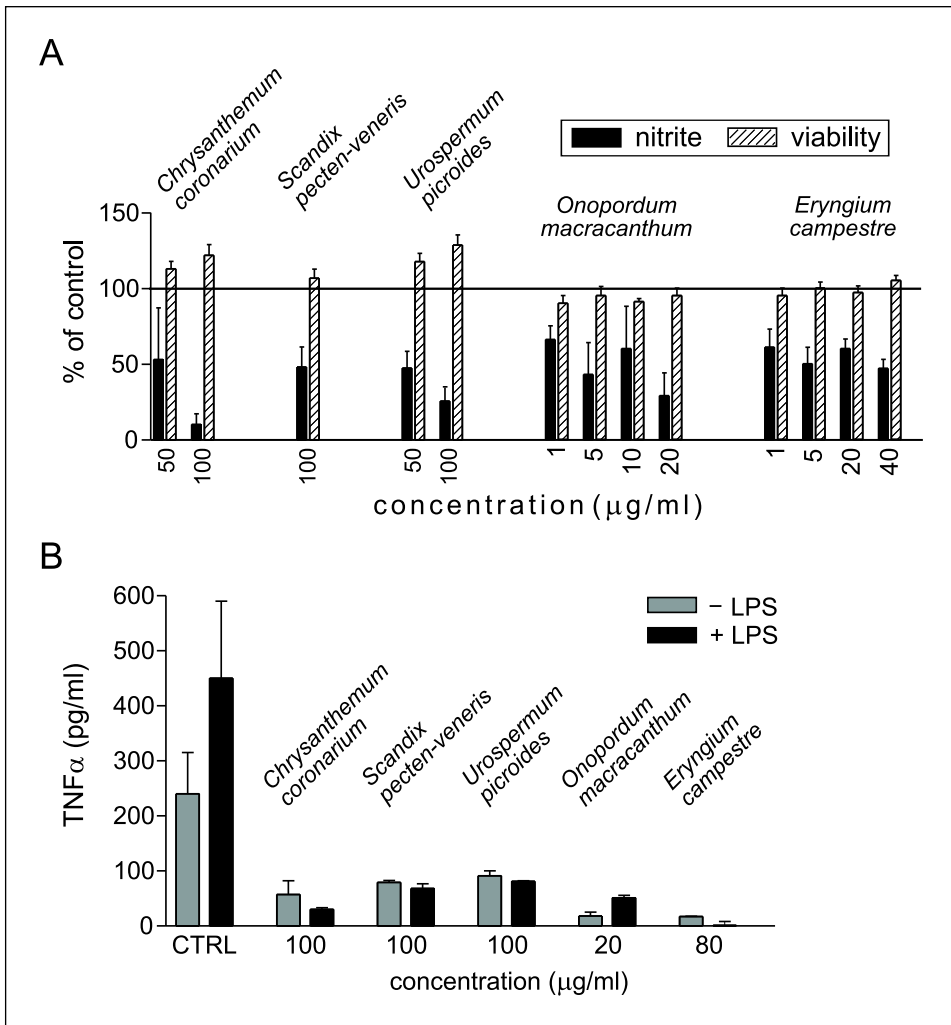


Fig. 2. Analysis of the influence of plant extracts on pro-inflammatory phenotype of cells of monocytic origin (P388D1). **(A)** Analysis of the inhibition of LPS-stimulated synthesis of nitric oxide. The nitrite concentration in the media of cells exposed to LPS in the absence of plant extracts was taken as 100%. Cell viability expressed as the value of A_{562} of the cells exposed to the cytokines in the absence of plant extracts was regarded as 100%. **(B)** Inhibition of the synthesis of TNF α by plant extracts. Data are shown as average \pm SD of three to four independent experiments **(A)**; and two experiments **(B)** (each in triplicate).

of the cytokine-inducible molecules responsible for trans-endothelial migration of immune cells to the sites of inflammation. Only the extract of *Onopordum macracanthum* completely inhibited TNF α -stimulated VCAM1 surface expression (Fig. 3A). The extracts of *Scandix pecten-veneris*, *Eryngium campestre*, *Artemisia alba* and *Merendera pyrenaica* (Fig. 3A, B) moderately decreased the levels of VCAM1 on the surface of MBE-SV cells, whereas the effects of other extracts were not significant (Fig 3A).

The extract of Onopordum macracanthum inhibits total protein synthesis in MBE cells

To exclude the possibility that some of the observed inhibitory effects of the plant extracts are due to inhibition of protein synthesis, we examined whether the extracts influenced the incorporation of radioactive methionine/cysteine into cell proteins. We observed that TNF α itself stimulated protein synthesis in MBE cells. The majority of plant extracts did not inhibit but rather moderately increased protein synthesis in both, unstimulated and TNF α -activated endothelial cells. Only the extract of *Onopordum macracanthum* did inhibit protein synthesis in MBE cells (Fig. 4).

Inhibition of iNOS mRNA levels

To ascertain that the plant extracts inhibit LPS or cytokine-mediated events leading to gene transcription, we analyzed iNOS mRNA levels in the cells exposed to the stimuli in the presence of the extracts. Both, in LPS-stimulated P388D1 and in MBE cells stimulated with TNF α and IL-1 β , all extracts inhibited iNOS mRNA levels, although with different efficiency (Fig. 5, 6). In P388D1 cells the extract of *Amaranthus cf. graecizans*. exerted the most potent effect. Majority of other extracts decreased the levels of iNOS mRNA in both cell types by 30-60%. The extract of *Onopordum macracanthum* did not significantly affect iNOS mRNA level in P388D1, although it strongly inhibited nitric oxide synthesis in those cells. This discrepancy could be easily explained by the observed inhibition of protein synthesis by *Onopordum macracanthum* extract. The *Onopordum macracanthum*-mediated inhibition of iNOS mRNA levels in MBE cells (Fig. 6) was not observed in another experiment (data not shown). The extracts of *Artemisia alba* and *Merendera pyrenaica*, which were examined only in MBE cells, strongly lowered cytokine-induced iNOS mRNA levels (Fig. 6).

Fractionation of plant extracts may result in identification of bioactive compounds

In order to identify the compounds exerting anti-inflammatory effect, the extracts of 5 plants were fractionated by HPLC and fractions tested for the possible cytotoxic activity and the ability to inhibit nitric oxide synthesis in MBE

cells. We were able to identify some fractions of all 5 examined extracts that significantly inhibited nitrite accumulation in the culture medium without affecting cell viability. The exemplary result of the tests performed using

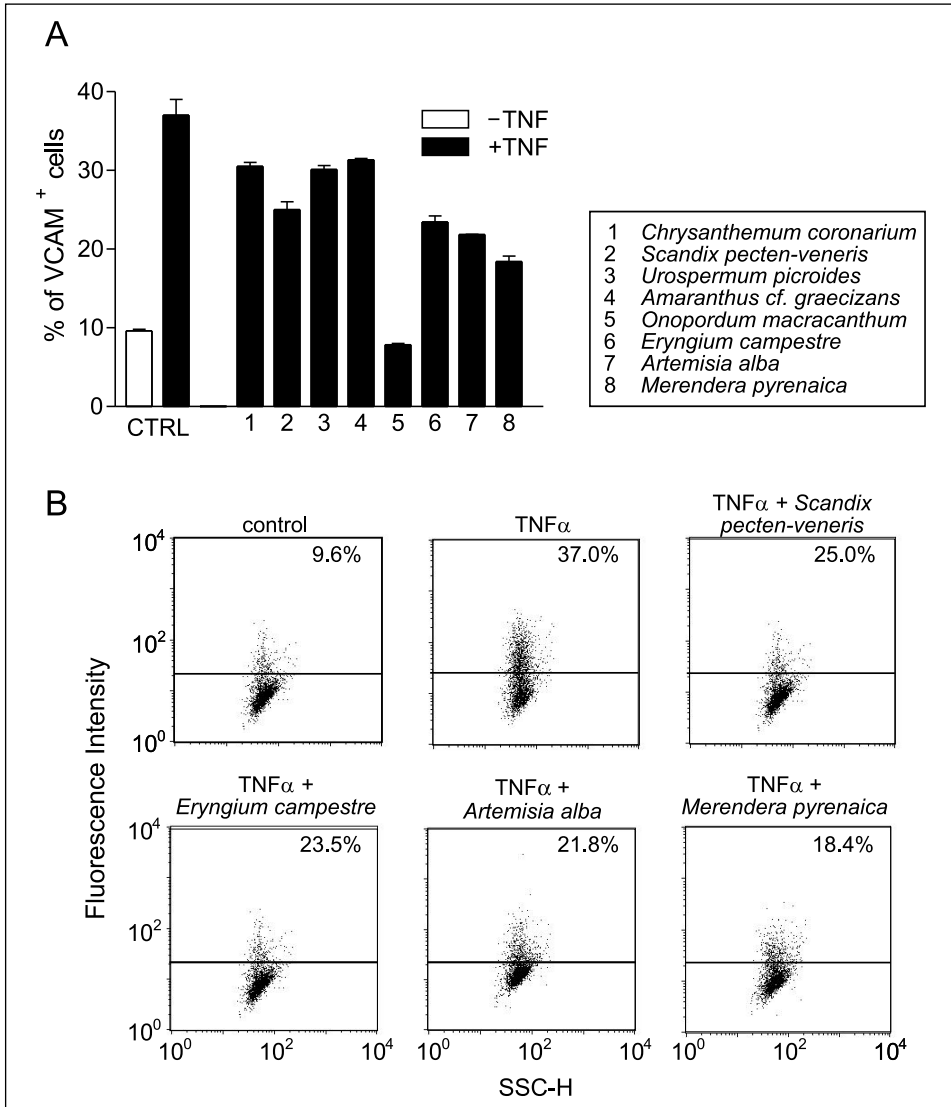


Fig. 3. Flow cytometric analysis of VCAM1 levels on the surface of MBE-SV cells incubated with TNF α in the presence of plant extracts. **(A)** Bars represent the average % of the positive cells \pm SD from one experiment done in duplicates, representative of two performed. **(B)** Comparison of the dot-plots (fluorescence intensity vs. side scatter) of the unstimulated cells, and the TNF α -stimulated cells, and the cells stimulated with TNF α in the presence of the selected plant extracts. Numbers indicate % of VCAM1-positive cells.

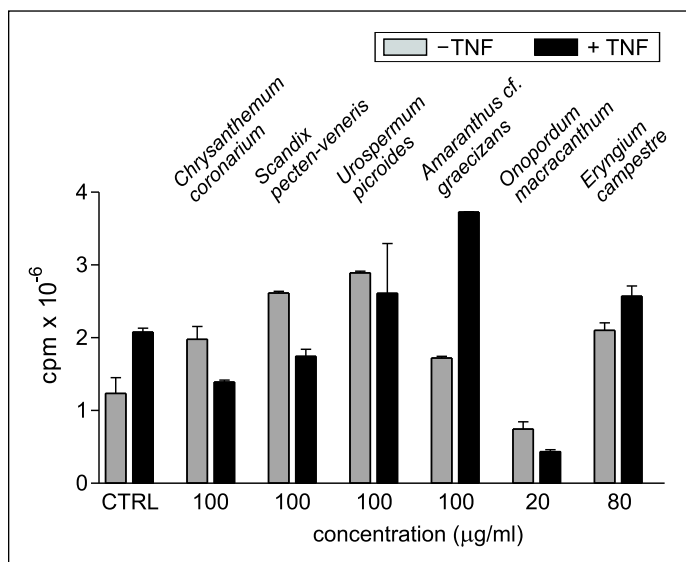


Fig. 4. Incorporation of ³⁵S-methionine/cysteine into the total cell protein in MBE-SV cells incubated for 24 h with the plant extracts in the presence or absence of TNF α . The values of radioactivity were normalized to the protein content in the samples. Data are shown as average \pm SD of two independent experiments performed in duplicates.

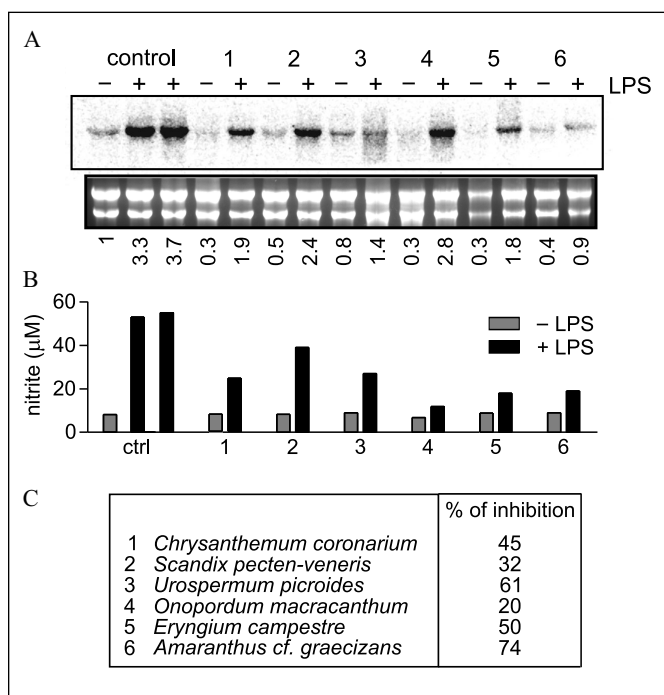


Fig. 5. (A) Northern blot analysis of iNOS mRNA levels in P388D1 activated for 24 h with LPS in the presence of plant extracts. The numbers represent quantification of autoradiography signals from the upper panel normalized to the intensity of ethidium bromide stained rRNA after background subtraction. (B) The levels of nitrite in the media of P388D1 cells used for RNA isolation and Northern blot analysis. (C) The magnitude of plant extract mediated decrease in iNOS mRNA levels. The autoradiography signal of iNOS mRNA in LPS-stimulated cells was taken as 100%.

fractions of the extract of *Eryngium campestre* is presented in Fig. 7. The fractions 15-23 contained compound(s) that very strongly inhibited the synthesis of nitric oxide. Chemical analysis of bioactive fractions done by another member

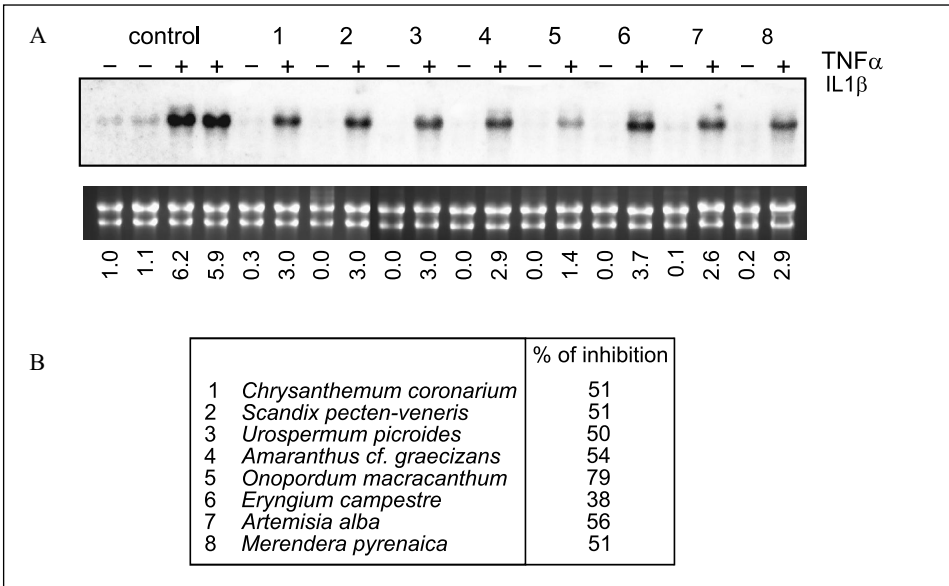


Fig. 6. (A) Northern blot analysis of iNOS mRNA levels in MBE-SV cells activated for 24 h with TNF α and IL-1 β in the presence of plant extracts. The numbers below represent quantification of autoradiography signals from the upper panel normalized to the intensity of ethidium bromide stained rRNA after background subtraction. **(B)** The magnitude of plant extract-mediated decrease in iNOS mRNA levels. The autoradiography signal of iNOS mRNA in cytokine-stimulated cells was taken as 100%.

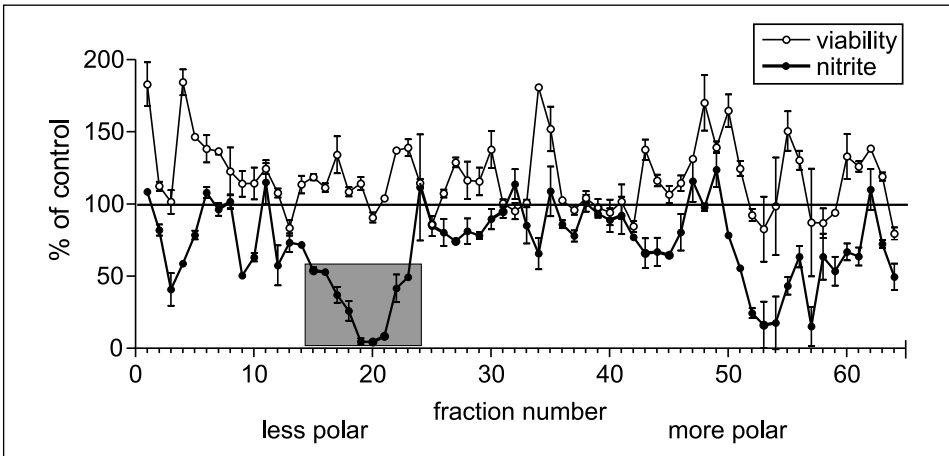


Fig. 7. Analysis of the biological activity of the fractionated extract of *Eryngium campestre*. The influence of 64 HPLC fractions on cell viability and cytokine-stimulated nitric oxide synthesis was examined in MBE-SV cells. The nitrite concentration in the media of cells exposed to the cytokines (TNF α + IL-1 β) in the absence of plant extract was taken as 100%. Cell viability expressed as the value of A₅₆₂ of the cells exposed to the cytokines in the absence of plant extracts was regarded as 100%. Data represent the average \pm SD from two experiments performed in triplicates.

of the Consortium “Local Food Nutraceuticals” may result in further characterization of anti-inflammatory compounds present in the studied plants.

DISCUSSION

Chronic inflammation is a complex, difficult to control, self-perpetuating process that is thought to be responsible for development of many serious diseases including rheumatoid arthritis, atherosclerosis and cancer. Numerous mediators of chronic inflammatory reaction such as chemokines, cytokines, adhesion molecules that might serve as a convenient therapeutic targets, have been identified. Nevertheless, due to the functional redundancy and pleiotropic effects of inflammatory mediators, it is difficult to pinpoint a single molecule or signaling pathway as the best candidate for the therapeutic intervention. For example, cell adhesion molecules including VCAM1 that mediate recruitment of leukocytes are also involved in their activation (15, 16). $\text{TNF}\alpha$, a pleiotropic cytokine produced by activated macrophages, stimulates synthesis of cell adhesion molecules and induces iNOS expression leading to production of biologically active nitric oxide (NO) (17). Activity of this simple compound ranges from suppression of adaptive immunity through modifications of cellular metabolism, to direct cytotoxic effects against pathogens (18-20). One of the major destructive activities of $\text{TNF}\alpha$ is related to stimulation of matrix metalloproteinases responsible for tissue degradation (17). $\text{TNF}\alpha$ neutralizing monoclonal antibodies, infliximab and adalimumab, were successfully introduced to the market as effective drugs alleviating symptoms of rheumatoid arthritis (21, 22). To date, there are several anti-inflammatory therapeutics considered for the commercial production or in early phases of clinical trials, that inactivate not a single cytokine but a range of inflammatory mediators through blocking activation of common transcription factors involved in their induction, such as NF- κ B and AP-1.

Recent recall of popular anti-inflammatory drugs, Vioxx and Celebrex, from the market because of the safety concerns (23, 24), underscored the necessity of finding more suitable replacements, especially in terms of health related hazards. Dietary plants are being recognized as a source of numerous bioactive compounds. Many of them were shown to possess antioxidant properties and inhibit redox-sensitive intracellular signaling pathways leading to cell activation. However, the search for new plants exerting anti-inflammatory properties continues, especially among the plants included in the diets regarded as health-beneficial ones. Clearly, this research has shown that such local food plants are a treasure trove for developing leads for new nutraceuticals and pharmaceuticals, especially for inflammatory conditions (25).

In the current study we have evaluated 121 extracts of plants that are a part of the traditional Mediterranean diet and selected 8 that express anti-inflammatory properties. However, all effects of the *Onopordum macracanthum*, such as

inhibition of TNF α and nitric oxide synthesis, as well as VCAM1 surface expression could be unspecific and result from the potent inhibition of total protein synthesis elicited by this extract. Unlike *Onopordum macracanthum*, other selected extracts neither affected cell viability nor inhibited protein synthesis when applied for 24 hours.

All seven remaining plant extracts strongly inhibited cytokine-induced iNOS-dependent nitric oxide synthesis in MBE cells. The activity of five of them was also examined in P388D1 cells and all diminished LPS-stimulated nitric oxide synthesis as well as production of TNF α . Northern blot analysis demonstrated that the extracts significantly diminished the levels of iNOS mRNA both in P388D1 and MBE cells possibly by influencing the process of *iNOS* transcription or iNOS mRNA stability. A correlation between the magnitude of decrease in iNOS mRNA levels and the inhibition of nitric oxide synthesis was observed for all extracts in P388D1, whereas some discrepancies occurred in MBE cells. For instance, the extract of *Eryngium campestre* at the concentration of 100 μ g/ml almost completely inhibited nitrite accumulation in the MBE culture media but decreased iNOS mRNA level by less than 40% (*Fig. 1* and *Fig. 6*). Since the regulation of iNOS quantity occurs not only at the transcriptional level but also involves iNOS mRNA translation and iNOS protein stability (26), it is possible that some of the studied extracts may affect both transcription and post-transcriptional processes. Although regulation of iNOS enzymatic activity is rather uncommon, the influence of plant extracts on this process cannot be excluded.

Extracts of four plants examined: *Scandix pecten-veneris*, *Eryngium campestre*, *Artemisia alba*, and *Merendera pyrenaica* partially inhibited TNF α -stimulated expression of VCAM1 on the surface of MBE-SV cells. Our preliminary results indicate that reduced surface expression of VCAM1 correlated with the moderately decreased VCAM1 mRNA levels in MBE-SV cells (a single experiment, data not shown).

At present, the mechanisms through which the plant extracts exert their effects are obscure. The experiments performed by our colleagues on other endothelial cells, HUVEC, revealed that the extracts of *Amaranthus cf. graecizans*, *Eryngium campestre* and *Artemisia alba* partially inhibited IL-1 β -mediated activation of transcription factors NF- κ B and AP-1. The extracts of *Scandix pecten-veneris* and *Urospermum picroides* influenced only the activation of AP-1 (27). The extract of *Merendera pyrenaica* was not included in their study. It is highly probable that also in our cell models the mechanism of anti-inflammatory action of these plant extracts is related to the inhibition of intracellular signaling pathways involved in the activation of the transcription factors. The activation of NF- κ B is crucial for induction of VCAM1 expression. Although AP-1 binding site was identified in VCAM1 promoter, the functional contribution of this factor in TNF α -induced VCAM1 expression is not clear (28). Transcription of TNF α is controlled by multiple regulatory elements such as NF- κ B binding site, cAMP-response element, and AP-1 binding site. It has been reported that maximal induction of TNF α

transcription requires the cooperation between NF- κ B and c-Jun complexes (29). The complexity of 5' flanking region of iNOS, the abundance of cis-acting elements together with the significant differences in the regulation of iNOS expression in distinct cell types and species suggest the existence of a few overlapping ways leading to the induction of iNOS expression (26). Yet, similarly to VCAM1 and TNF α , the activation of NF- κ B plays a primary role in the induced transcription of iNOS (26, 30). Thus, these plant extracts that diminish the magnitude of NF- κ B activation may influence the expression of VCAM1, TNF α , and iNOS. However, since the plant extracts-mediated inhibition of VCAM1 surface expression was less pronounced than the inhibition of TNF α and iNOS-dependent nitric oxide synthesis, the effects of plant extracts are presumably more complex.

At this stage of our study it is also difficult to speculate what kind of bioactive compounds might be responsible for the observed effects of the extracts. The majority of identified so far phytochemicals with anti-oxidative and anti-inflammatory activities belong to the vast family of polyphenols (3, 31-34). The content of total polyphenols was determined in the extracts of the studied plants (14). The extracts of *Scandix pecten-veneris*, *Amaranthus cf. graecizans* and *Eryngium campestre* contained moderate or low amounts of polyphenols, 131, 26 and 55 mg/g of extract, respectively, whereas the extracts of *Chrysanthemum coronarium*, *Urospermum picroides*, *Artemisia alba* and *Merendera pyrenaica* had relatively high polyphenols content: 235, 246, 229 and 207 mg/g of extract, respectively. Whether these compounds are responsible for the anti-inflammatory activity of plant extracts remains to be elucidated. However, other compounds, as for example isoflavones and omega-3-fatty acids that were identified in some edible plants of Mediterranean region are also good candidates for anti-inflammatory constituents of the studied species (35).

There is a limited amount of data on the anti-inflammatory activities of extracts or compounds derived from plants related to the species examined in this study. The topically applied hexane extract containing phytosterols of *Eryngium foetidum* reduced phorbol ester-induced acute and chronic auricular oedema in mouse (36). However, the inhibition of carrageenin-induced paw oedema in rats by the hydrophilic extract of *Eryngium maritimum* was not attributed to the steroid-like compound (37). The esters of triterpenes isolated from edible flower extract of *Chrysanthemum morifolium* were shown to inhibit phorbol ester-induced inflammation in mice (38). Another species of this genus, *Chrysanthemum parthenium* is rich in parthenolide (39), an anti-inflammatory sesquiterpene lactone (40).

The screening of fractions of the selected plant extracts allowed the identification of those with the most potent anti-inflammatory activity (Fig. 7) and the future analysis of their chemical content may lead to identification of specific anti-inflammatory compounds.

The result of the presented work should be regarded as preliminary and interpreted with caution. First, we observed significant differences in biological activity of selected extracts between repeated experiments. One possible explanation could be different stability of various compounds with additive or converse effects present in a particular plant extract. It is also possible that the effect of a given extract strongly depends on the cell culture condition as for example on a cell culture density, growth rate or other unidentified parameters. Moreover, even when anti-inflammatory properties of some plant extracts seem to be indisputable in the *in vitro* experiments, the results have to be verified *in vivo* in animal models since bioactive compounds such as polyphenols or other phytochemicals may have limited bioavailability and may also be extensively metabolized (41). Although our results demonstrate promising anti-inflammatory properties of the extracts of *Chrysanthemum coronarium*, *Scandix pecten-veneris*, *Urospermum picroides*, *Amaranthus cf. graecizans.*, *Eryngium campestre*, *Artemisia alba* and *Merendera pyrenaica*, further studies are required to confirm the pharmacological relevance of the findings.

Acknowledgements: We thank Prof. Aleksander Koj for all helpful discussions and the careful review of the manuscript. The studied plants were collected by following scientists: Dr. Zaxarias Kypriotakis, Iraklio, Crete, Greece, (*Chrysanthemum coronarium* L., *Scandix pecten-veneris* L., *Urospermum picroides* (L.) Scop. Ex F. W. Smith, and *Amaranthus cf. graecizans* L.); Drs. Cristina Inocencio and José Fajardo, Murcia, Spain (*Onopordum macracanthum* Schousboe); Dr. Alonso Verde, Murcia, Spain, (*Eryngium campestre* L.) and Dr. Christian Brandt, London, UK, (*Artemisia alba* Turra, *Merendera pyrenaica* (Pourret) Fourn). This work was supported by the European Commission grant (QLK1-CT-2001-00173) and by grant (No. 158/E-338/SPB/5.PR UE/DZ 383/2003) from the State Committee for Scientific Research (Warsaw, Poland).

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Received: January 31, 2005

Accepted: February 15, 2005

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