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Full Length Research Paper

Aqueous extract of *Achillea millefolium* L. (Asteraceae) inflorescences suppresses lipopolysaccharide-induced inflammatory responses in RAW 264.7 murine macrophages

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Yarrow *Achillea millefolium* L. (Asteraceae), is a perennial herb highly recognized in traditional medicine for its anti-inflammatory properties. However, studies on molecular mechanisms underlying these properties are scarce. The present work focuses on examining the effect of aqueous extract of *A. millefolium* L. inflorescences on the inflammatory responses of RAW 264.7 macrophages challenged with LPS. Applied at a concentration range of 25 - 300 µg/ml, the extract did not affect cell viability. LPS-induced NO production was strongly suppressed and correlated with a dose-dependent down-regulation of iNOS protein expression. The extract exerted no significant effect on PGE_2 synthesis and protein COX-2 levels. The secretion of GM-CSF and IL-10 was decreased, while the production of TNF-α and IL-1β was enhanced. The levels of IL-6 were not significantly altered. The anti-inflammatory effects of the extract were partially mediated by the suppression of the activation of transcription factor NF-κB and p38 MAPK signaling cascade. In addition, at the higher concentrations tested, the extract demonstrated good free radical scavenging activity and ability to decrease the levels of intracellular ROS. The present work corroborates the traditional use of *A. millefolium* L. infusions as antiphlogistic drug.

Key words: *Achillea millefolium* L., anti-inflammatory, cytokines, nitric oxide, NF-κB, MAPK signaling.

INTRODUCTION

Inflammation is a hallmark of many diseases such as rheumatoid arthritis, cancer, diabetes and atherosclerosis (Banning, 2005; Krupinski et al., 2008; Khansari et al., 2009; Wu and Zhou, 2009). Two types of anti-inflammatory drugs, steroidal and non-steroidal, are available for treatment. While the first type is more specific, the second type has fewer side effects. This explains the never-ending quest for drugs combining both characteristics. The uniqueness of plant secondary metabolism makes plants one of the richest sources for isolation of such drugs. A classical approach to identify potential target plant species is the use of ethnobotanical leads.

Yarrow *A. millefolium* L. (Asteraceae), a perennial herb native to Europe, is highly regarded for its medicinal activities, including anti-inflammatory properties. The plant has been traditionally used externally for treatment of wounds, burns, swollen and irritated skin and as herbal teas: to relieve gastro-intestinal inflammations. Nowadays, yarrow can be found in herbal pharmacies as tinctures and capsules containing dry flowers or aerials. The plant is included as a component of a variety of industrial tea mixtures and is also an ingredient of phytoremedies (e.g. Amersan).

Studies have shown that two classes of secondary metabolites, isoprenoids and phenolics, contribute mainly to the anti-inflammatory properties of *A. millefolium* L.

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However, a few molecular targets of the active compounds have been identified so far. The fraction of sesquiterpene lactones exerts an inhibitory effect on arachidonic acid metabolism (Kastner et al., 1993), which contributes to the topical anti-inflammatory properties of the herb (Kastner et al., 1993; Maswadeh et al., 2006). Flavonoids inhibit the activity of neutrophyl elastase (Benedek et al., 2007), a key regulator of cell signaling during inflammation (Wiedow and Meyer-Hoffert, 2005). Dicaffeoylquinic acid, on the other hand, targets matrix reorganization by suppressing the activity of metalloproteinases-2 and -9 (Benedek et al., 2007). The latter effects, demonstrated by *in vitro* enzymatic assays, probably contribute to the efficacy of yarrow infusions for treatment of gastro-intestinal inflammations. However, more studies using appropriate *in vitro* models and/or *in vivo* experiments are required to support the internal use of the herb as an antiphlogistic drug.

Macrophages play a central role in the initiation and propagation of the inflammatory process and serve as interface between innate and adaptive immunity. A model system based on macrophages challenged with a prototypic inflammagen, bacterial lipopolysaccharide (LPS), has been traditionally used to study the anti-inflammatory potential of many plant extracts and phytochemicals and to define the mode of their action (Basu and Hazra, 2006; Fang et al., 2008: Verma et al., 2009). The recognition of LPS occurs through Toll-like receptor 4 (TLR4), which upon engagement recruits at least four different adaptor proteins: myeloid differentiation primary-response protein 88, TIR domain-containing adaptor molecule, TIR domain-containing adaptor protein inducing interferon-β and TRIF-related adaptor molecule (Akira and Takeda, 2004). This event leads to nuclear translocation of Nuclear Factor-κB (NF-κB), activation of Mitogen-Activated Protein-Kinases (MAPKs) and further culminates in a rapid change in the expression of numerous genes such as those coding for cytokines, degradative enzymes and a wide range of small-molecule inflammatory mediators (Takeda et al., 2003).

The present work employs LPS-challenged RAW 264.7 murine macrophages as an *in vitro* model system to study the molecular mechanisms underlying the anti-inflammatory properties of *A. millefolium* L. aqueous extract. The effects on NF-κB and MAPK signaling, cytokine secretion, nitric oxide (NO) and prostaglandin (PG) production and the antioxidant activity of the extract, are reported in this study.

MATERIALS AND METHODS

Plant material

A. millefolium L. plants were harvested manually in 2008 in Rhodope Mountains (Bulgaria) at the time of full blossom and authenticated by Dr. D. Ninova (Department of Botany, University of Plovdiv, Bulgaria). A voucher specimen is kept in our laboratory (CIDV, The Biodesign Institute at Arizona State University, USA) for further reference. Aqueous extract of dried inflorescences was prepared by boiling the finely dried material for 10 min in water. After incubation for 30 min at room temperature, the extract was passed through a paper filter. The yield of the dried extract as the percent weight of the dried plant material was 20 ± 1%. The extract was cold-sterilized using Millipore 45 µm filters (Millipore, Billerica, MA), and kept in aliquots at -20°C until use.

Cell line

RAW 264.7 murine macrophages cell line (TIB-71) was purchased from American Type Culture Collection (ATCC). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in a humidified incubator containing 5% CO_2. The subculture procedure was performed according to ATCC recommendations. For all experiments, cells were subjected to no more than 20 cell passages.

Cell viability assay

RAW 264.7 macrophages were plated in 24-well plates at a cell density of 5×10^5 cells/well and treated for 24 h with various concentrations of the extract (25, 50, 100, 200 and 300 µg/ml). Cell viability was quantified by 2-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells were incubated with MTT at a final concentration of 0.5 mg/ml for 4 h at 37°C and 5% CO_2, the medium was then removed and the formazan precipitate formed by the viable cells was solubilized in dimetylsulfoxide (DMSO). An aliquot of 100 µl from each sample was transferred to a 96-well plate and the absorbance at 540 nm was measured on a microplate reader (Dynex Technologies, Chantilly, VA).

NO, prostaglandin E_2 (PGE_2) and cytokine analyses

All analyses were performed using a 24-well format. RAW 264.7 macrophages were plated at a density of 5×10^5 cells/well and treated with various concentrations of the extract in the absence or presence of 1 µg/ml LPS. Twenty four hours later, the medium was collected. The released NO was measured as its end product, nitrite, by a colorimetric assay based on a diazotization reaction using the Griess reagent system (Promega, Madison, WI). Nitrite concentration was determined by a standard curve prepared with sodium nitrite dissolved in the medium. PGE_2 production was measured using Prostaglandin E_2 Express EIA Monoclonal Kit (Cayman Chemical Company, Ann Arbor, MI). Samples were assayed directly without purification after a 10-fold dilution in the medium. Cytokine analysis was performed using a custom mouse Bio-Plex panel (BioRad Laboratories, Hercules, CA) and Bio-Plex suspension array system.

Measurement of intracellular reactive oxygen species (ROS) and free radical scavenging activity

The level of intracellular ROS was determined by labeling with 2',7'-dichlorofluorescein-diacetate (DCFH-DA) dye (Sigma Chemicals Co., St. Louis, MO). The dye diffuses in the cells, gets trapped by deacetylation and in the presence of hydrogen peroxide, peroxynitrite and hydroxyl radicals becomes oxidized to yield 2',7'-dichlorofluorescein. For the assay, cells were seeded in 96-well plates (5×10^4 cells/well) and treated with 1 µg/ml LPS and different plant extract concentrations. Twenty four hours later, cells were washed and incubated with 20 µM DCFH-DA for 25 min at 37°C in

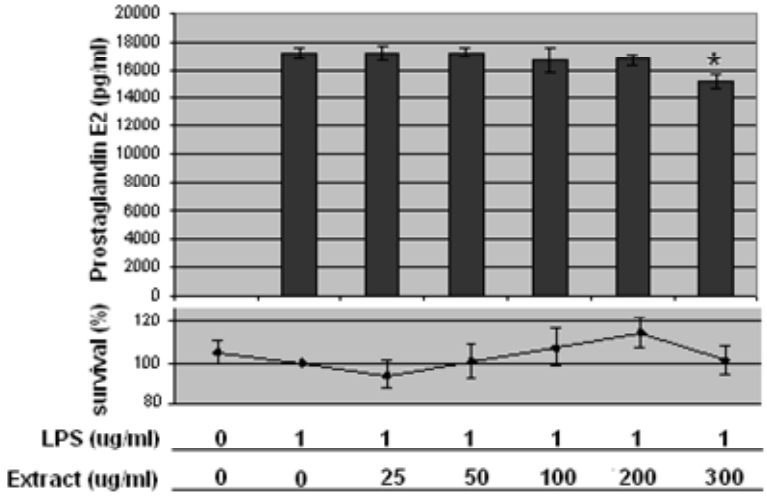

Figure 1a. Effect of *A. millefolium* L. aqueous extract on PGE_2 production: RAW. 264.7 macrophages were treated simultaneously for 24 h with LPS and a range of plant extract concentrations, and PGE_2 levels and cell viability were examined by EIA and MTT assays, respectively. Data are means ± SD (n = 3); statistical significance* (P < 0.05) is based on the difference when compared to the respective positive control.

a CO_2 incubator (5% CO_2). After another wash with warm PBS, the fluorescence (ex 485 nm, em 525 nm) was read using a Spectra Max 190 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). 1,1-Diphenyl-2-picrylhydrazyl (DPPH) scavenging effect was measured according to Dinis et al. (1994). The reaction was performed in 1 ml freshly prepared 0.1 mM methanol solution of DPPH (Sigma Chemicals Co., St. Louis, MO) and various concentrations of plant extract. The absorbance at 517 nm was measured at three time points (0, 2 and 5 min after mixing the plant extract with the DPPH solution) and the scavenging effect was calculated against controls in which the plant extract was replaced with the same amount of vehicle (water).

Western blot analyses

RAW 264.7 cells were seeded in 6-well plates (1 × 10^6 cells/well) and treated with various concentrations of plant extract and 1 µg/ml LPS for 24 h for probing with rabbit iNOS (Sigma Chemicals Co., St. Louis, MO), goat COX-2 and mouse β-actin antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA), or were pre-treated for 1 h with various concentrations of the extract followed by a 15 min treatment with 0.1 µg/ml LPS for probing with rabbit phospho-p38, rabbit phospho-p42/44, rabbit phospho-SAPK antibodies (Cell Signaling Technology, Danvers, MA), goat phospho-IkB-α and mouse β-actin antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Following two washes with PBS buffer, cells were lysed with cold lysis buffer (25 mM HEPES buffer pH 7.4, containing 150 mM NaCl, 1% (w/v) Triton X100, 0.5 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT, 10µl/ml Sigma phosphatase inhibitor mixture, 1 mM PMSF, 10 µg/ml leupeptin, 5 µg/ml aprotinin, 10 µg/ml pepstatin A). Cell debris was removed by centrifugation at 10,000 g for 10 min at 4°C, and protein concentration in the supernatants was measured by Bradford (1976). Total cell lysates with normalized protein content were resolved by SDS PAGE (12% gels) and transferred onto 0.45 µm PVDF membranes. The blots were blocked with TBST (20 mM Tris HCl buffer pH 7.5, 150 mM NaCl, 0.05% v/v Tween 20) containing 5% (w/v) dry nonfat milk for 1 h at room temperature, washed (2 × 15 min) with buffer only, and incubated at 4°C overnight with each of the primary antibodies specified above (1:1000 dilution in TBST buffer containing 1% (w/v) dry milk). Blots were then washed again, incubated with the relevant HRP-conjugated secondary antibody (1:3000 dilution) for 1 h at room temperature, washed again with TBST (2 × 15 min), and developed using an Amersham ECL detection kit (GE Healthcare, Waukesha, WI).

Statistical analyses

Data were expressed as means ± S.D. Statistical analyses were performed with one-way ANOVA followed by Tukey post test; values of P < 0.05 were considered statistically significant.

RESULTS

Effect of *A. millefoilum* L. aqueous extract on viability and LPS-induced PGE_2 and NO production of RAW 264.7 murine macrophages

A 24 h treatment of RAW 264.7 murine macrophages with *A. millefolium* L. aqueous extract at a 25 - 300 µg/ml concentration range had little to no effect on cell viability. Cell survival at the highest concentration tested was 98.19 ± 0.86%. Applied simultaneously with 1 µg/ml LPS for 24 h, the extract did not exert pronounced inhibitory effect on LPS-induced PGE_2 production. Moderate suppression (by ~ 12%) was observed only after co-treatment with the highest concentration examined (Figure 1a). However, no correlated down-regulation of protein levels of COX-2, an inducible form of cyclooxgenase directly involved in prostaglandin synthesis (Harris et al., 2002), was detected (Figure 1d). In contrast, the extract exerted strong and dose-dependent effect on NO production. Treatment with a concentration of 50 µg/ml led to a

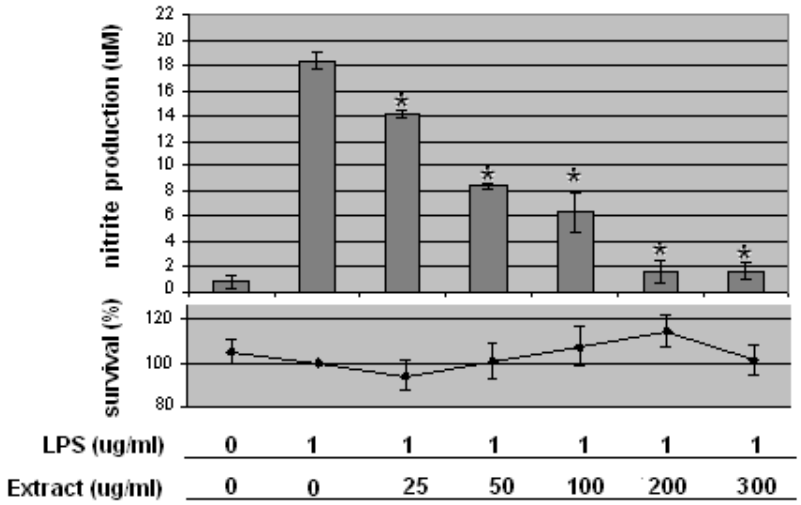

Figure 1b. Effect of *A. millefolium* L. aqueous extract on NO production: RAW. 264.7 macrophages were treated as above, and NO production and cell viability were examined by Griess and MTT assays, respectively. Data are means ± SD (n = 3); statistical significance* (P < 0.05) is based on the difference when compared to the respective positive control.

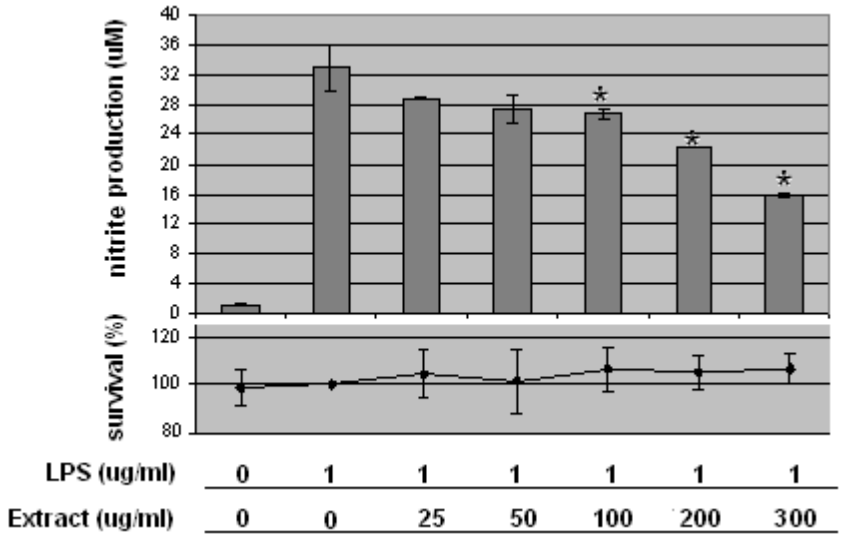

Figure 1c. Effect of *A. millefolium* L. aqueous extract on NO production: RAW. 264.7 macrophages were pre-treated for 24 h with a range of plant extract concentrations, washed, challenged with LPS for 24 h, and NO production and cell viability were examined as in B. Data are means ± SD (n = 3); statistical significance* (P < 0.05) is based on the difference when compared to the respective positive control.

reduction by ~ 55%, while the highest concentration tested, 300 µg/ml, decreased NO levels to less than 10% of those of the LPS-exposed cells (Figure 1b). The observed reduction of NO pool reflected the strong inhibitory effect of the extract on the protein levels of the inducible nitric oxide synthase (iNOS), an enzyme directly involved in LPS-stimulated NO synthesis. (Figure 1d).

Pre-treatment of the macrophages for 24 h with *A. millefolium* L. infusion followed by its removal and a 24 h challenge with 1 µg/ml LPS, also led to a dose-dependent inhibition of the induced NO synthesis although at lower levels: reduction of NO production by half required exposure to the highest extract concentration examined, 300 µg/ml (Figure 1c).

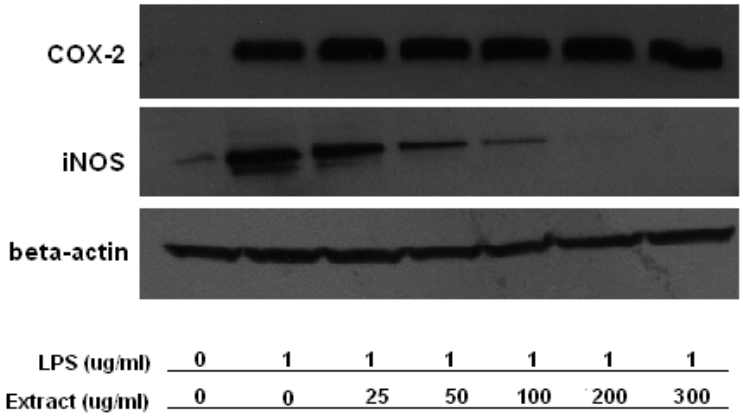

Figure 1d. Effect of *A. millefolium* L. aqueous extract on COX-2 and iNOS protein expression: RAW. 264.7 macrophages were treated as in A., and COX-2 and iNOS expression was monitored by Western blot analysis; beta-actin was used as an internal control. Immunoblots for Figure 1d are representative of three independent experiments.

Effect of *A. millefolium* L. aqueous extract on LPS-induced cytokine production of RAW 264.7 murine macrophages

The effect of the extract on LPS-induced cytokine secretion was evaluated by Bio-Plex suspension array system which allows for simultaneous measurement of different analytes in a single sample. Two different concentrations, 25 and 200 µg/ml, were applied to verify dose-dependency of the effects. No statistically significant changes were observed for IL-6 levels (Figure 2a). There was a dose-dependent inhibition of GM-CSF production (Figure 2b), while, upon treatment with 200 µg/ml extract, the secretion of TNF-α and IL-1β was enhanced by ~16 and ~223%, respectively (Figure 2c and 2d). Interestingly, the levels of IL-10 were distinctly down-regulated (Figure 2e). Cells exposed to the extract alone secreted cytokines at levels indistinguishable from the control at both concentrations tested (data not shown).

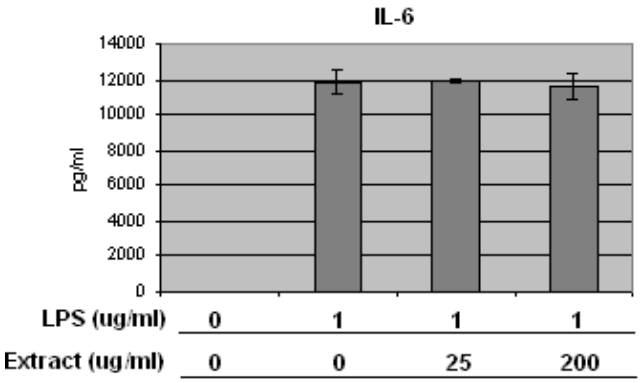

Figure 2a. Effect of aqueous extract of *A. millefolium* L. on LPS-stimulated production of IL-6 of RAW 264.7 macrophages. The amounts of cytokines released in the culture medium after simultaneous 24 h treatment of the cells with LPS and a range of plant extract concentrations were quantified with a custom mouse Bio-Plex cytokine panel using a Bio-Plex suspension array system (BioRad). Data are means ± SD (n = 2); statistical significance* (P < 0.05) is based on the difference when compared to the respective positive control.

Effect of *A. millefolium* L. aqueous extract on LPS-induced NF-κB and MAPKs activation of RAW 264.7 murine macrophages

Classic activation of NF-κB, a key transcription factor in inflammation, involves its translocation from the cytoplasm to the nucleus and requires release from the inhibitory protein IκB (Baeuerle, 1998). This occurs *via* phosphorylation of IκB by IKK and further triggers IκB degradation through the ubiquitin system (Brown et al., 1995, Didonato et al., 1996). *A. millefolium* L. extract suppressed IκBα phosphorylation (Figure 3), thereby preventing the release of NF-kB from the NF-kB:IκB complex.

MAPK signaling regulates the activity of a number of other transcription factors contributing to the amplification of inflammatory responses and also is activated by phosphorylation events. Targets of MAPK kinases are tyrosine and threonine residues on specific tripeptide motifs of MAPKs. Therefore, activation of the three main MAPK signaling cascades, those of extracellular signal-regulated kinase 1 and 2 (ERK1/2), p38 MAPK and stress-activated protein kinases (SAPK1/2), was followed by probing with antibodies recognizing their phosphorylated forms. The results presented in Figure 3 showed that while the extract exerted no effect on ERK1/2 and SAPK1/2 signaling, it strongly inhibited p38 activation.

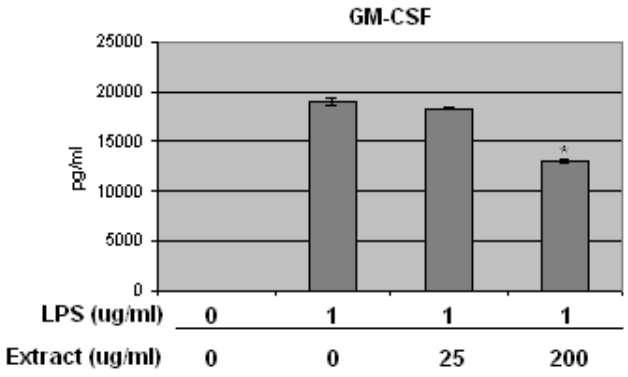

Figure 2b. Effect of aqueous extract of *A. millefolium* L. on LPS-stimulated production of GM-CSF of RAW 264.7 macrophages. The amounts of cytokines released in the culture medium after simultaneous 24 h treatment of the cells with LPS and a range of plant extract concentrations were quantified with a custom mouse Bio-Plex cytokine panel using a Bio-Plex suspension array system (BioRad). Data are means ± SD (n = 2); statistical significance* (P < 0.05) is based on the difference when compared to the respective positive control.

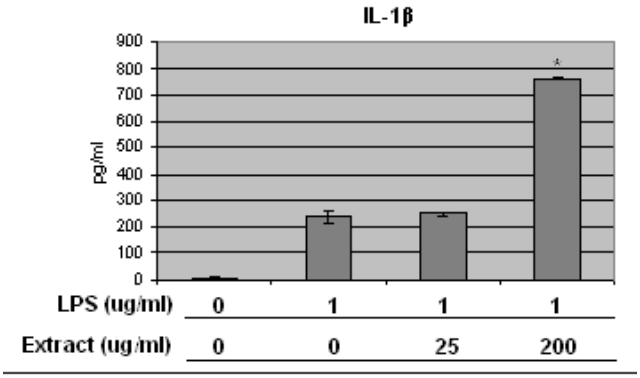

Figure 2d. Effect of aqueous extract of *A. millefolium* L. on LPS-stimulated production of IL-1β of RAW 264.7 macrophages. The amounts of cytokines released in the culture medium after simultaneous 24 h treatment of the cells with LPS and a range of plant extract concentrations were quantified with a custom mouse Bio-Plex cytokine panel using a Bio-Plex suspension array system (BioRad). Data are means ± SD (n = 2); statistical significance* (P < 0.05) is based on the difference.

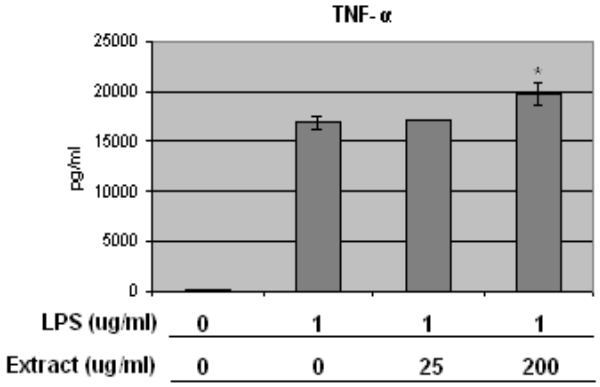

Figure 2c. Effect of aqueous extract of *A. millefolium* L. on LPS-stimulated production of TNF-α of RAW 264.7 macrophages. The amounts of cytokines released in the culture medium after simultaneous 24 h treatment of the cells with LPS and a range of plant extract concentrations were quantified with a custom mouse Bio-Plex cytokine panel using a Bio-Plex suspension array system (BioRad). Data are means ± SD (n = 2); statistical significance* (P < 0.05) is based on the difference when compared to the respective positive control.

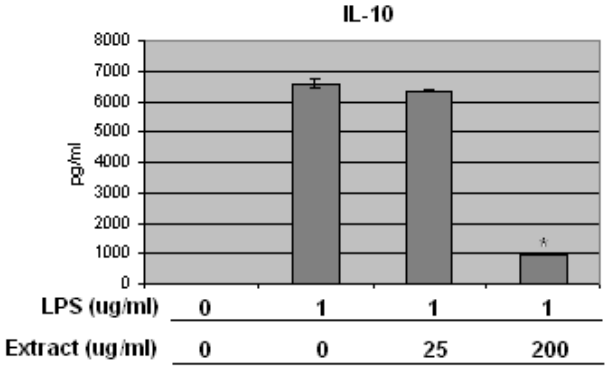

Figure 2e. Effect of aqueous extract of *A. millefolium* L. on LPS-stimulated production of IL-10 of RAW 264.7 macrophages. The amounts of cytokines released in the culture medium after simultaneous 24 h treatment of the cells with LPS and a range of plant extract concentrations were quantified with a custom mouse Bio-Plex cytokine panel using a Bio-Plex suspension array system (BioRad). Data are means ± SD (n = 2); statistical significance* (P < 0.05) is based on the difference when compared to the respective positive control.

Antioxidant activity of *A. millefoilum* L. aqueous extract

The DPPH assay was employed to test for compounds with antioxidant activity in *A. millefolium* L. aqueous extract. In the presence of free radical scavengers or hydrogen donors, the violet color of the stable free radical DPPH changes to yellow and the change can be monitored spectrophotometrically. The ability of the extract for bleaching the DPPH solutions was pronounced at concentrations of 200 µg/ml or higher (Figure 4a). Exposure of RAW 264.7 macrophages to these concentrations also significantly inhibited LPS-induced intracellular ROS production as evidenced by the results of the fluorescent DCFH-DA assay. Treatment with the highest concentration tested, 300 µg/ml, reduced ROS levels by ~ 45% (Figure 4b).

DISCUSSION

Following an ethnobotanical lead, the present study

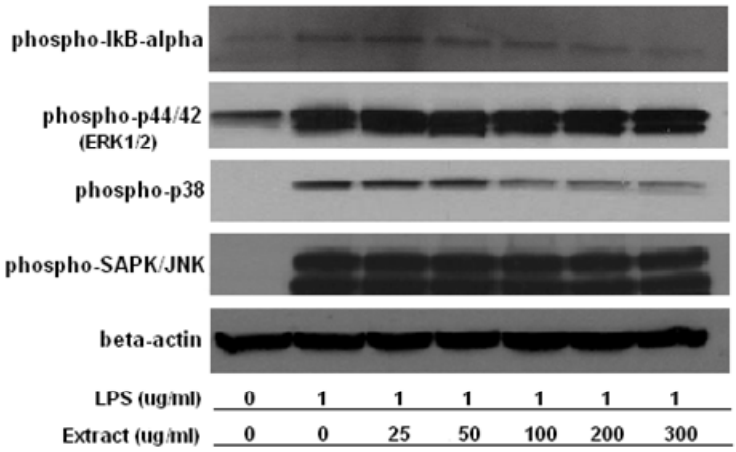

Figure 3. Effect of aqueous extract of *A. millefolium* L. on LPS-induced phosphorylation of IκB-α, ERK1/2, p38, and SAPK/JNK. RAW 264.7 macrophages were pretreated for 1 h with different extract concentrations, and then stimulated with 0.1 µg/ml LPS for 15 min. Nuclear-free whole cell lysates were separated by SDS-PAGE on 12% homogenous gels, and probed with phospho-specific antibodies. Beta-actin was used as a loading control. Blots are representative of three independent experiments.

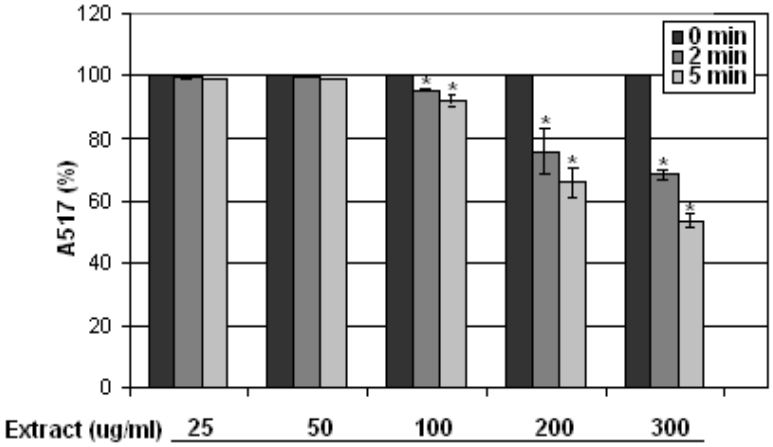

Figure 4a. Antioxidant activity of aqueous extract of *A. millefolium* L.: free radical scavenging activity of the extract in a range of concentrations as measured by the DPPH assay. Data are means ± SD (n = 3); statistical significance* ($P < 0.05$) is based on the difference when compared to the respective control sample.

tested for anti-inflammatory activities of the *A. millefolium* L. aqueous extract using LPS-challenged RAW 264.7 murine macrophages as a model system. We demonstrated that the extract had no detrimental effect on the cell viability at a wide concentration range. This result corroborates previous observations for the low *in vitro* (Giorgi et al., 2009) and *in vivo* (Cavalcanti et al., 2006) cytotoxicity of aqueous extracts of the *Achilea* species. In our test system, the extract inhibited the activation of the main transcription factor, NF-κB, by suppressing IκB phosphorylation. In addition, it impacted negatively the activation of p38 MAPK signaling cascade. These events possibly contribute to the observed down-regulation of iNOS, as NF-κB plays a central role in LPS-mediated iNOS induction (Xie et al., 1994), while p38 MAPK signaling augments this process (Chen and Wang, 1999). Suppression of NF-κB activation is a mechanism underlying the ability of many plant extracts to modulate the expression of iNOS (Woo et al., 2005; Ha et al., 2008; Sarkar et al., 2008). Compounds belonging to different

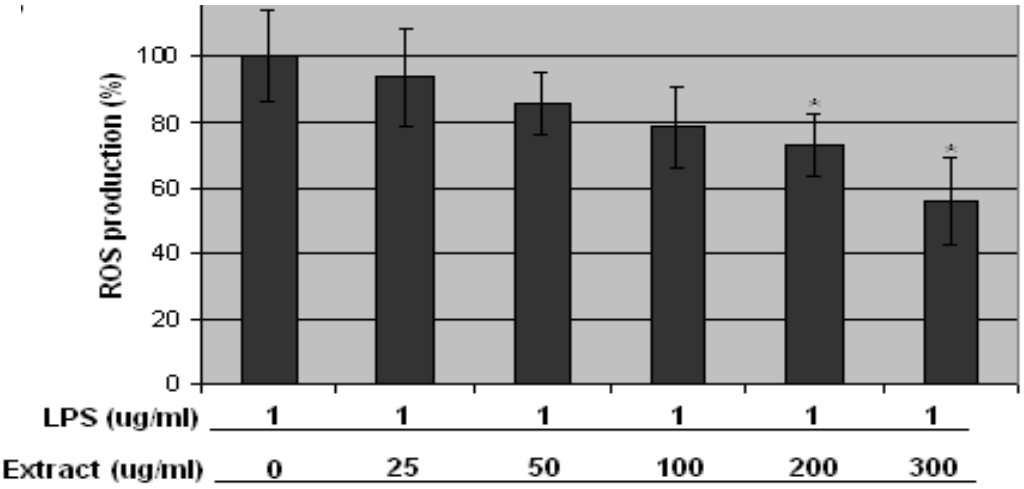

Figure 4b. Antioxidant activity of aqueous extract of *A. millefolium* L.: effect on intracellular ROS production of cells treated simultaneously for 24 h with 1 µg/ml LPS and different extract concentrations as measured by the DCFH-DA assay. Data are means ± SD (n = 3); statistical significance* ($P < 0.05$) is based on the difference when compared to the respective control sample.

classes of secondary metabolites, such as phenolics and terpenoids, show inhibitory properties (Bremner and Heinrich, 2002). This chemical diversity reflects the fact that the pathways of NF-κB activation, canonical and non-canonical, are multi-component and multi-step processes and as such, they can be intersected at different stages. Thus, while curcumin, a phenolic diketone isolated from *Curcuma longa* L., inhibits IKK-1 and IKK-2 activities (Pan et al., 2000), *Amica* spp.-derived sesquiterpene lactone helenalin alkylates p65 subunit of NF-κB (Lyss et al., 1998).

The activity of iNOS exclusively regulates NO over-production in inflammation (Szabo, 1995) and down-regulation of iNOS expression leads to reduction of NO levels. Our results confirm this correlation. The fact that the reduction of NO was much stronger when the extract was simultaneously applied with LPS compared to its use as a pre-treatment before the LPS stimulation, might be attributed to reversible binding of the active plant compounds to their molecular targets.

In contrast to the effect on iNOS, *A. millefolium* L. aqueous extract did not down-regulate COX-2 protein levels at the concentration range tested. This might be due to its inability to affect the activation of all four transcription factors involved in COX-2 control, namely NF-κB, C/EBP, CREB and AP-1. Complete inhibition of COX-2 requires simultaneous suppression of the three MAPK cascades (Hou et al., 2005) and our study clearly demonstrated that ERK1/2 and SAPK1/2 signaling arms remain unaffected. CDK712, a tetrahydroisoquinoline alkaloid that inhibits NF-κB activation and does not affect SAPK1/2 cascade, also suppresses iNOS, but does not down-regulate COX-2 expression. This selectivity is mainly attributed to the compound-mediated inhibition of JAK-2/STAT-1 pathways and induction of heme oxygenase-1 (HO-1) gene expression, which modulates JAK-2/STAT-1 activity (Tsoyi et al., 2008). Further studies are needed to clarify if the seemingly different effects of *A. millefoilum* L. infusions on iNOS and COX-2 levels are related to effects on JAK-2/STAT-1 signaling.

Despite its lack of effect on COX-2 expression, the extract slightly reduced the levels of PGE_2 at the highest concentration examined, 300 µg/ml. A direct inhibition of the activity of COX-2 or other enzymes involved in PGE_2 biosynthesis by the plant compounds, and/or modulation of arachidonic acid release from membrane phosphatidylinositol (Shibata et al., 1988) may account for this effect.

A. millefolium L. extract exerted different effects on the levels of the examined cytokines. Reduced GM-CSF secretion could be linked to extract-mediated inhibition of NF-κB activation (Harrington et al., 1991). The inhibitory effect on IL-10 levels, on the other hand, likely occurs through impact on p38 MAPK signaling which regulates the activity of transcription factor Sp 1 (Ma et al., 2001) playing a key role in LPS-induced IL-10 expression (Brightbill et al., 2000). Two of the studied pro-inflammatory cytokines, TNF-α and IL-1β, were up-regulated upon the extract treatment despite that binding sites for NF-κB have been identified in their promoter regions. This outcome probably reflects the fact that other transcription factors (Godambe et al., 1995; Kominato et al., 1995; Delgado et al., 1998) and additional levels of regulation also contribute to the control of TNF-α and IL-1β gene expression. Thus, the transport of TNF-α mRNA from the nucleus to the cytoplasm is controlled by ERK1/2 cascade (Dumitru et al., 2000). However, according to our results, ERK1/2 signaling does not seem to be affected by *A. millefolium* L. extract.

Antioxidant activity of *Achillea* spp. has been documented in several studies (Konyalioglu and Karamenderes,

2005; Vitalini et al., 2006; Giorgi et al., 2009), and is corroborated by our results. The low total phenolic content in *Achillea* inflorescences (Giorgi et al., 2009) may explain the relatively moderate capacity of *A. millefolium* L. infusions to bleach DPPH. The extract showed potential to reduce the levels of intracellular ROS at the highest concentrations tested, which indicates for a presence of compounds that possibly inhibit the activity of the $O_2^{\bullet-}$ - generating enzymes, NAD/NADH oxidase and xanthine oxidase. Reduction of ROS levels might be another mechanism by which *A. millefolium* L. extract exerts its inhibitory effect on NF-κB activation, as this activation is an oxidative stress-responsive process (Gloire et al., 2006). Yarrow compounds could target the interaction of TLR4 with a NADPH oxidase in RAW 264.7 macrophages, thus following a pattern similar to the one demonstrated for HEK293T cells (Park et al., 2004). The inhibition of ROS production by the extract might also contribute to its suppressive effect on p38 MAPK activation. This suggestion is based on the fact that the formation of the complex between the TRAF6 adaptor molecule and apoptosis signal-regulating kinase 1 (ASK1) which is a prerequisite for p38 MAPK activation through TRAF6-ASK1 axis, requires LPS-induced generation of ROS (Matsuzawa et al., 2005).

In conclusion, the present study confirms the antiphlogistic activity of *A. millefolium* L. aqueous extract. The potential of the extract to suppress LPS-induced inflammatory responses in RAW 264.7 murine macrophages is based on the synergistic action of plant compounds that act as free radical scavengers, target NF-κB and p38 MAPK signaling and further affect the activity of inflammation-related enzymes and production of inflammatory mediators; other possible mechanisms should not be excluded, either. The extract exerted some pro-inflammatory effects, such as enhanced secretion of TNF-α and especially of IL-1β. Further “fingerprinting” could eliminate these effects, thus improving the anti-inflammatory properties of the extract which could eventually lead to the isolation of novel drugs to combat acute and chronic inflammation.

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