Journal of Medicinal Plants Research Vol. 3(9), pp. 686-698, September, 2009
Available online at http://www.academicjournals.org/JMPR
ISSN 1996-0875© 2009 Academic Journals

Full Length Research Paper

Inhibition of endotoxin-induced pro-inflammatory markers by water extracts of *Onopordum cynarocephalum* and *Achillea damascena*

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Accepted 31 July, 2009

Water extracts of *Onopordum cynarocephalum* (*Oc*) and *Achillea damascena* (*Ad*), traditionally used in Lebanon to treat various medical conditions including inflammatory diseases, were evaluated for anti-inflammatory activities employing an *in vitro* model of endotoxin (ET)-induced inflammation in mammary epithelial SCp2 cells and an *in vivo* model of ET-induced paw edema in male rats. Initially, both extracts inhibited the activity of fetal bovine serum derived gelatinase A and B in a concentration-dependent manner. In SCp2 cells, water extracts of *Oc* and *Ad* at non-cytotoxic concentrations of 0.5% and 1%, respectively, decreased the expression of ET-induced inflammation markers interleukin-6 (IL-6) protein and mRNA, as well as gelatinase A and B activity via NF-κB. Furthermore, mRNA and protein levels of β-casein, an SCp2 differentiation marker, were suppressed 48 h post ET-stimulation and were not reversed by either of the plant extracts. In the *in vivo* model intraperitoneally injected water extracts of *Oc* or *Ad* in rats resulted in significant reduction of ET-induced paw edema starting at 12 and 8 h respectively. This new insight into the *Oc* and *Ad* mode of action contribute towards a better understanding of the claimed anti-inflammatory activities reported in folk medicine literature.

Key words: Inflammation, IL-6, gelatinases, NF-κB, mammary cells, *Onopordum cynarocephalum*, *Achillea damascene*.

INTRODUCTION

Medicinal plants have been acknowledged as potential sources of new compounds of therapeutic value (Matu and Van Staden, 2003). When compared to modern medicine, herbal medicines remain popular especially since the former fail to be free from adverse effects and fail to cure some chronic conditions (Phillipson, 2003). In developing countries, it is estimated that about 80% of the population rely on traditional medicine for their primary health care (Matu and Van Staden, 2003). Herbal medicines represent an estimated $60 billion a year global market and some 20% of the overall drug market (U.N. agency report, 2004), hence the need to better understand and to screen medicinal plants for bioactive compounds as a basis for further pharmacological studies.

Over the last 10 years, a significant body of evidence has emerged indicating that naturally occurring substances derived from higher plants are of potential interest for therapeutic interventions in several inflammatory diseases such as rheumatoid arthritis, allergy, asthma, inflammatory bowel disease and others. (Calixto et al., 2003). Initially, numerous studies have focused on the role immune cells play in the initiation and propagation of inflammation. However, current studies aimed to decipher the involvement of non-immune cells, mainly endothelial and epithelial cells, in the inflammatory

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process, suggests an important role of these cells in the innate immune host defense (Skerett et al., 2004; Cook-Mills and Deem, 2005; McClenahan et al., 2006; Griesbeck-Zilch et al., 2007; Kalari et al., 2009; Günther et al., 2009).

The aim of this study is to evaluate the anti-inflammatory activities of 2 aqueous extracts of *Onopordum cynarocephalum (Oc)* and *Achillea damascene (Ad)* known for their use in folk medicine in Lebanon. Towards this aim, we used an *in vitro* mammary epithelial cell culture model and an *in vivo* paw edema inflammatory model (Talhouk et al., 2008; Saliba et al., in press) and demonstrated that water extracts of both plants possessed potential anti-inflammatory bio-activities.

MATERIALS AND METHODS

SCp2 cell culture and treatment

SCp2 mouse mammary epithelial cells of low passage number (17 through 21) kindly provided by P.Y. Desprez, Geraldine Brush Cancer Research Institute, San Francisco, CA were seeded at 1 x 10^6 cells/well (6 -well tissue culture plates) in growth media and placed in a humidified incubator (95% air, 5% CO_2) at 37°C. This growth media consisted of Dulbecco's Modified Eagles Medium and Nutrient Mixture F12 Ham (DMEM/F12) with 5% heat inactivated fetal bovine serum (FBS) (Gibco, Paisley, Scotland), insulin (5 µg/ml, Sigma, St.Louis) and gentamycin (50 µg/ml). 24 h after plating, cells were washed 3 times with phosphate-buffered saline (PBS) and were placed in differentiation medium (DM) which consists of FBS-free DMEM/F12 media, containing insulin (5 µg/ml), hydrocortisone (1 µg/ml, Sigma, St. Louis), ovine prolactin (3 µg/ml, Sigma, St. Louis) and gentamycin (50 µg/ml). Cells were incubated until confluent after 2-3 days. Cells were then shifted to DM supplemented with 1% FBS and treated with 10 µg/ml endotoxin (ET) (Safieh-Garabedian et al., 2004). Samples were collected at 9, 24 and 48 h post ET-treatment.

Treatment with plant extracts

Plant extracts were prepared according to Harborne (1998). A water extract (Decoction) of the aerial part of the plants was prepared by soaking air-dried ground material (4 mm^2 mesh) in already boiling water for 20 min. The ratio (w/v) of plant material to water was $^1/_8$ g/ml for *Ad* is $^1/_7$ g/ml for *Oc* as recommended by folk practitioners. The resulting solution was then filtered through 3 mm Whatman number 2 filter paper sterilized using the 0.2 µm non-pyrogenic sterile-R filter and then stored at - 20°C. Different doses of the plant extracts 0.5, 1, 2, 3, 4 and 8% (volume extracts/volume media) were added to the cultured cells either 30 min before or upon treatment with ET (referred to as Pre- or Co- treatment respectively), or alternatively added for 1, 2 or 4 h and the cultured cells were then washed 3 times with PBS before addition of ET (referred to as Wash Treatment).

SCp2 cell viability as assessed by the trypan blue exclusion method

Cells were plated in a 6-well plate at 1 × 10^5 cells/ml with GM overnight and the following day, shifted to DM. The third day, cells were treated, in triplicates, with the plant extracts at different concentrations. At the end of the treatment, cells were washed with warm 1× PBS before trypsinization and centrifugation. The supernatant was discarded and the pellet was resuspended in 1 ml fresh medium (containing 5% serum). An aliquot of 50 µl of cell suspendsion was mixed with an equal volume of trypan blue and the cells were counted using a hemocytometer. Dead cells stain blue and viable cells are clear. The percentage of dead cells is then determined using the formula:

% dead cells = dead cells / total number of cells × 100.

Interleukin-6 quantification using enzyme linked immuno sorbent assay (ELISA)

Media were sampled from cultures treated with ET (10 µg/ml) and different plant extract concentrations, and from control cultures, at different time points (referred to as "conditioned media (CM"). Protease Inhibitor (Complete™, a broad spectrum inhibitor of serine, cysteine and metalloproteases, Roche Diagnostics GmbH) was added at a concentration of 40 µl (1 tablet dissolved in 2 ml water) per 1 ml of sample. The samples were stored at - 70°C until further use. Mouse Interleukin-6 (mIL-6) Immunoassay ELISA Kit (BioSource International, Inc., USA) was used for the quantitative determination of the IL-6 in cell culture conditioned media. The optical density (O.D) of the plates was measured at a wavelength of 450 nm on an ELISA reader (Thermolabsystems, Multiskan EX). All the samples were run in triplicates.

Substrate-gel electrophoresis (Zymography assay) to assay for gelatinase activities

CM were sampled from cultures at different time points as described before and stored at - 70°C. Gelatinase activity in the medium samples was analyzed using the method described by Talhouk et al. (2008). Briefly, equal sample volumes mixed in 3:1 ratio (V/V) with 4× sample buffer were loaded and run on 7% polyacrylamide gels impregnated with gelatin (4.5 mg/ml). The gels were run in 1× electrophoresis running buffer (0.0025 M Tris-HCL, pH 8.3, 0.192 M glycine, 0.1% SDS). After electrophoresis, gels were washed once for one hour with wash buffer (substrate buffer with 2.5% Triton × -100) at room temperature and then incubated for 24 h in substrate buffer (50 mM Tris-HCL, 5 mM $CaCl_2$, 0.02% NaN_3, pH 8.0) at 37°C. The gels were then stained for 2 h at room temperature in 0.05% Comassie blue R-250 (Sigma, St. Louis, Missouri, USA), in 50% methanol and 10% acetic acid and destained in distilled deionized water for 16 h. The gelatinases were visualized as clear white bands on darkly stained blue gels. Photographs of substrate gels are shown as negative images.

In order to assay for the inhibitory activity of the plant water extracts on the gelatinases found in FBS, equal volumes of 20% FBS mixed with 4× sample buffer were loaded as described above. The gels were cut into fragments of 2 lanes each and then washed with 2.5% Triton ×-100 solution in substrate buffer and incubated in substrate buffer with 3, 5, 10 and 20% concentration of plant extracts. Staining was performed as described above.

Western blot analysis of casein expression (SCp2 cells differentiation marker)

SCp2 cells were seeded in 100 mm petri dishes at 3.0 x 10^5 cells/ml (kept on plastic) and at 5.0 × 10^5 cells/ml (to be dripped with EHS-matrix [Growth factor reduced Matrigel, derived from Engelbreth-Holm-Swarm (EHS) tumor, Collaborative Biomedical Products,

Bedford, MA]). 24 h later, cells kept on plastic were shifted to DM whereas those to be dripped with EHS-matrix were shifted to DM containing diluted EHS-matrix (1.5% vol /vol) as described in Talhouk et al. (2008). The media were changed on a daily basis and cells were treated with endotoxin on day 4 and harvested on day 6 after plating. Cells were lysed with lysis buffer (50 mM Tris-HCL, pH 7.5, 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, 40 µl/ml protease inhibitor) at 48 h post ET treatment. Cell extracts were centrifuged at 15,000 Xg, 4°C. The supernatants were collected and stored at - 70°C. Proteins were quantified using the Bio-Rad Assay. Western Blot analysis for β-casein was conducted as described by Safieh-Garabedian et al. (2004).

RT-PCR for casein

Total cellular RNA was extracted from cultured SCp2 cells on day 6 of culture using RNeasy kit (Sigma, St. Louis, MO) and 1 µg of mRNA was reverse transcribed into first strand cDNA using RT-PCR kit (Reddy Mix version Abgene, Promega). The primers for β-casein (5'-GTGGCCCTTGCTCTTGCAAG-3' and 3'-AGTCTGAGGAAAAGCCTGAAC-5') and β-actin (5'-CGCCTGCGCCTGGTCGTCGACA-3' and 3'-GTCACGCACCGATTTCCCGCT-5') as well as PCR cycling parameter were as described in Talhouk et al. (2005).

Cytokines mRNA levels as determined by ribonuclease protection assay (RPA)

Total cellular RNA isolated as described above was used to assay for the mRNA levels of nine cytokines (IL-12p35, IL-12p40, IL-10, IL-1α, IL-1β, IL-1Ra, MIF, IL-6 and IFNγ). RPA was performed using the mCK-2 RPA kit for mouse cytokines (Ambion, Austin, TX). *In vitro* transcription system (promega) was used to generate the radiolabeled RNA probes according to the manufacturer's protocol. For each hybridization reaction, 5 µg of total RNA samples were incubated with 20 µl of probe mixture containing 3.7×10^5 cpm of each of the 9 mouse cytokine probes in addition to 2 housekeeping genes (L32 and GAPDH). Negative control containing 5 µl of probe mixture incubated with 2 µg of yeast tRNA (Ambion) was included. The probe set was hybridized in excess to target RNA in solution, for 12-p16 h at 56°C after which free probe and other single-stranded RNA were digested with 100 µl of the RNase cocktail containing RNase A + T1 mix in an RNase buffer and incubated for 45 min at 30°C. Digestion was stopped by adding 18 µl of proteinase K in a solution of proteinase K buffer and incubating for 15 min at 37°C. RNA was extracted from each reaction with Tris-saturated phenol and chloroform: isoamyl alcohol. The protected RNA was then precipitated using 4 M ammonium acetate and ice-cold 100% ethanol and incubated for 30 min at - 70°C. After that, the RNA was centrifuged for 30 min at 4°C and the pellets were washed with ethanol, air-dried, resuspended in Gel Loading Buffer II (Ambion), heated at 95°C for 3 min and resolved on a 5% acrylamide 8 M urea denaturing gel at 400 V for 3.5 h. An aliquot of the probes diluted 10× was applied to the gel as a control. Protection of these probes was sequence-specific since yeast tRNA failed to protect any of the probes from digestion.

NF-κB electrophoretic mobility shift assay (EMSA)

SCp2 cells, cultured on EHS-drip, were washed twice in 5 ml ice-cold PBS and the cells were collected (gently scraped by a rubber policeman) and centrifuged at 420 g for 5 min at 4°C. Cell membranes were lysed and the nuclei were released, by resuspending the pellet in 250 µl buffer A (10mM Tris-HCl pH 7.8, 10 mM KCl, 1.5mM $MgCl_2$ and 1 tablet complete protease Inhibitors 30 ml buffer). The suspension was left on ice for 10 min followed by 45 s homogenization at a moderate speed (15000 r.p.m.), using a polytrone (Kinametica, Littau-Luzern, Switzerland). The nuclei were collected by centrifugation at 4500 g for 5 min at 4°C, followed by lysis and resuspension in 100 µl buffer B (20 mM Tris-HCl, pH 7.8, 420 mM KCl, 1.5 mM $MgCl_2$, 20% glycerol and 1 tablet complete protease inhibitors 30 ml buffer), with gentle agitation at 4°C for 30 min. The debris was cleared by centrifugation at 10000 *g* for 30 min at 4°C and the supernatant containing nuclear proteins was stored at - 70°C until used. On the day of the assay, protein quantification, using the microtiter Bradford assay, was performed for the samples, using BSA as a standard. EMSAs were performed using a ^{32}P-radiolabeled deoxyoligonucleotide sequences (from Sigma-Genosys, Cambridge, UK) of NF-kB-binding DNA: W-22, 5'-AGTTGA*GGGGACTTTCCC*AGGC- 3' (consensus sequence italicized) and M-22 (1 -pb missense control), 5'-AGTTGAGGCGACTTTCCCAGGC- 3'. After end labeling with polynucleotide kinase purifying and annealing probes, identical amounts of radioactivity (2×10^4 counts/min) were added to the binding reactions containing 10 µg cell nuclear protein extracts in a final volume of 40 µl in DNA-binding buffer (20 mM HEPES pH 7.9, 1 mM $MgCl_2$ and 4% Ficoll) containing 0.15 µg poly (dl-dC) as a non-specific competitor. The mixtures were incubated for 30 min before separation on non-denaturing 4% polyacrylamide gels at room temperature by electrophoresis in Tris–borate–EDTA buffer. As a control of the specificity of the band, non-labeled oligonucleotide competitors were added in 100 - fold molar excess immediately before the addition of a radiolabeled probe of the same sequence. Distribution of ^{32}P-labeled bands was visualized by autoradiography and the autoradiograms were scanned and quantified using NIH Image 1.62 software (http://rsb.info.nih.gov/nih-image/). The arbitrary values obtained from the scanning were plotted as percentage of the negative (-ET) control at 30 min.

Paw edema

Adult male Sprague-Dawley rats (Charles River, Celco and Como, Italy) were used in all experiments with 5 rats per group. The animals (200 - 250 g) were housed under optimum conditions of light and temperature (12 h light and 12 h dark cycle; 22 ± 2°C), with food and water provided *ad libitum*. All experiments were carried out with strict adherence to ethical guidelines (Zimmerman, 1983). Localized inflammation was induced as described by Talhouk et al. (2004). For each plant extract, 20 rats were grouped into 4 groups. The rats in the first group (control) were injected with de-ionized distilled water (400 µl) intraperitoneally (i.p.) 30 min prior to intraplantar (i.pl.) ET injection (1.25 µg extracted from *Salmonella typhosa* / 100 µl of sterile and pyrogen-free de-ionized distilled water) in the left hind paw. The rats in the second and the third group were injected intraperitoneally with 2 doses of water plant extract of *Oc* and *Ad* (400 and 200 µl, respectively) 30 min prior to ET (100 µl) injection in the left hind paw. The fourth group was injected by plant extract of *Oc* and *Ad* (400 µl) intraperitoneally with no ET injections in the paw. Paw thickness was measured using a caliber at different time points for all groups (n = 5 each) starting before the injections and then at 0, 4, 8, 12, 24, 28 and 32 h following the injection.

Statistical analysis

The bioassay results from individual plant species were analyzed as a Completely Randomized Design with 2 factors, time and

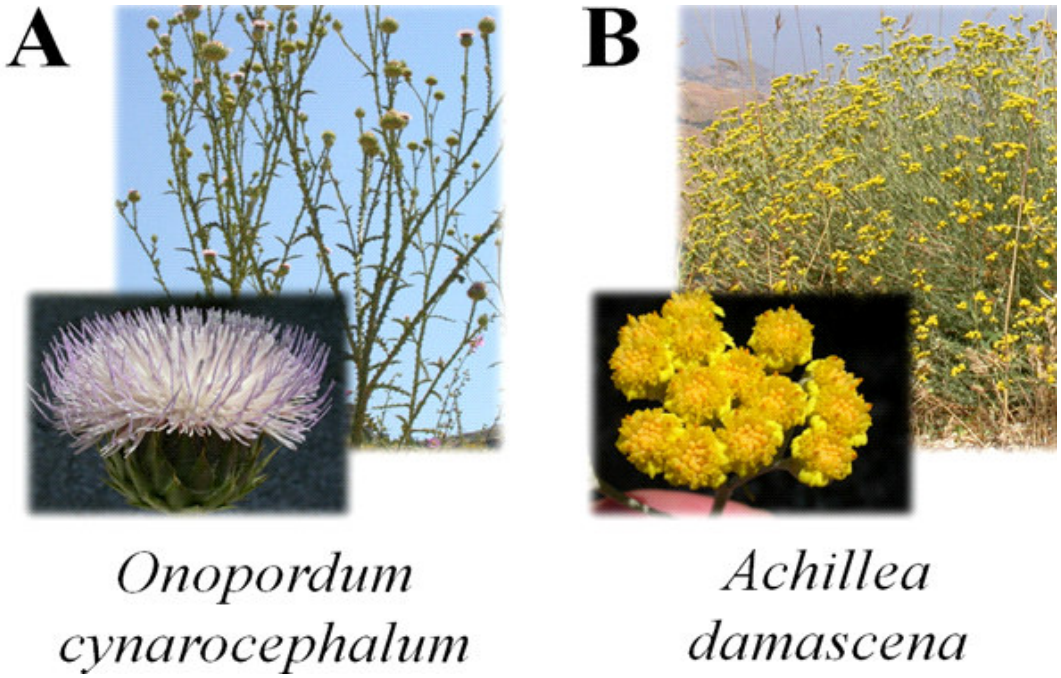

Figure 1. (A) Onopordum cynarocephalum is a plant endemic to Lebanon. It mainly occurs in Juniyah to Khirbat (Sidon) and Tall, Ayn-Zahalta, Hasrun, and Jabal al-Rihan. It is also reported in Anti-Lebanon at the southern base of Mount Hermon. The entire plant is greenish, somewhat cobwebby and minutely glandular-viscid with wings pinnatiparted into strong lanceolate-spines. The lower leaves are oblong-lanceolate and pinnatisect or parted into short, spiny lobes, while the upper leaves are smaller and decurrent. The heads are ovate, 8 to 10 cm in length and the scales of involucres are glabrous and glossy with oblong-triangular outer scales and lanceolate inner ones. The flowering is during the months of May and August, and it grows on hillsides and in fields. [Arabic name: shorter than flowerets *Aqsun harshafi al-ra's* (Talhouk et al. 2008)] (B) Achillea damascena is found in Lebanon particularly in Bsharri, Ihdan, Diman and Hasrun. It is also reported in Anti-Lebanon above Ba'albak, in Wadi-Karn, and in Rashayya to Zabadani. Its involucres are 4 - 5 times longer than its rays that are sometimes 6 - 7 units. The leaves are hoary and often shortened. [Arabic name: Akhilia zat al-alf waraqah (Talhouk et al. 2008)].
Photos Courtesy of Khaled Sleem 2002, IBSAR Nature Conservation Center for Sustainable Features, American University of Beirut, Lebanon). From: Flora of Syria, Palestine, and Sinai. George Post. 2nd edition, edited by John Dinsmore, pp.47, 97. 1932.

treatments, replicated for several times. Analysis of variance was carried out using SPSS (Statistical Package for Social Sciences) version 11 and all means of the appropriate treatments were separated by Duncan's Multiple Range Test (DMRT) ($p \leq 0.05$) using MSTAT-C version 2.1.

RESULTS

A. *In vitro* studies

Assessment for optimal plant extracts concentration

To assess the toxicity of water extracts from traditionally used folk medicinal plants *Onopordum cynarocephalum* (*Oc*) and *Achillea damascena* (*Ad*) (Figure 1), SCp2 cells were treated with varying concentrations of the plant extracts and the number of dead cells 9, 24 and 48 h post ET treatment was determined using the Trypan blue exclusion method (Figure 2). Concentrations below 0.5% for *Oc* and 1% for *Ad* were shown to be non-cytotoxic and comparable to control non-treated cells. Gradual increase in cell death was noted with higher concentrations and prolonged exposure to the plant extracts (Figures 2A, 2B). Therefore, to minimize exposure of cells to the plant extracts, cells were treated with 0.1% and 0.5% *Oc* or 0.5% and 1% *Ad* respectively for 1, 2 or 4 h and then washed off before ET-stimulation. Similarly, viability of cells was checked at 9, 24 and 48 h post ETstimulation. No cytotoxic effect was noted when cells were subjected to such wash treatments even at 48 h post ET treatment (Figure 2C, 2D).

The effect of the plants extracts on et-induced IL- 6

To check if *Oc* and *Ad* extracts were biologically effective, their ability to suppress ET-induced IL-6 in SCp2 cells

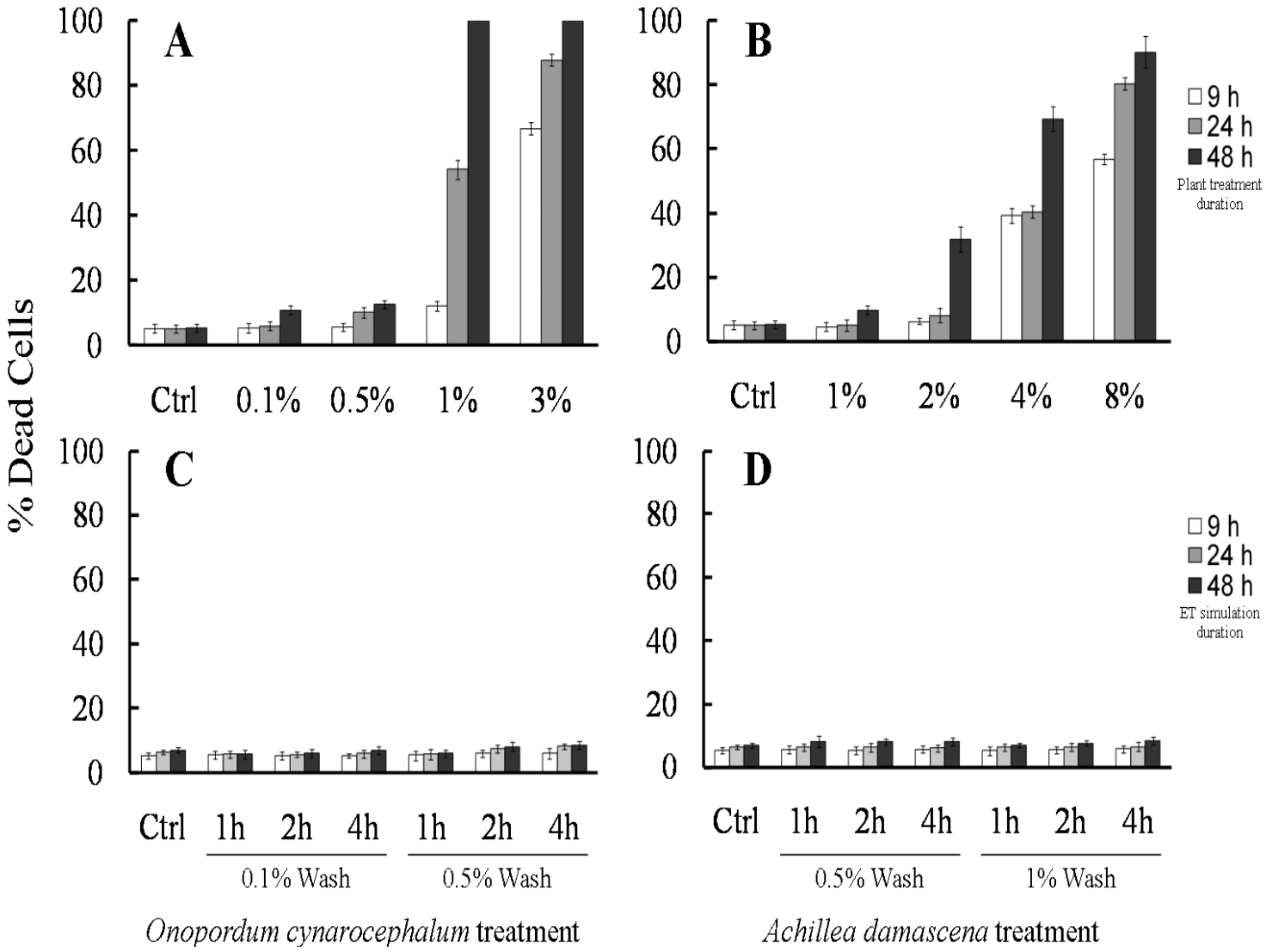

Figure 2. Viability of SCp2 cells with the different *Onopordum cynarocephalum (Oc)* and *Achillea damascena (Ad)* wash-treatment and ET treatment times. Effect of different concentrations of water extracts from (A) *Onopordum cynarocephalum* and (B) *Achillea damascena* on the viability of SCp2 cells. Cells were treated on day 3 of culture for 9, 24 and 48 h with different concentrations of plant extracts in DM supplemented with 1% FBS. Cells were also treated with (C) 0.1 and 0.5% *Oc* or (D) 0.5 and 1% *Ad* for 1, 2 or 4 h each and then washed off prior to 9, 24 and 48 h of ET-stimulation. The values depicted are average values (± SD) of duplicate measurements of 3 independent experiments. Means with the same letter, within each treatment group, were not significantly different according to one-way ANOVA ($p \leq 0.05$).

was determined. The effect of plant extracts on levels of IL-6 produced by SCp2 cells as a result of ET stimulation was analyzed after treating cells with plant extracts at non cytotoxic concentration via one of 3 methods:

(i) Cells were treated with the plant extracts 30 min prior to ET stimulation and left up to sampling time of conditioned media (CM). This treatment was referred to as “Pretreatment” (Pre).
(ii) Plant extracts were co-added with ET and up to sampling time of CM; this treatment was referred to as “Co-treatment” (Co).
(iii) Plant extracts were added for 1, 2 or 4 h and then washed off prior to ET-stimulation of cells.

This latter treatment was referred to as “Wash Treatment” (Wash). CM samples were collected from all treatments 9 h post ET-stimulation and analyzed for IL-6 levels. As depicted in Figure 3, ET increased IL-6 levels by 7-folds compared to the levels observed in control non ET-stimulated cells. *Oc* at 0.1% and 0.5% did not affect the basal levels of IL-6 in non ET-stimulated SCp2 cells.

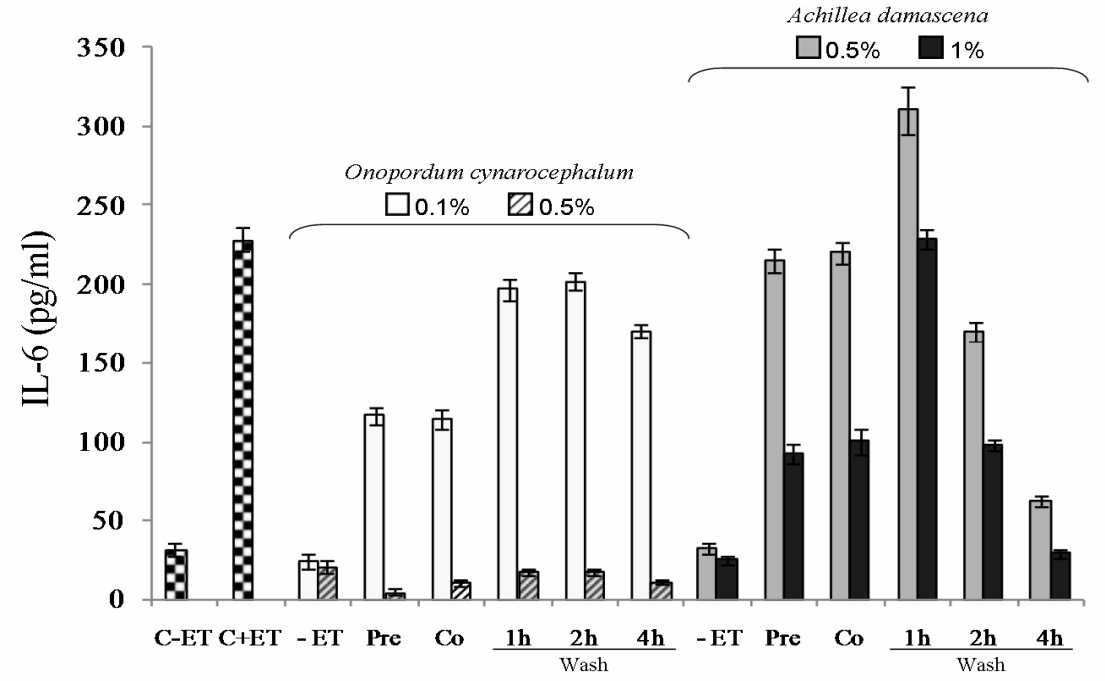

Figure 3. The effect of different concentrations and modes of treatment of *Onopordum cynarocephalum (Oc)* and *Achillea damascena (Ad)* on ET-induced IL-6 in SCp2 cells. Cells in culture were provided with DM supplemented with 1% FBS on day 3. CM samples were collected 9 h post ET-stimulation. Cells were treated with 0.1 and 0.5% *Onopordum cynarocephalum* or 0.5 and 1% *Achillea damascena* without ET [-ET], thirty minutes prior to ET [Pre] treatment, directly with ET [Co] treatment or treated for 1, 2 or 4 h and then washed off prior to ET- stimulation [Wash]. C-ET and C+ET referred to CM from non- and ET-stimulated cells respectively. The values depicted are average values (± SD) of IL-6 from CM of 3 culture plates. Means with the same letter were not significantly different according to one-way ANOVA ($p \leq 0.05$). An adsorption treatment was performed to assess the ability of extracts to interfere with the ELISA reading. The extract was mixed with CM taken 9 h post ET-stimulation. Sustained levels of adsorption treatment as compared to ET-stimulated cells indicated no interference (data not shown).

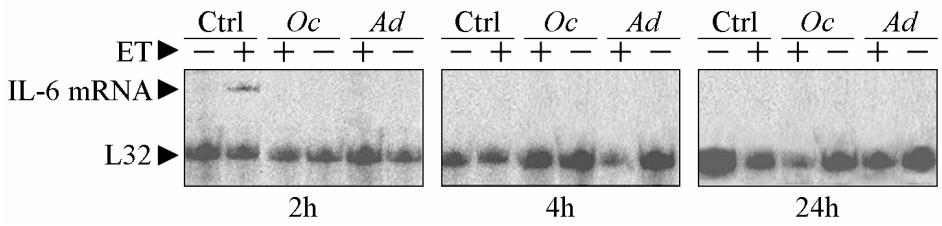

Figure 4. Ribonuclease protection assay for IL-6 mRNA. SCp2 cells were treated on day 3 with ET, *Oc* + ET and *Ad* + ET. Inhibiting effects of IL-6 mRNA expression at 2, 4 and 24 h post ET-stimulation are shown. Protected ribosomal L32mRNA was used as a control.

In contrast, 0.1% *Oc* was able to significantly reduce ET-induced IL-6 whether pre- or co-treated with ET, but has marginally significant effect if subject to "Wash Treatment" for 1, 2 or 4 h. However, 0.5% *Oc* whether pre-, co- or wash-treated, was able to inhibit significantly ET-induced IL-6 to levels comparable to control non ET-stimulated cells. This inhibition was evident during the first hour of subjecting the cells to "Wash Treatment". Taken together, 0.5% *Oc* wash-treated for 1 h prior to ET-stimulation was adopted throughout the rest of the study and this was denoted as "*Oc*+ET", while "*Oc*" alone refers to 0.5% *Oc* wash-treated for 1 h without subsequent ET-stimulation.

Similarly, and as shown in Figure 3, *Ad* at 0.5 and 1% had no effect on basal levels of IL-6 in non ET-stimulated SCp2 cells, while 0.5% *Ad* had no effect on ET-induced IL-6 if pre- or co-treated; however, it exerted marginal but significant increase in ET-induced IL-6 levels if subject to "Wash Treatment" for 1 h (Figure 3). The IL-6 levels significantly decreased with longer "wash treatment"

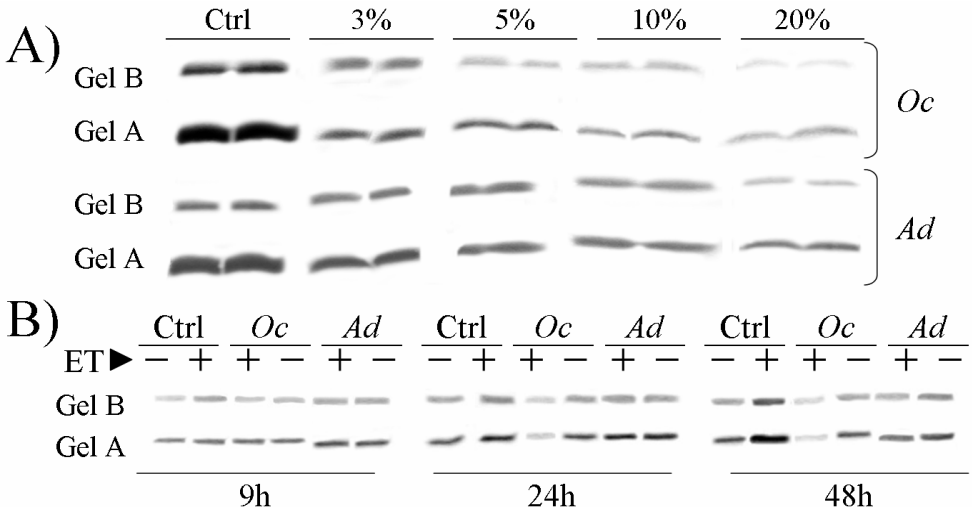

Figure 5. The inhibitory effect of the plant extracts *Onopordum cynarocephalum* (*Oc*) and *Achillea damascena* (*Ad*) on (A) FBS-derived gelatinase A (Gel A) and B (Gel B). Different concentrations of the plant extracts (3, 5, 10 and 20%) were incubated with 20% FBS resolved on gelatin-imbedded polyacrylamide gels. (B) Gelatinase inhibitory effect on the ET-induced gelatinase A (Gel A) and B (Gel B) expressed by SCp2 cells that were treated on day 3 with ET, *Oc* + ET and *Ad* + ET in DM supplemented with 1% FBS. CM samples were collected 9, 24 and 48h post ET-stimulation.

times of 2 and 4 h respectively. On the other hand, 1% *Ad* was able to decrease ET-induced IL-6 whether pre-, co- or wash-treated but was most effective if wash-treated for 4 h. Taken together, 1% *Ad* wash-treated for 4 h prior to ET-stimulation denoted as "*Ad*+ET" was adopted throughout the rest of the study, while "*Ad*" alone refers to 1% *Ad* wash-treated for 4 h but without subsequent ET-stimulation.

The effect of *Oc*+ET and *Ad*+ET on ET-induced cytokines

Ribonuclease protection assay (RPA) was performed to detect the IL-6 mRNA expression after ET, Oc+ET and Ad+ET treatment of SCp2 cells. The most evident regulation upon ET-stimulation was obtained at 2 h with IL-6 mRNA (Figure 4), where ET induced a 2.5 fold increase in IL-6 mRNA levels as opposed to control non ET-stimulated cells. Treatment with *Oc+ET* and *Ad+ET* was also shown to inhibit ET-induced IL-6 mRNA at 2 h post ET-stimulation (Figure 4). mRNAs for IL-12p35, IL-12p40, IL-10, IL-1α, IL-1β and IFNγ couldn't be detected under these experimental conditions. MIF and IL-RamRNAs showed marginal regulation based on preliminary experimentation (data not shown).

The effect of the plants extracts on gelatinase activity

Both *Oc* and *Ad* extracts exhibited dose-dependent inhibition of the activity of FBS-derived gelatinase A and B (Figure 5A). *Oc* extracts at a concentration of 5, 10 or 20% (V/V) demonstrated a more potent inhibition for both gelatinases than *Ad* extracts (Figure 5A). Substrate-gel electrophoresis showed that both gelatinases in CM samples increased 48 h post ET-stimulation as compared to non-stimulated samples (Figure 5B). *Oc*+ET inhibited both gelatinases at 24 and 48 h post ET-stimulation as compared to stimulated control samples (Figure 5B), while *Ad*+ET reduced the activity of both gelatinases only 48 h post ET-stimulation (Figure 5B). Moreover, at 9, 24 and 48 h, *Oc* and *Ad* had no effect on the basal levels of gelatinases as compared to control non ET-stimulated cells (Figure 5B).

The effect of *Oc*+ET and *Ad*+ET on ET-induced NF-κB activation

Safieh-Garabedian et al. (2004) demonstrated that 10 µg/ml ET stimulated maximum activation and translocation of NF-κB in CID-9 cells within 1 h post ET-stimulation. In this study, EMSA was performed to examine the nuclear translocation and activation of NF-κB in SCp2 cells in response to ET-stimulation for 0.5, 1 and 3 h. The data demonstrated that ET stimulated the activation of NF-κB in SCp2 cells starting from 0.5 h and peaking at 1 h with no evident activation of NF-κB at 3 h (Figure 6). Next, cells were treated with *Oc*+ET or *Ad*+ET and the effect on NF-κB was examined. *Oc* had no effect on NF-κB activation in non ET-stimulated cells, whereas

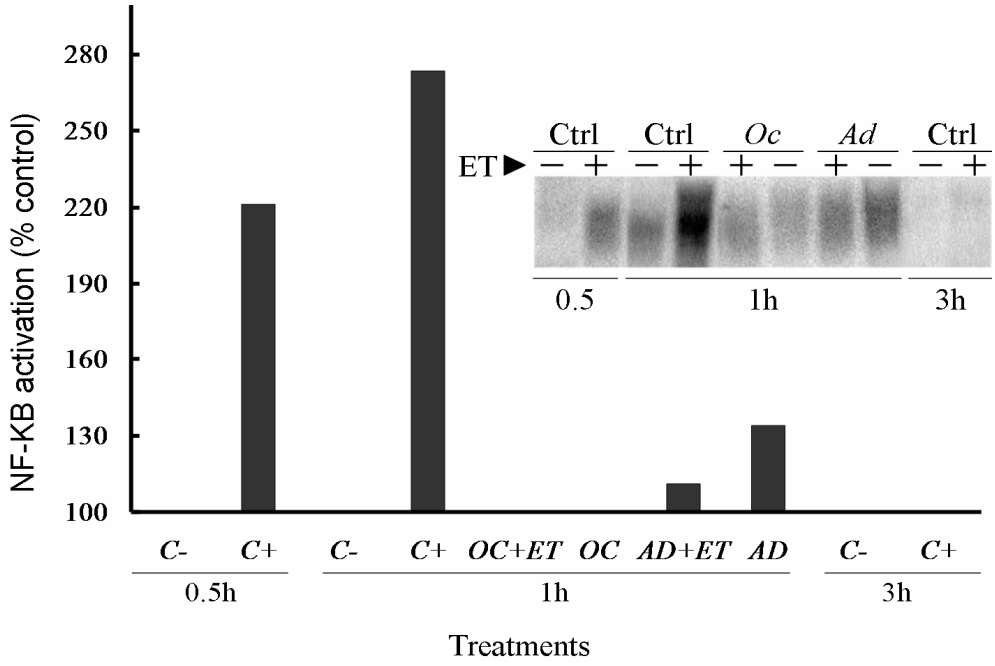

Figure 6. (A) A representative EMSA exhibiting the activation of NF-κB with ET-stimulation at 0.5, 1 and 3h, and the effect *Oc* + ET and *Ad* + ET at 1 h prior ET-stimulation. The maximum activity was observed at 1 h post ET-stimulation which was inhibited by *Oc* + ET and *Ad* + ET. (B) Graphic analysis of ET-induced NF-kB activation showing the effect of *Oc* + ET and *Ad* + ET, as determined by densitometric quantification of the shifted bands, plotted as percentage of control at each time point. C- and C+ referred to non and ET-stimulated cells, respectively.

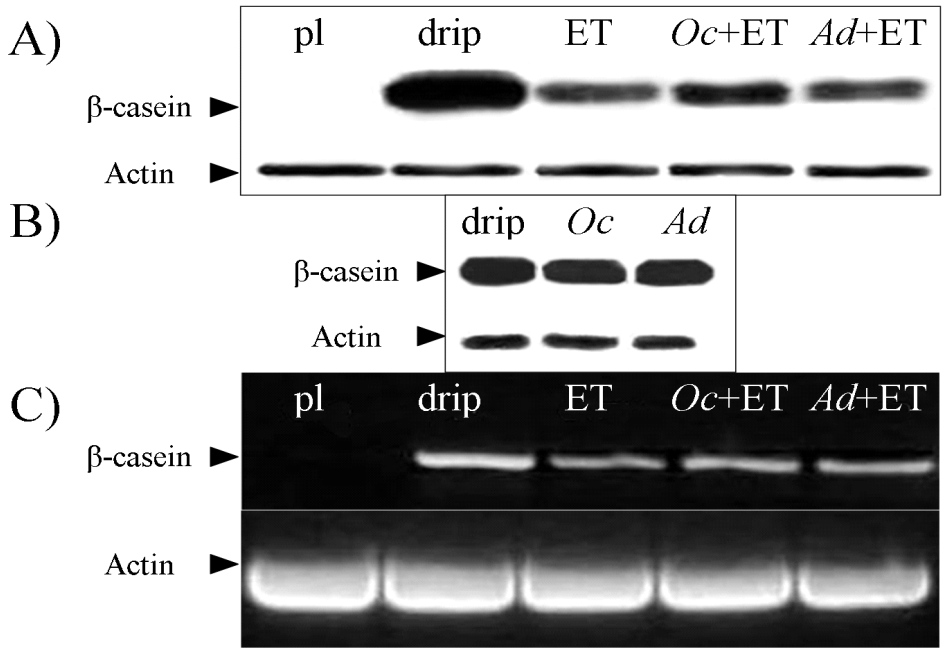

Figure 7. Western blot analysis (A and B) and RT-PCR (C) for β-casein expression in SCp2 cells showing the effect of ET, *Oc* + ET and *Ad* + ET 48 h post ET-stimulation. Cells were plated on EHS-drip (except Pl), treated on day 4 in DM + 1% FBS and harvested on day 6. "Pl" and "Drip" referred to non ET-stimulated cells plated on plastic and drip respectively. Equal loading was determined by actin western blots for (A) and (B) and actin mRNA for (C).

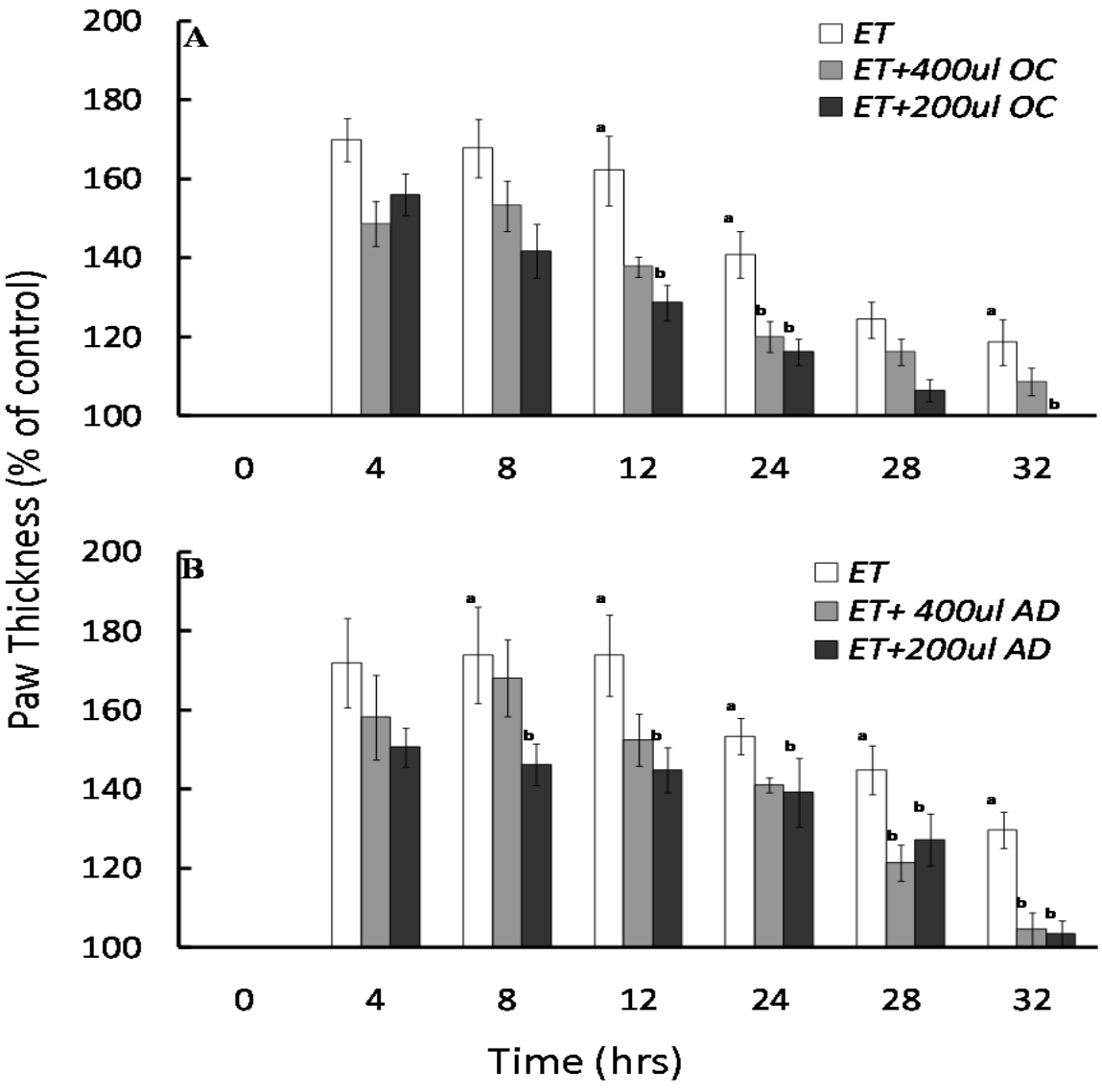

Figure 8. The effect of water extracts from (A) *Onopordum cynarocephalum* (*Oc*) and (B) *Achillea damascena* (*Ad*) on ET-induced paw edema in rats. Rats received intraplantar injections (i.pl.) of ET with or without intraperitoneal injections (i.p.) of different volumes of the plant extract (200 and 400 µl). The values plotted as percentage of control at time zero, depicted an average (± SD) of 5 measurements for each treatment group. Means with same letter, within the same time point, were not significantly different according to one-way ANOVA ($p \leq 0.05$). Absence of letters indicated insignificant difference.

Oc+ET completely inhibited ET-induced NF-κB activation. On the other hand, *Ad* had marginal effect on NF-κB activation as compared to control non ET-stimulated cells, whereas *Ad*+ET inhibited considerably ET-induced NF-κB activation (Figure 6).

The effect of *Oc*+ET and *Ad*+ET on β-casein expression

CID-9 cells, plated on EHS-drip for 4 days and treated with 10 µg/ml ET, slowed down regulation of β-casein expression both at the level of protein and m-RNA (Safieh-Garabedian et al., 2004). To study the effect of ET and the *Oc* and *Ad* plant extracts on the differentiation marker (β-casein) of mammary epithelial cells, SCp2 cells were plated on EHS-drip and on plastic and treated on day 4 with ET, *Oc*+ET or *Ad*+ET. β-Casein western blot (Figure 7A) and RT-PCR (Figure 7C) performed 48 h post ET-stimulation, showed that non ET-stimulated SCp2 cells expressed β-casein only when plated on EHS-drip (drip) as compared to cells plated on plastic

(pl). In addition, ET inhibited cell differentiation as revealed by a significant decrease in β-casein protein and mRNA levels (Figures 7A, 7C). Treatment with *Oc*+ET or *Ad*+ET didn't reverse ET-suppressed β-casein expression to normal levels (Figures 7A, 7C). Moreover, *Oc* and *Ad* had no effect on β-casein expression 48 h post ET-stimulation (Figure 7B).

In vivo studies: The effect of the plants extracts on ET-induced paw edema

2 doses of 200 and 400 µl from 1/7 (w/v) *Oc* (114 and 228 mg/kg body weight respectively) and 1/8 (w/v) *Ad* (100 and 200 mg/kg body weight respectively) were administered intra peritoneal (i.p.) 30 min prior to intra plantar (i.pl.) ET injection in male rats. Paw edema started at 4 h post ET-injection and declined gradually as of 12 h after injection, reaching 40-50 % recovery after 32 h (Figures 8A, 8B). Compared to control ET-induced paw edema, ET-induced paw edema in rats receiving i.p. injection of *Oc* or *Ad* exhibited a significant reduction of edema thickness starting from 12 and 8 h respectively. Intraperitoneal injection of *Oc* or *Ad* was able to reverse ET-induced paw edema to normal thickness by 32 h post ET-treatment and hence significantly earlier than control ET-injected rats (Figure 8A for *Oc* and 8B for *Ad*). Both doses of *Oc* and *Ad* significantly reduced ET-induced paw edema and no significant difference was noted between the 2 doses used.

DISCUSSION

Medicinal plants have been widely used in folk medicine and remain a rich source of novel therapeutic agents. However, the majority of plant species have not been investigated chemically or biologically (Cragg et al., 1997). In the present study, we demonstrate that water extracts from two traditionally used medicinal plants, *Onopordum cynarocephalum* (*Oc*) and *Achillea damascena* (*Ad*), exhibited potential anti-inflammatory activities *in vitro* and *in vivo* models of acute inflammation induced by ET.

The choice of these 2 species was based on a review of the local folk literature which points out the anti-inflammatory potentials and therapeutic properties that the genus Onopordum (*Aqsun harshafi al-ra's*) and Achillea (Akihilia *Zat al-alf Waraqah*) possess (Akeil, 1997; Mkarzel, 1997; Ruwaiha, 1981) and on a preliminary screening study done in our laboratory investigating twenty nine indigenous medicinal plants for their low cytotoxicity, gelatinase inhibitory activity and their ability to suppress ET-induced IL-6 levels (Talhouk et al., 2008).

Trypan blue exclusion staining showed that the optimal working concentrations of *Oc* and *Ad* that did not affect the viability of SCp2 cells were 0.5 and 1% respectively hence, these concentrations were used throughout the study to minimize the exposure of cells to plant extracts and to ensure that viability of cells was not affected while maintaining the extract's bio-activity. Cells were treated for 1, 2 or 4 h with the plant extracts and then washed off prior to ET-stimulation. Furthermore, considering the inhibition pattern of ET-induced IL-6 displayed for each plant extract, the adopted *Oc*+ET and *Ad*+ET treatments limited the exposure time and rendered the viability counts similar to those of control untreated cells. Moreover, this "Wash Treatment" method eliminated the possibility that the plant extracts are blocking ET binding to its receptor or extracts sequestering ET in the media. For example, the inhibitory effect of Catalposide isolated from the stem bark of *Catalpa ovata*, on the pro-inflammatory cytokine production and mRNA expression as well as NF-kB activation in ET-stimulated macrophages was found to be mediated by Catalposide's ability to block binding of ET to CD14 on the surface of cells (An et al., 2002).

In the SCp2 mammary epithelial cell culture model used in the present study, significant amount of secreted IL- 6 can be detected upon stimulating the cells with 10 µg/ml ET. Accordingly, only the modulation of ET-induced IL-6 at both the protein and mRNA levels was tested. ET clearly induced the activation of NF-kB within the first hour, up-regulated IL-6 mRNA expression within the first 2 h and the secretion of IL-6 thereafter into the culture medium of treated SCp2 cells. These results are in line with similar findings reported in the literature (Safieh-Garabedian et al., 2004, Ajuwon et al., 2004; Vona-Davis et al., 2003; Heyen et al., 2000; Libermann and Baltimore, 1990). The marginally significant increase in IL-6 levels noted with *Ad*+ET subjected to "Wash Treatment" for 1 h may be due to the different constituents present in the extract at different concentrations. Some of these constituents may have immuno-suppressive activity while others may be immunostimulant at different times of incubation (Rezaeipoor et al., 1999). For example, Achillea was suggested to work in 3 ways, reflecting the different properties of its constituents: sesquiterpene lactones to stimulate immune function; achillein as an antimicrobial and anti-inflammatory agent; and azulene to reduce inflammation (O'Donnell, 1999). However, Oc+ET and *Ad*+ET exhibited significant inhibition for ET-induced IL-6 which was associated with IL- 6 mRNA and NF-κB inhibition. This suggests that the anti-inflammatory potential of the plant extracts might be in part due to an impaired NF-κB DNA-binding activity or to an upstream interference in the NF-κB activation pathway like the stabilization of the NF-κB associated inhibitory peptide IκB (Ishii et al., 2003). Few studies showing IL-6 inhibition by plant extracts investigated the effect of the extracts at the level of mRNA and transcription factors. For example, Ishii et al. (2003) reported that an

aqueous acetone extract obtained from the pericarps of *Mallotus japonicus*, inhibited IL-6 cytokine production and mRNA expression via suppression of NF-κB activation in lypopolysaccharide (LPS) stimulated macrophages. This suppression was due to attenuation in the LPS-induced degradation of IκB-α protein (Ishii et al., 2003).

The involvement of gelatinases A (MMP-2) and B (MMP-9) and their inhibitors in inflammation and hyperalgysia have been previously established (O'Conor and FitzGerald, 1994; Talhouk et al., 2000). These 2 gelatinases are the dominant MMPs released by most epithelial and endothelial cells (Hanemaaijer et al., 1993; Matrisian, 1992). When tested for their abilities to inhibit gelatinase activities found in FBS, water extracts of *Oc* and *Ad* demonstrated dose-dependent inhibition for both gelatinases. This inhibition may be explained as either a direct binding effect of the chemicals in the plant extracts to these gelatinases rendering them inactive, or as a result of having chelators in the extracts for important MMP cofactors like Zinc or Calcium (Shian et al., 2003; Cheng et al., 2003; Mannello et al., 2001). Previous studies in our laboratory have shown that CID-9 cells plated on EHS-drip and treated daily with 10 µg/ml ET increased the activity of gelatinases A and B throughout 6 days in culture (Safieh-Garabedian et al., 2004). In the present study, SCp2 cells were also able to increase the activity of gelatinases B and A starting from 24 and 48 h respectively post ET-stimulation. Again, *Oc*+ET and *Ad*+ET were able to inhibit the activity of ET-induced gelatinases although with varying kinetics. *Oc*+ET was able to inhibit the activity of ET-induced gelatinases 24 h post ET-stimulation, while *Ad*+ET produced a less potent inhibition after 48 h post ET-stimulation. This inhibition suggests that the plant extracts can probably interfere in the biosynthesis or activation of these gelatinases, since the inhibition was still evident at 24 and 48 h post ET-stimulation although the extracts were initially washed off. Previous work in our laboratory demonstrated that 10 µg/ml ET stimulated maximum activation and translocation of NF-κB in CID-9 cells within one hour of exposure to ET (Safieh-Garabedian et al., 2004). Mammary epithelial SCp2 cells showed a similar pattern of NF-κB activation upon ET stimulation. Oc+ET and *Ad*+ET treatments showed inhibition of ET-induced NF-κB activation, supporting previous findings that demonstared NF-κB inhibition in many plants exhibiting anti-inflammatory potentials (Calixto et al., 2003; Merfort, 2003; Tsai et al., 1999; Bork et al., 1997). The slight activation of NF-κB in cells treated with 1% *Ad*, washed after 4 h and then left for an additional 1 h in media supplemented with 1% FBS can be explained as serum mediated activation or interference of some other immunostimulants found in the plant extract. Alternatively, as mentioned above, *Ad* specific immunostimulants could be inducing the residual NF-κB activation. In some studies, basal and transient NF-κB activation was shown to represent low residual stimulation by serum-derived mitogens (Sasu and Beasley, 2000).

Previous studies on the mammary gland reported that NF-κB was involved in regulating β-casein expression (Doppler et al., 2000). NF-κB activation inhibits the prolactin-induced β-casein expression by 2 mechanisms. NF-κB was able to inhibit the STAT 5 by tyrosine phosphorylation which is an essential step for STAT 5 activation that leads to β-casein gene expression (Yang et al., 2000). In addition, NF-κB can inhibit STAT 5 from binding to the β-casein gene promoter region by allosteric hindrance due to overlapping binding sites for STAT5 and NF-κB in the β-casein gene promoter (Doppler et al., 2000; Geymayer and Doppler, 2000). In support of these findings, we have shown that ET-stimulated SCp2 cells activated NF-κB and suppressed β-casein mRNA and protein levels. Considering the ability of *Oc*+ET and *Ad*+ET to block the activation of NF-κB, one would expect the extracts to reverse β-casein expression to normal levels. However treatment with the studied plant extracts was not able to reverse ET-suppressed β-casein protein and mRNA expressions to normal levels. Preliminary data in our lab showed the ability of other plant extracts to reverse β-casein expression. Further studies are thus required to explain the effect of *Oc*+ET and *Ad*+ET on β-casein expression.

In this study, paw edema was noted at 4 h post ET-injection and declined gradually 12 h thereafter, reaching to 40-50 % recovery after 32 h. Both plant extracts were given thirty minutes prior to ET injection. Both doses of *Oc* showed significant inhibition of paw edema 12 h post ET-injection. While only the lower dose of *Ad* showed significant inhibition of paw edema 8 h post ET-injection. Whereas, the higher dose of *Ad* reduced paw edema significantly at a later time (28 h post ET-injection). This can be explained by the different constituents of the plant extract which could have immuno-suppressive or immuno-stimulant activities at different times and concentrations (Rezaeipoor et al., 1999) and thus would account for the net delayed inhibition of the extract at the higher dose. Overall, both extracts significantly reduced paw edema.

In conclusion, the results of this study indicate that referring to folk literature is a valuable approach to identify plants with bio-active potentials. The 2 tested water extracts of *Oc* and *Ad* showed potential anti-inflammatory bio-activities in *in vitro* and *in vivo* models of ET-induced inflammation. The extracts inhibited ET-induced IL-6, gelatinases and NF-κB activation in SCp2 mammary epithelial cells. However, they were not able to reverse ET-suppressed β-casein expression in SCp2 cells. Furthermore, the extracts exhibited significant anti-edematous effect in ET-induced paw edema model. The nature of these bio-active compounds and their mechanism of action were not determined and will be subject for further investigations.

ACKNOWLEDGMENTS

The authors would like to thank Mohamed El-Zein for his critical reading of the manuscript, Farah Nassar and Pamela Nasr for the preparation of the manuscript and Randa Al-Ashy and Iman Chakroun for their help in EMSA and RNP assays. This work was supported by the American University of Beirut-University Research Board (AUB-URB), the HiTech FZE, Dubai, through IBSAR Nature Conservation Center for Sustainable Futures, the Lebanese National Council for Scientific Research (LNCSR) and the Mercy Corps/Lebanon Program (award number RA:M01/12). The authors are also grateful for Dr. Nayef Saadeh, Department of Human Morphology, Faculty of Medicine, American University of Beirut, for his guidance on animal studies.

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