

Full Length Research Paper

Anti-inflammatory bio-activities in water extract of *Centaurea ainetensis*

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Of a selected 29 plants endemic to Lebanon and claimed to have anti-inflammatory effects, according to Lebanese folk literature, *Centaurea ainetensis* was chosen to further characterize its claimed anti-inflammatory activity using in-vitro and in-vivo assays. Water extract from *C. ainetensis*, at non-cytotoxic concentrations, inhibited in a dose dependant manner interleukin-6 (IL-6) and gelatinases A and B produced by endotoxin (ET)-treated CID-9 cells grown in conditions that favor differentiation, on EHS-drip, at 9, 24 and 48 h post ET treatment. The inhibition was detected in doses as low as 3% (of a 1:8 decoction) and reached maximal levels of inhibition at 8% similar to control non ET-treated cells. *C. ainetensis* extract at these concentrations did not affect CID-9 cell's expression of β -casein, a milk protein expressed by functionally differentiated mammary cells in culture. *In vivo* studies showed that *C. ainetensis* extract reversed ET-induced paw edema and thermal hyperalgesia in rats as demonstrated by hot plate and heat hyperalgesia pain tests.

Key words: Inflammation, IL-6, gelatinases, mammary cells, *Centaurea ainetensis*.

INTRODUCTION

Natural products of plants are major players in traditional medicine practices in developing countries, and recently such products, in the form of herbal medicine, have found their way as alternatives for pharmaceutical drugs in Western countries. Many herbal-based remedies are believed to have a range of biomedical efficacies including treatment of inflammation, hyperlipemia, arteriosclerosis, osteoporosis and bone resorption, and some are reported to have beneficial effect in cardiovascular diseases, immune deficiency, central nervous system disorders, and cancer (Liu et al., 2005 ; Jin et al., 2006 ; Luo 2006 ; Radad et al., 2006). Bioactive compounds from plant extracts are classified according to their chemical structure into several families: flavonoids and polymeric

flavonoids, caretenoids, monophenolic alcohols, monoterpenes, phenolic acids, and tannins, which may be used as health-beneficial food supplements and therapeutics (Strzelecka et al., 2005; Talhouk et al., 2007).

Lebanon, a Middle Eastern country on the Mediterranean Coast, known for its rich floral diversity and unique trade route through history, has had a resourceful literature of folk medicine and herbal remedies for a wide range of diseases including, but not limited to, inflammatory diseases.

Centaurea, a genus of about 500 species of herbaceous plants of the composite family (Asteraceae) and native to the Mediterranean region, is widely used in Middle Eastern folk medicine (Ruwaiha, 1981; Awadallah, 1984; Salah and Jager 2005; Talhouk et al., 2007). In fact, antifungal activities were reported in constituents of *Centaurea thessala* ssp. *drakiensis* and *Centaurea attica* ssp. (Skaltsa et al., 2000), while chloroform extract from *Centaurea musimomum* showed antiplasmodial effects

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(Medjroubi, 2005). Methanolic extract from *Centaurea diffusa* demonstrated antibacterial activities (Skliar et al., 2005) and similarly other species have been used for the treatment of various ailments. For instance, *Centaurea cyanus* and *Centaurea scabiosa* are used against coughs, as liver-strengthening, itch eliminating, and ophthalmic remedies, *Centaurea calcitrapa*, *Centaurea solstitialis* and *Centaurea melitensis* have hypoglycemic effects, and *Centaurea calcitrapa*, *Centaurea iberica* and *Centaurea jacea* have antipyretic effects (Kumarasamy et al., 2003). Although medicinal folk literature ascribes the use of water extracts of *Centaurea* for treatment of ailments (Ruwaiha 1981; Awadallah 1984), no previous reports attribute bio-activities of *Cenaturea* genus to its water extracts, except for an earlier study (Massó and Adzet, 1976) that reported hypoglycaemic effect in diabetic rats receiving chronic oral treatment of water extracts of *Centaurea aspera*.

The aim of this study was to identify indigenous plants of the region known for their medicinal value, and through preliminary screening efforts, characterize the claimed anti-inflammatory effect of the candidate plant that showed least cytotoxicity effect. *C. ainetensis*, Bois. [= *C. eryngioides* Lam. var. *ainetensis* Bois.] (Post and Dinsmore, 1932), a native plant of Lebanon, which grows at an elevation of 1200 – 1800 m in the northern part of the country, was selected as the candidate plant. Water extract of *C. ainetensis*, did not alter, *in vitro*, the differentiated phenotype of mammary epithelial cells, and inhibited, in a dose response manner, ET-induced IL-6 and gelatinases A and B levels. *In vivo* the extract reduced ET-induced paw edema and thermal hyperalgesia in rats.

MATERIALS AND METHODS

Cell culture

CID-9 mouse mammary cell strain (kindly provided by M. J. Bissell, Lawrence Berkeley National Laboratory, Berkeley, CA) were cultured as previously described (Safieh-Garabedian et al., 2004). Briefly, all cells were grown in culture plates in Dulbecco's Modified Eagles Medium and Ham F12 Nutrient Mixture (DMEM/F12, Gibco, Paisley, Scotland) with heat inactivated 5% fetal bovine serum (FBS), (Gibco, Paisley, Scotland), insulin (5 µg/ml, Sigma, St. Louis), and 1% penicillin/streptomycin mixture (Gibco, Paisley, Scotland) in a humidified incubator (95% air 5% CO₂) at 37°C (Forma Scientific Inc. Ohio, U.S.A.). The above-described medium referred to as "growth medium" was changed every other day until the cells formed a confluent monolayer. The cells were then replated on 6 well tissue culture plates at 1.2x10⁶ cells / well or 1.7x10⁶ cells/well for EHS matrix. For all experiments described, cells were plated in growth media the first day, and the next day media was replaced with differentiation medium, consisting of DMEM/F12 medium containing insulin (5 µg/ml), hydrocortisone (1 µg/ml, Sigma, St. Louis) and ovine prolactin (3 µg/ml, Sigma, St. Louis), and referred to hereafter as cells grown on plastic or with 1.5% EHS matrix in differentiation medium (referred to hereafter as cells grown on EHS-drip). Cells were allowed to grow until confluency (day 3 or 4). Then, the media was supplemented with 1% FBS (1 ml/well), and cells were treated with 10 µg/ml of ET (Lipopolysaccharide from *Salmonella typhosa*, Sigma, St. Louis)

after addition of plant extract as indicated. Samples were collected at 9, 24 or 48 h post ET treatments.

Treatment with plant extract

As recommended in herbal remedies air-dried ground flower head of *C. ainetensis* (4 mm square mesh) was soaked in boiling water (decoction) 1:8 (w/v) for 20 min. The resulting solution was filtered through 3 mm Whatman filter and then sterilized using the 0.2 µm non-pyrogenic sterile-R filter and stored at -20°C until used. Different doses (volume extract/volume media) of water extracts, as indicated in respective experiments, were added to the cells 30 min prior to ET treatment. Three replicates were maintained for each treatment.

ELISA

The BioSource International, Inc., USA Immunoassay Kit, Mouse Interleukin-6 (mIL-6) Enzyme Linked Immuno Sorbent Assay (ELISA) was used for the *in vitro* quantitative determination of the IL-6 in the cell culture conditioned media. The minimum detectable dose of IL-6 is < 3 pg/ml as described by the manufacturers.

The O.D of the plates was measured at a wavelength of 450 nm on ELISA reader (Thermolabsystems, Multiskan EX). Media were sampled from cultures treated with ET (10 µg/ml) and different plant extract concentrations, and from control cultures, at different time points. Protease Inhibitor (Complete; a broad spectrum inhibitor of serine, cysteine, and metalloproteases; Roche Diagnostics GmbH) was added at a concentration of 40 µl (of 1 tablet dissolved in 2 ml water) per 1 ml of sample. The samples were stored at -70°C until the day of the assay. All the samples were run in triplicates and the difference between the readings were plotted as error bars for each experiment.

Substrate-gel electrophoresis (Zymography)

Conditioned media were collected from cultures treated with ET for either 9, 24, and 48 h and from control untreated cultures. Similarly, media were collected from ET-treated cultures whose media were supplemented with 3, 5, or 8% plant extract 30 min before ET treatment. The collected samples were stored in cryotubes at -70°C until zymography. Gelatin substrate gel electrophoresis or zymography was done as previously described by Talhouk et al. (2004). Samples were loaded on 7% polyacrylamide gels impregnated with gelatin (4.5 mg/ml). Gels were washed twice with wash buffer (substrate buffer with 2.5% Triton X-100) at room temperature, and then incubated for 24 h in substrate buffer (50 mM Tris-HCL, 5 mM CaCl₂, 0.02% NaN₃, pH 8.0) at 37°C. The gels were then stained for 2 h; at room temperature; in 0.05% Coomassie blue R-250 (Sigma, St. Louis, Missouri, USA), in 50% methanol, and 10% acetic acid, and destained in distilled deionized water for 16 h.

Protein extraction and Western Blotting

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 9, 21 or 48 h post ET treatment. Cell extracts were centrifuged at 15,000Xg, 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 as described by Safieh-Garabedian et al. (2004).

In vivo studies; Paw edema and hot plate pain test

Adult male Sprague-Dawley rats at 6 - 7 wks of age were used in all

experiments. The animals (200 - 250 g) were housed under optimum conditions of light and temperature. All experiments were carried out with strict adherence to ethical guidelines (Zimmerman, 1983). Localized inflammation was induced as described by Talhouk et al. (2004). Twenty rats were grouped into four groups. The rats in the first group (control) were injected by de-ionized distilled water (400 μ l) intraperitoneally (i.p.) 30 min prior to intraplantar (i.pl.) ET injection (100 μ l) of 1.25 μ g/100 μ l sterile and pyrogen-free physiologic solvent of de-ionized distilled water in the left hind paw. The rats in the second and the third group were injected intraperitoneally by aqueous crude extract of *C. ainetensis* (400 and 200 μ l, respectively; that is, a 1:8 decoction of 200 and 100 mg respectively of dried plant material per Kg body wt) 30 min prior to ET (100 μ l) injection in the left hind paw. The fourth group was injected by *C. ainetensis* (400 μ l) 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, 3, 5, 7, 9, 12, 20, 25 and 30 h following the injection.

The hot plate (HP) test was used to assess the thermal pain threshold. All rats were subjected to the HP test for three consecutive days prior to ET injection and then HP test was performed for all groups (n = 5 each) at 0, 3, 6, and 9 h following the injection. The test was performed as previously described by Talhouk et al. (2004). The animals were individually placed on a heated plate ($53 \pm 0.2^\circ\text{C}$) and the latency of the first sign of paw licking, or jumping was recorded. The latencies recorded in each group were averaged for each time interval.

Statistical analysis

Analysis of variance (ANOVA) with post hoc analysis with the least significant difference (LSD) as well as Student-Newman-Keuls, and Tukey tests was used to analyze the IL-6 concentration, the paw thickness and the response latency to ET using SPSS program (Version 15.0). Comparison of means between the control and ET groups at each time point was also performed using SPSS program. The results are represented as means \pm SE.

RESULTS

C. ainetensis was chosen for further studies of anti-inflammatory activities from among a group of 29 plants typically used in Middle Eastern folk medicine. The taxonomic verification of plants was made based on herbarium specimens at Post Herbarium, American University of Beirut, Lebanon (Post and Dinsmore, 1932). The selection of the 29 plants and then later the singling out of *C. ainetensis* was based on two approaches that we have undertaken: First, in selecting the plants we reviewed local medicinal folk text books and related literature (Ruwaiha, 1981; Awadallah, 1984) for identifying native plants with proposed anti-inflammatory activities. This was followed by a process of validation of the selected plants by interviewing known registered folk practitioners and/or herbalists in Greater Beirut Area and Mount Lebanon that were recommended by the Lebanese Chamber Of Commerce, Ministry of Economy and several folk doctors that herbalists recognize as being the most sought after by individuals seeking herbal-based medication. Noteworthy is the fact that in Lebanon herbalist may assume the role of a folk doctor and vice versa. The se-

cond approach for selecting a candidate plant, *C. ainetensis* in this case, entailed an initial screening of extracts from the 29 plants, for bioactivities such as low cytotoxicity to cells in culture, inhibitory activity for gelatinases A (matrix metalloproteinase [MMP]-2 or 72 kDa gelatinase) and B (MMP-9 or 92 kDa gelatinase), and inhibitory activity to IL-6 production by ET-stimulated mammary epithelial cells in culture. Table 1 lists the selected plants for our study in alphabetical order, and their common folk Arabic names.

Water extracts of the above listed plants were tested for their cytotoxicity using the Cyto Tox 96 Non Radioactive Cytotoxicity Assay Kit, on four different cell lines, grown on plastic substratum, namely, MCF-7 (cancerous mammary cell line), MCF-10A (normal mammary cell line), T-84 (cancerous colon cell line) and HCT-116 (cancerous colon cell line) and a normal mammary cell strain, the CID-9 cells. Furthermore, plant extracts were tested for their ability to modulate IL-6 levels produced by ET-treated CID-9 cells and for their ability to inhibit gelatinases A and B, known markers of inflammation as described by Safieh-Garabedian et al. (2004) and Talhouk et al. (2004). *C. ainetensis* (Figure 1) was selected as the plant whose extract exhibited least toxicity, reduced IL-6 levels produced by ET-treated CID-9 cells and inhibited gelatinases A and B. According to the aforementioned and to reports in folk literature describing the actual use of *Centaurea* species extracts in treating inflammatory-based ailments (Ruwaiha, 1981; Awad Allah, 1984; Talhouk et al., 2007), further studies were carried to characterize the bioactivity.

C. ainetensis extract inhibited ET-induced IL-6

To assess the potential anti-inflammatory bio-activity of *C. ainetensis* water extracts and how that would affect differentiation markers of the cells, we cultured CID-9 cells in differentiation permissive medium on EHS-drip.

Water extract of *C. ainetensis* inhibited IL-6 production by CID-9 cells on EHS-drip, in a dose dependant manner by interfering in its synthetic pathway (Figure 2). The IL-6 inhibition noted by the *C. ainetensis* plant extract is not due to the extract's sequestering the ET, making it unavailable to the cells. This was tested by treating the cells on EHS-drip with 10% plant water extract followed by its aspiration and washing the treated cells twice with PBS prior to addition of 1% ET (10 μ g/ml). IL-6 ELISA assay showed that after 9 h IL-6 levels in such cells were significantly lower than ET-treated cells and did not differ from control cells. Furthermore, the extract does not bind the secreted IL-6 rendering it unavailable for ELISA detection, since incubating IL-6 containing conditioned medium with *C. ainetensis* plant extract did not alter the levels of detected IL-6 (Figure 2A). The inhibition of IL-6 was sustained up to at least 48 h in culture. Water extract at 3, 5 and 8% (vol/vol) from *C. ainetensis* inhibited, in a dose dependant manner, IL-6 production by ET-treated

Table 1. Plants selected for the study and listed in alphabetical order. Scientific names in left hand column. Arabic names are from M. Nehmeh. *Dictionnaire Atymologique de la Flore du Liban*. Beirut : Librairi du Liban Editeur, 2000.

Plant name	Arabic name of the plant
<i>Achilla damascena</i>	Akhilia zat al-alf waraqah
<i>Alchemilla diademata</i>	Kamaliyyah mukallalah
<i>Anthemis hebronica</i>	Bahar al-kalil
<i>Anthemis scariosa</i>	Bahar ghishai
<i>Calendula officinalis</i>	Adharyun makhzani, kahlah
<i>Calendula palestina</i>	Bayd al-qitt/Adharyun filastini
<i>Centaurea ainetensis</i>	Qanturyun aynata/shawk al-dardar
<i>Centaurea erengoides</i>	Qantaryun
<i>Cirsium "sp."</i>	Quswan
<i>Echinops gaillardoti</i>	Qarqafan, Bardaward
<i>Eryngium deserlorum</i>	Shindab Sahrawi
<i>Heliotropium rotundifolium</i>	Ikkrir-mustadir al waraq
<i>Hieracium sp.</i>	Saqriyyah, hashishah al-ghurab
<i>Lotus carmeli</i>	Lutus al karmal
<i>Melissa inodora</i>	Turunjan adim al-ra'ihah
<i>Nepeta curviflora</i>	Qatram muqawwas al-zahr
<i>Onopordum cynarocephalum</i>	Aqsun harshafi al-ra's
<i>Origanum libanoticum</i>	Mardaqush lubnani
<i>Phlomis syriaca</i>	Ayzarah suriyyah
<i>Phlomis damascena</i>	Ayzarah
<i>Ranunculus cuneatus</i>	Hawdahan isfini
<i>Ranunculus Constantinopolitanus</i>	Hawdhan fa'ri
<i>Salvia robifolia</i>	Quwaysah ullaqqiyyah al-waraq
<i>Serratula pusilla</i>	Warkhah qazimah
<i>Taraxacum "sp."</i>	Tarakhshaqun
<i>Tamus orientalis</i>	Fasharshin sharqi
<i>Urtica fragilis</i>	Qurrays qasif
<i>Verbascum blanchetum</i>	Busir bkanche
<i>Verbascum leptostychem</i>	Busir nahil al-sunbulah



Figure 1. *C. ainetensis* is a plant endemic to Lebanon with claimed medicinal value (Arabic name; Qanturyun aynata or Shawk al-dardar). The plant grows in Dayr-ul-Ahmar to Aynata region at elevations of 1200 – 1800 m above sea level, respectively). It is also reported in Anti-Lebanon Mountain range above Ayn-Burday at 1250 – 1300 m. The entire plant is grey with curly hair; its stems are short, erect and are 1 or 2 headed. The heads are small oblong, rounded at the base and not truncate. The tube of anthers is purplish, not yellow; achene more densely hairy about the hilum. The flowering is during the months of May and June. It grows in stony, sterile or bushy places. A specimen is deposited at the herbarium of the American University of Beirut, Beirut, Lebanon (photos courtesy of Khaled Sleem 2002, Crop Production and Protection Department, Faculty of Agriculture and Food Science, American University of Beirut. Beirut, Lebanon). From: Flora of Syria, Palestine, and Sinai. George Post (2nd Edition, John Edward Dinsmore, 928pp, 1932).

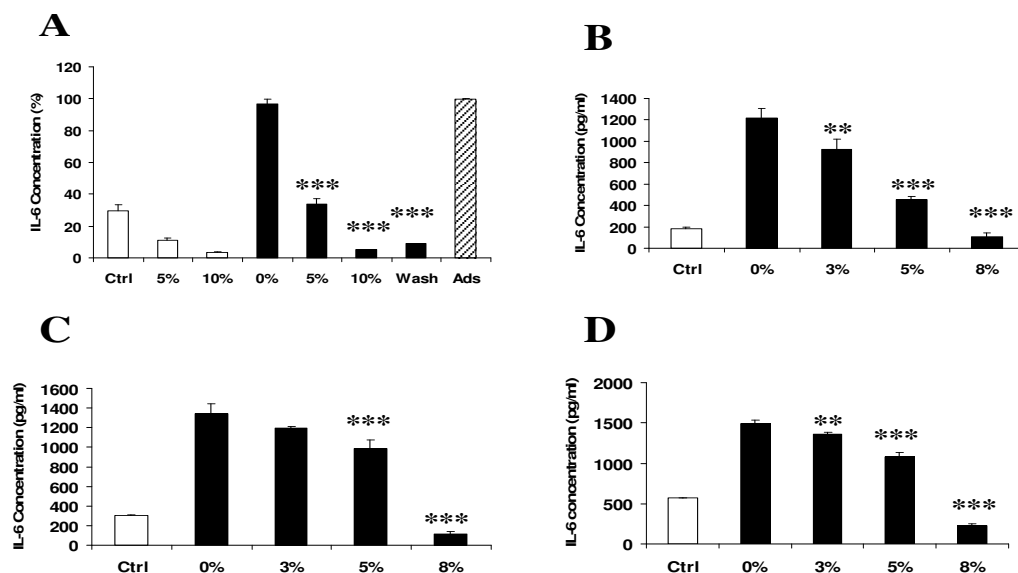


Figure 2. *C. ainetensis* water extract inhibits in a dose-dependant manner IL-6 levels produced by CID-9 cells cultured on EHS-drip. A) Water extract at 5 and 10% (of a 1:8 vol/vol decoction) reduced IL-6 levels in ET-treated or non-treated cells (Ctrl). Treatment of cells with 10% extract then washing it off (Wash) prior to ET treatment still inhibited IL-6 levels (Figure 2A), indicating the inhibitory effect is not due to plant extract interfering with ET binding to cells. Adsorption (Ads) bar is the control demonstrating that the extract does not bind IL-6 in conditioned medium and thus do not interfere with ELISA read out. Conditioned medium of ET-treated cells assayed after its incubation with 10% of the plant extracts (vol/vol) for 30 min, show the sustained levels of IL-6 indicating that the plant extract does not interfere with the ELISA assay. (B-D) Cells exposed to different concentrations of *C. ainetensis* extract (0, 3, 5 and 8% vol/vol) half an hour before ET treatment for 9 h (B), 24 h (C), and 48 h (D) after stimulation with 1% ET showed significant reduction in IL-6 levels. The values depicted are the means (\pm S.D.) of a triplicate treatment. Statistical significance of the difference from Ctrl+ET (i.e. 0% plant extract) is with * $P < 0.05$, ** $P < 0.01$ or *** $P < 0.001$.

CID-9 cells plated on EHS-drip at 9, 24 and 48 h post stimulation with 1% ET (Figure 2B, C, and D respectively). However, it is noted that at 5% of the extract the extent of inhibition of IL-6 secretion is decreased beyond 9 h and the IL-6 at 24 or 48 h rise to levels exceeding 70% of control IL-6 levels in ET-treated cells. Plant extract at 8% suppressed IL-6 levels in excess of 85% up to 48 h.

***C. ainetensis* extract inhibited ET-induced gelatinases**

Water extract of *C. ainetensis* inhibited, in a dose dependant manner, gelatinases A and B produced by ET-treated CID-9 cells. CID-9 cells in culture produce abundant levels of gelatinase A in ET-treated and untreated cells, however, gelatinase B is markedly up regulated in ET-treated cells and most notably in conditioned medium of ET-treated cells at 24 and 48 h. Water extract of *C. ainetensis* inhibited gelatinases A and B in conditioned media at 9, 24 and 48 h post ET-treatment. Partial inhibition of gelatinase B was observed with 3% plant extract and complete inhibition was noted with 8% concentration. Gelatinase A was partially inhibited at 48 h with 5% plant extract and at 24 and 48 h with 8% plant extract (Figure

3). Similarly, such extract inhibited gelatinase A and B activity of FBS and in a dose dependant manner at 3, 5 and 8% (data not shown).

Water extract of *C. ainetensis* did not perturb β -casein expression

β -casein expression was monitored 9 h after treatment of non ET-treated cells with 5 and 10% (vol/vol) of plant water extract alone or 1% ET-treated cells with plant water extract at 5, 8 and 10% concentration (vol/vol). Water extract did not modulate the expression of β -casein, a marker of differentiation, in non-ET treated or ET-treated CID-9 cells plated on EHS-drip (Figure 4A and B). Cells on EHS-drip up-regulate their β -casein expression compared to those on plastic. Its expression in cells on EHS-drip declines in response to ET-treatment at 48 h (Safieh-Garabedian et al., 2004). In Figure 4C we show that whereas ET does not alter β -casein levels in cells on plastic, it does down-regulate β -casein expression at 48 h post ET-treatment. Plant extract at 8% does not reverse the ET-induced decline in β -casein levels (Figure 4C) even though extract did reverse the IL-6 and gelatinase levels induced by ET (Figures 2 and 3). Similar results

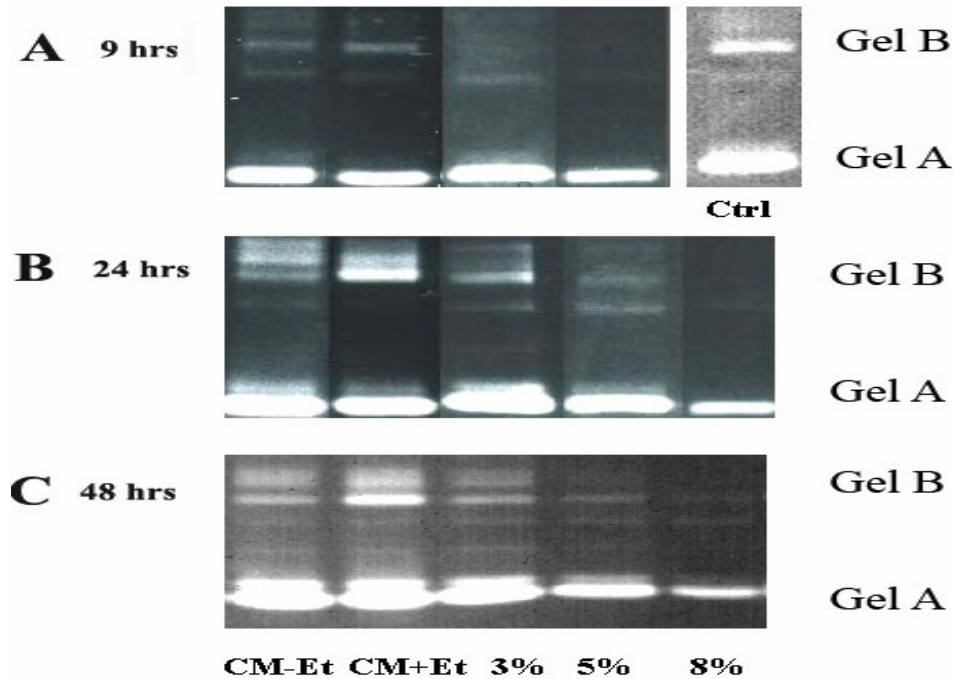


Figure 3. Water extract from *C. ainetensis* inhibits gelatinases expressed by ET treated CID-9 cells cultured on EHS-drip. Cell culture conditioned media (CM) collected from cells untreated (CM-ET) or treated with ET (CM+ET) at 9 h (A), 24 h (B), and 48 h (C) after treatment and subjected to zymography showed that the plant extract contains gelatinase inhibitory activity. (A) Cells treated with ET for 9 h showed minor increase in the expression of gelatinase B (Gel B) but not gelatinase A (Gel A). The basal level of gelatinase B activity decreased in cells treated with 5 and 8% plant extract. Note that 8% of *C. ainetensis* showed some inhibition for gelatinase A activity. (B) Cells treated with ET for 24 h showed increased expression of gelatinase B. This increased activity in ET-treated cells decreased gradually in a dose dependant manner and was completely inhibited at 8%. Partial inhibition of gelatinase A was noted at 8%. (C) Cells treated with ET for 48 h sustained their increased expression of gelatinase B. Similar to 24 h, gelatinase B and gelatinase A were inhibited in a dose dependant manner. Gelatinase B was completely inhibited at 8% plant extract. Ctrl lane; are gelatinase B and gelatinase A activities from FBS.

were observed in SCp2 cells, an epithelial sub-clone of CID-9 cells (data not shown).

***C. ainetensis* extract reverses ET-induced paw edema and hyperalgesia**

The fact that water extract of *C. ainetensis* were not cytotoxic, did not affect β -casein expression, but down regulated IL-6 expression, and inhibited gelatinase activity, suggested that these extracts possess anti-inflammatory and/or analgesic activity. Thus, *in vivo* studies were initiated to validate the *in vitro* findings. *C. ainetensis* water extract reduced paw edema (local inflammation) induced by intraplantar injection of ET in Sprague Dawley rats (Figure 5A). Plant extract also reversed thermal hyperalgesia (Figure 5B). ET injected paws showed increased paw thickness as early as 3 h post ET injection. This peaked (6.2 mm) at 5 - 9 h and decreased slightly at 12 h to return to baseline levels (4.2 mm) beyond 20 h of ET-treatment. Water extract of *C. ainetensis* showed signi-

ficant inhibition of paw edema throughout the experiment, and up to 12 h the paw thickness did not exceed 5.4 mm in rats that have received i.pl. injections of ET in the left paw and i.p. injections of 400 or 200 μ l of the plant extract. At 20 h, and up to 30 h, no significant difference was noted in paw thickness of either ET-treated or non-treated animals (Figure 5A).

Hyperalgesia was monitored up to 9 h post ET-injection in rats that have been pretreated with i.p. injections of 400 μ l of *C. ainetensis* extract. ET induced thermal hyperalgesia was reversed by pre-treatment with 400 μ l of *C. ainetensis* extract as early as 3 h and this was maintained up to at least 9 h (Figure 5B). The response latency of ET-treated rats subjected to $53 \pm 0.2^\circ\text{C}$ on a hot plate was reduced from 17 s at 0 h to approximately 11 s at 3 - 9 h post ET-treatment. Rats pre-treated with plant extract had paw latency between 14 - 16 s at 3 - 9 h post ET-treatment. Rats, receiving 400 μ l of *C. ainetensis* extract with no ET injections in the paw, showed no signs of either paw edema or a change in thermal pain threshold

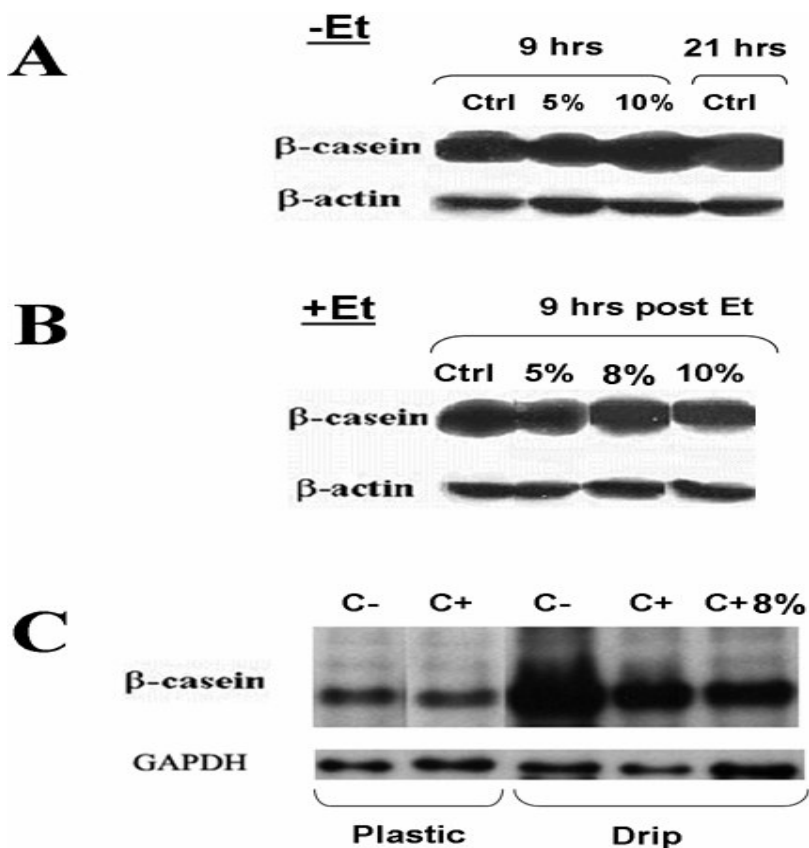


Figure 4. *C. ainetensis* water extract does not modulate β -casein expression by CID-9 cells cultured on EHS-drip. **A)** β -casein expression in non ET-treated (-Et) CID-9 cells 9 h after exposure to 5 and 10% (vol/vol) *C. ainetensis* water extract. **B)** β -casein expression in CID-9 cells 9 h post ET-treatment (+Et) after exposure to 5, 8 and 10% (vol/vol) of plant extract. **C)** β -Casein expression in CID-9 cells on plastic or EHS-drip (Drip) in control non ET-treated (C-) and ET-treated (C+) cultures. C+8% are cells to which 8% plant extract was added. Ctrl, is casein level in cells not exposed to plant extract. β -actin and GAPDH demonstrate equal loading of proteins.

DISCUSSION

The use of herbal medicine has become a popular practice and is widely used by folk medicine practitioners in Lebanon and elsewhere in the region. Descriptions and names of such plants endemic to the region were published in local medicine folk books (Ruwaita, 1981; AwadAllah, 1984). Strategies for selecting medicinal plants for research studies typically consisted of interviewing folk practitioners and collecting information from locally published folk medicine texts prior to verifying their bio-activities as previously described by Hamill (2003). Following a similar strategy to select candidate plants we also referred to local published texts and studies in herbal medicine and to "Registered" folk practitioners and herbalists in the Greater Beirut Area and Mount Lebanon. After collecting the information, a list of 29 plants was compiled for the study. All the selected plants are commonly used as herbal remedies. Of the compiled list, *C. ainetensis* was selected as the plant of choice to further characterize

its anti-inflammatory effects since its water extract (i.e. decoction) was non-cytotoxic and suppressed known inflammatory mediators such as IL-6 levels and gelatinases A and B activity (Talhouk -et al., 2000; 2004). The genus *Centaurea* was reported earlier on for its anti-inflammatory activities. Studies on the aerial parts of *Centaurea chilensis* identified two elemanolide esters, the 2-methylpropanoate and 2-methyl-2-propenoate of 11,13-dehydromelitensin. The mixture of these two substances exhibited anti-inflammatory activity in the carrageenan-induced paw edema assay (Negrete et al., 1993). Another study reported that polysaccharides extracted from *Centaurea cyanus* flower-heads had anti-inflammatory properties as shown by different pharmacological experiments including inhibition of carrageenan, zymosan and croton oil-induced edemas, inhibition of plasma hemolytic activity, and/or induction of anaphylatoxin activity (Garbacki et al., 1999). Recently, Gürbüz and Yesilada (2007) identified two guaianolide type sesquiterpene lactones in the chloroform extract of the aerial parts of *C. solstitialis*

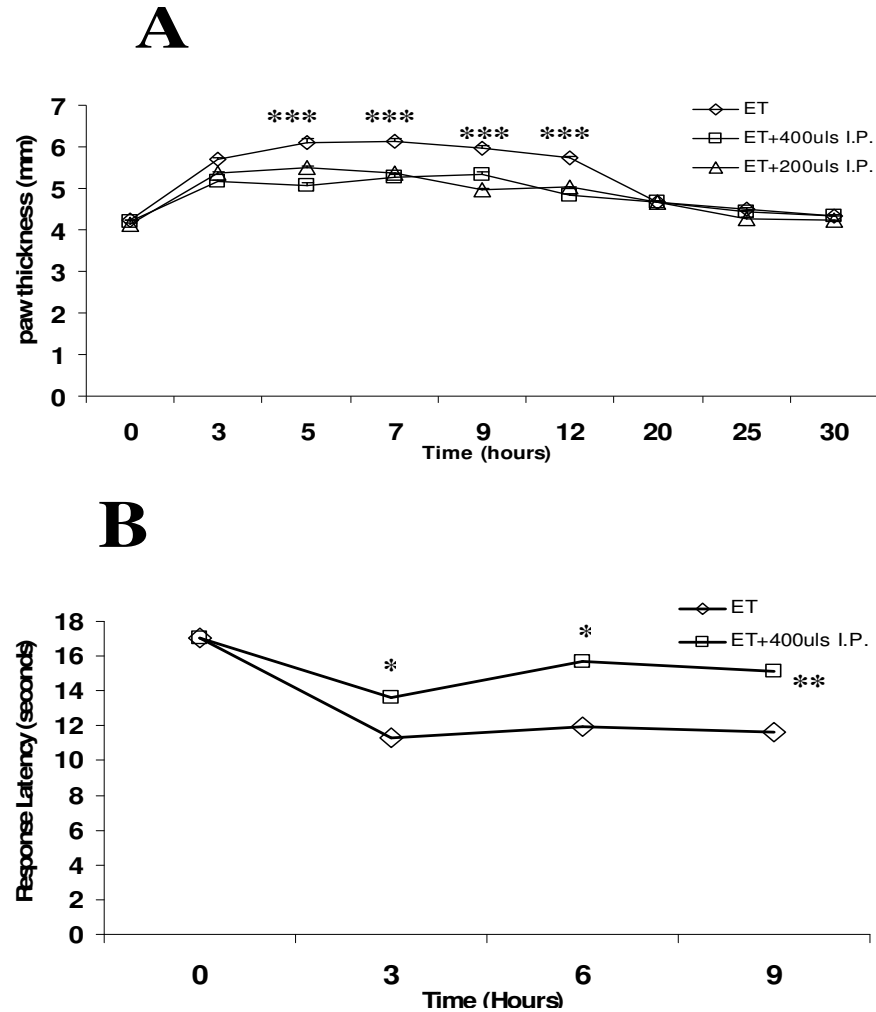


Figure 5. *C. ainetensis* water extracts reduced paw edema and hyperalgesia. ET Intraplantar injection of adult male Sprague Dawley rats induced local and reversible paw edema. As shown, intraperitoneal (I.P.) injections of different volumes of plant extract (400 and 200 µl) reduced paw edema (A) and hyperalgesia (B). The values depicted are the means (\pm S.D.) of $n = 5$ animals per treatment. Statistical significance of the difference from ET-treated animals (ET) is with $*P < 0.05$, $**P < 0.01$ or $***P < 0.001$.

ssp. solstitialis (Asteraceae) as having an anti-ulcerogenic activity. This is the first study to report anti-inflammatory activity in *C. ainetensis*.

To develop further our understanding of the proposed anti-inflammatory effects of water extract from *C. ainetensis*, it was necessary to characterize its noted effects both *in vitro*, in ET-treated CID-9 cells cultured in differentiation-permissive conditions on EHS-drip (Safieh-Garabedian et al., 2004), and *in vivo*.

Culture studies demonstrated that conditioned medium collected at 9, 24 and 48 h post-ET treatment contained decreasing amounts of IL-6, within each time interval, for cells receiving 3, 5 and 8% plant extract respectively. The data further demonstrated that *C. ainetensis* extract does not bind ET or IL-6 in conditioned medium and hence any decrease in IL-6 levels, as detected by ELISA, is due to the extract's inhibition of IL-6 secretion from CID-9 cells

or down regulation of IL-6 gene expression. Preliminary data in IL-1 treated Mode K intestinal cells suggests the latter, since *C. ainetensis* water extract interfered with NF- κ B activation and its subsequent translocation to the nucleus (F. Homeidan, American University of Beirut, Lebanon, personal communication), an event pre-requisite to the induction of IL-6 gene expression and other inflammatory mediators such as gelatinases.

Water extract from *C. ainetensis* showed dose dependent inhibition of gelatinases A and B proteolytic activity detected in conditioned medium of ET-treated cells at 24 and 48 h post ET-treatment. Similarly, water extract incubated with a 20% FBS solution, prior to zymography, inhibited gelatinases A and B of FBS (data not shown). Based on the facts that increased MMP activation, notably gelatinase B, is detected at 24 h post ET-treatment, and that TNF- α (Safieh-Garabedian et al., 2004) and IL-6

production by ET-treated CID-9 cells increased within 6 to 9 h post ET treatment may infer that TNF- α stimulates the binding of NF- κ B to a consensus kB-oligonucleotide, thus increasing the stability of MMP-9 transcripts and MMP-9 activity (Lee et al., 2001; Wendy et al., 2001). We speculate that MMP inhibition, noted in culture by the water extract of *C. ainetensis* is partially an indirect one via the inhibition of cytokines production. However since it also inhibits gelatinases in FBS it also suggests that it does interfere directly with gelatinases A and B activity.

The ability of the water extract from *C. ainetensis* to inhibit cytokine IL-6 and gelatinases A and B, both markers of inflammation and dedifferentiation, prompted us to investigate the effect of extract on β -casein expression, a marker of differentiation, by CID-9 cells. Our investigation showed that 5 and 10% of the water extract from *C. ainetensis* had no effect on β -casein expression by ET-treated and non-treated CID-9 cells plated on EHS-drip, up to 9 h of treatment. This suggested that the inhibitory effects on IL-6 and gelatinases are specific. Interestingly, our earlier studies showed that β -casein protein levels were down regulated at 48 h post ET-treatment (Safieh-Garabedian et al., 2004) in contrast to the noted elevation in cytokine and gelatinase levels. Here we show that water extract of *C. ainetensis* does not reverse the down regulation of β -casein expression at 48 h.

In this study we have used CID-9 cell strain which is predominantly made up of epithelial cells and fewer myo-epithelial and fibroblast cells (Schmidhauser et al., 1990). However, to better define the target cell population that the water extract of *C. ainetensis* is affecting we used the SCp2 mammary epithelial cell model, a sub-clone of CID-9 cells (Desprez et al., 1993). Similar to CID-9 cells, SCp2 cells produce IL-6 post ET treatment. Water extract from *C. ainetensis* inhibited IL-6 production by ET-treated SCp2 cells suggesting that the inhibitory effect of the bio-active compound is mediated via, at least partially, the mammary epithelial cells component of CID-9 cells (data not shown).

Previous studies demonstrated that inhibitors of hyperalgesia and inflammation down regulated both gelatinases and inflammatory cytokines, including IL-6 (Talhok et al., 2000; 2004). The fact that *C. ainetensis* extract down regulated gelatinases and IL-6 in ET-treated CID-9 cells suggested that it possesses anti-inflammatory and possible analgesic activities. Our *in vivo* data, using the established animal model of Kanaan et al. (1996), confirmed this as shown by the reversal, in rats, of ET-induced paw edema and heat hyperalgesia. Similar observations were previously reported in extracts of several plants (Talhok et al. 2007).

In conclusion; we referred to folk literature and folk herbalists to identify plants with bio-active potentials. Of the selected 29 plants endemic to Lebanon and region that were screened, the *C. ainetensis* was the plant chosen for further characterization of its potential anti-inflammatory effect. We show that water extract from *C.*

ainetensis contains bio-active compounds that exhibit anti-inflammatory and analgesic activities. The nature of these compounds and their mechanism of action are subject of a separate study.

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