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In vitro antimicrobial and anti-inflammatory activity of Jordanian plant extracts: A potential target therapy for *Acne vulgaris*

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Acne vulgaris **is a chronic inflammatory disorder in which** *Propionibacterium acnes* **plays a critical role in its development when it overgrows in the pilosebaceous unit. The present study was conducted to evaluate the antimicrobial and anti-inflammatory activity of fourteen Mediterranean plant extracts separately and in combinations for the treatment of acne. The methanolic plant extracts were tested against** *P. acnes* **by determining the minimum inhibitory concentration (MIC). The extracts that showed high antimicrobial activity were studied for their anti-inflammatory effect. They were evaluated for their ability to suppress the pro-inflammatory cytokines IL-1β, IL-8, IL-12 and the TNF-α release from PBMCs and mTNF-α from mouse splenocytes using ELISA assay.** *Rosmarinus officinalis* **was found to exhibit the highest antimicrobial activity amongst all of the tested extracts (MIC 0.5 mg/ml) followed by** *Origanum syriacum* **(MIC 1 mg/ml) and** *Punica granatum* **(MIC 1.25 mg/ml). 100 and 50 µg/ml of** *Chrysanthemum coronarium, Origanum syriacum* **or** *Inula viscosa* **extracts showed the most effective inhibition for the cytokine release. Six extracts had shown both antimicrobial and anti-inflammatory activities with apparent dose-dependent responses making them potential agents for the treatment of acne. Two mixtures prepared from the plants with the highest antimicrobial activity had shown synergistic antimicrobial and anti-inflammatory effect.**

Key words: Cytokines, minimal inhibitory concentration (MIC), enzyme-linked immunosorbent assay (ELISA), peripheral blood mononuclear cells (PBMCs), splenocytes, *Propionibacterium acnes.*

INTRODUCTION

Acne vulgaris is one of the most common skin diseases that affect areas containing a large number of sebaceous follicles (including the face, back and chest) resulting in severe inflammatory lesions on those areas (Leyden, 1997; Williams et al., 2012). Abnormal ductal keratinize-tion and an elevated rate of sebum excretion can also be associated with the disease. When sebum is accumu-lated in the pilosebaceous channel, it leads to abnor-malities of the microbial flora and inflammation that can facilitate the proliferation of bacteria on the skin (Koreck

et al., 2003; Youn, 2010). *Propionibacterium acnes* is a Gram-positive, non-sporeforming, pleomorphic and anaerobic bacterium that is a commensal human skin microorganism and known to play a role in the pathogenesis of acne (Liu et al., 2011).

The pathogenesis of acne vulgaris is multifactorial involving infection of the pilosebaceous unit with *P. acnes* and a cytokine-mediated inflammatory response. It starts as a non-inflammatory comedo and proceeds to inflam-matory reactions with pustules, granulomas and cystic

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Table 1. List of the studied plants.

Scientific name	Parts used	Family	Local name	Uses in traditional medicine
Achillea biebersteinii Afan.	Flowers	Asteraceae	Qaysum	Carminative &Insect repellant
A. andrachne L.	Bark			
A.s andrachne L.	Flowers	Ericaceae	Kaykab	Asthma
A. andrachne L.	Leaves			
Cichorium pumilum Jacq.	Flowers	Asteraceae	Hindiba	For liver diseases
Chrysanthemum coronarium L.	Flowers	Asteraceae	Busbas	Dermal disease and vermifuge
I. viscosa L.	Leaves	Asteraceae	Ut-tayoon	Tuberculosis, anemia and malaria
I. viscosa L.	Flowers			
Lactuca serriola L.	Leaves	Asteraceae	Khas barri	Calmative, catarrhs and cough
Opuntia ficus-indic (L.) Mill.	Flowers	Cactaceae	Saber	Skin moisturizer
Origanum syriacum L.	Leaves	Labiateae	Za'ter	Carminative, pectoral, antitussive and aperative
Punica granatum L.	Flowers	Punicaceae	Romman	Diarrhea and nematodes
P. granatum L.	Peels			
Rosmarinus officinalis L.	Leaves	Lamiaceae	Haslban	Antiseptic, Antispasmodic and diuretic

lesions. These factors provide a potential target for acne treatment in addition to targeting one of the causative microbes *P. acnes* (Hamnerius, 1996; Leyden, 2001).

P. acnes is implicated in the development of inflammatory acne by the activation of complements and ability to metabolize sebaceous triglycerides into fatty acids, which chemotactically attract neutrophils. Previous studies showed that the proliferation of *P. acnes* leads to inflammatory lesions and severe acne by stimulating the production of proinflammatory cytokines such as tumor necrosis factor-alpha (TNF- α), interleukin-8 (IL-8), IL-12 and IL-1β by human monocytic cell lines and freshly isolated peripheral blood mononuclear cells (PBMCs) from acne patients (Vowels et al., 1995; Dessinioti and Katsambas, 2010). Also, studies showed that the release if IL-1 α, TNF- α and GM-CSF was significantly increased in human keratinocytes (the first line of defence in the skin) stimulated with *P. acnes* compared to the non-stimulated ones (Graham et al., 2004). Keratinocytes and sebocytes may act as immune cells that can recognize pathogen and might have an important role in the activation of both innate and adaptive immune responses (Koreck et al., 2003).

A variety of acne treatments exist, including topical antibiotics, oral antibiotics, medications with bacteriostatic and anti-inflammatory properties that are effective for treating mild to moderate inflammatory acne. However, long term use of antibiotics against acne might exacerbate antibiotic resistance towards *P. acnes* (Degroot and Friedlander, 1998; Swanson, 2003). To overcome this, there is a great need for new types of antimicrobial and anti-inflammatory agents to which the bacteria have not been exposed. The use of medicinal plants has been extensively studied as alternative agents for the treatments of different diseases including acne. In developing countries and Jordan in particular, the use of medicinal plants in traditional medicine is very common where a large portion of the population relies on it for health care.

In the present study, ten medicinal plants which have been used extensively in traditional medicine in Jordan were examined for their activities against acne by testing their ability to inhibit the growth of *Propionibacterium acnes* and the release of pro-inflammatory cytokines. Some of the plants were tested for the effect of their different parts (like flowers, bark, peels or leaves). Also, two combinations of extracts that showed the highest antimicrobial activity were evaluated for their anti-microbial and anti-inflammatory effect. Those that show both antimicrobial and anti-inflammatory activities are considered as possible therapeutic agents in the treatment of acne vulgaris.

MATERIALS AND METHODS

Plant material

The plants used in this study were either collected in the wild or cultivated in gardens in Jordan. The taxonomic identity of each plant was authenticated by Mohammad Al-Gharaibeh, a botanist in the Department of Natural Resources and Environment, Faculty of Agriculture, Jordan University of Science and Technology (JUST), Irbid-Jordan. Voucher specimens were deposited there (Table 1).

Plant extraction

The plant samples were dried carefully under shade at room temperature and then grounded to powder and stored in airtight bottles. Suitable amounts of the powdered plant materials were soaked in methanol for 72 h at 37°C room temperature with continuous stirring. The crude methanolic extract was filtered using glass microfiber fiber (grade GF/B 1 µm Whatman, GE Healthcare)

and then the solvent was evaporated to dryness under reduced pressure using rotary evaporator (Heidolph laborota, Germany). The residues were further subjected to dryness by incubating them for 8 days at 30°C. The crude extracts were either used directly or stored in an air-tight container for further use.

Microorganism strain

The antimicrobial activity was tested on *Propionibacterium acnes* (ATCC 6919) an anaerobic bacterium that plays a role in acne vulgaris. The *P. acnes* was stored at -20°C in brain heart infusion broth with 20% glycerol.

Evaluation of antimicrobial activity

The inocula of the bacteria for agar diffusion and agar dilution methods were prepared from an overnight culture in brain heart infusion broth. The suspensions were adjusted to 0.5 McFarland standard turbidity (~10^8 CFU/ml) to be used for inoculation. The incubation was made under anaerobic conditions in anaerobic jar using Anaerogen (Oxoid Ltd, Hampshire, England).

The antimicrobial activity of the plants' methanolic extracts was initially evaluated against *P. acnes* using the agar diffusion cup plate method (Oskay and Sari, 2007), where volumes of saturated solutions of extracts in dimethylsulfoxide (DMSO) equal were used to fill the wells.

The plates were incubated overnight at 37°C under anaerobic conditions in an anaerobic jar using Anaerogen bags (Oxoid Ltd, Hampshire, England). Wells filled with DMSO were used as negative controls. Extracts producing zones of inhibition more than those produced by DMSO were considered to have antimicrobial activity and their minimum inhibitory concentrations (MIC) were evaluated using agar dilution method (Assaf et al., 2013). In this method, the prepared Muller Hinton agar plates containing the extracts were inoculated with 10 µl microbial suspension ($1×10^6$ CFU/ ml). The inoculated plates were incubated for 24 h at 37°C under anaerobic conditions in anaerobic jar using Anaerogen bags.

Positive controls for all microorganisms were prepared by using DMSO instead of the extract solution. After incubation, the plates were inspected visually. The MIC was defined as the minimum concentration that resulted in no growth or less than five colonies. The test was considered not valid unless the positive controls showed significant microbial growth. Same procedures were applied when two-component or three-component extract mixtures were tested using an equal volume from each extract.

Peripheral blood mononuclear (PBMCs) cells isolation

Venous blood was collected from healthy volunteers into heparinized vacutainer tubes (Becton Dickinson, Mountain View, Calif.). The heparinized blood was diluted 1:1 with PBS (pH 7.2) and layered on a Ficoll-Hypaque gradient (Lymphoprep; Accurate Chemical Corp., Westbury, N.Y). The gradient was centrifuged at 2500 rpm for 15 min at room temperature, and the buffy coat containing the peripheral blood mononuclear cells (PBMC) was collected and washed twice in PBS.

Cell culture and experimental animals

To determine the effect of the plant extracts *in vitro*, PBMCs were either pretreated, to induce inflammatory response, or left untreated before their stimulation with the plant extracts. Also, for their effect on splenocytes, an animal model was used. Specific-pathogen-free

6-8-week-old Balb/c mice were housed in an animal room at a temperature of 23 ± 1°C and a humidity of 55 ± 5%, with a 12/12-h light/dark cycle. The mice were fed a standard laboratory diet with tap water *ad libitum*. The mice were sacrificed by cervical dislocation; splenocytes were prepared by dissociation into single-cell suspension by mechanical disruption between glass slides. Erythrocytes from spleen were depleted by ammonium chloride solution buffer 0.84% and subsequently washed three times in RPMI 1640.

Plant extracts that showed antimicrobial activity were used in this part. Also, when the extract from different parts of the same plant (that is, leaves, flower or bark) showed an effect, then the most effective part and commonly used in the folk medicine was used. Accordingly, the peels were used from *Punica granatum*, flowers from *Chrysanthemum coronarium* and the leaves were used from *Rosmarinus officinalis*, *Arbutus andrachne*, *Origanum syriacum* and *Inula viscosa*. Two mixtures from the extracts that showed highest antimicrobial activity were also evaluated for their anti-inflammatory activity. Mixture one was made from *R. officinalis* leaves and *O. syriacum* leaves in a 1:1 ratio, while mixture two was made from the leaves of *R. officinalis*, *O. syriacum* and *A. andrachne* in a 1:1:1 ratio.

PBMCs and splenocytes were cultured in RPMI 1640 medium (Lonza, Euro-clone) containing 10% heat-inactivated fetal bovine serum (FBS) (Gibco, USA), HEPES Buffer (10 mM), L-glutamine (2 mM), Gentamicin (50 µg/ml), penicillin (100 U/ml), and streptomycin sulfate (100 mg/ml) (Lonza, Germany). Cell count and viability were assessed by trypan blue dye exclusion. To examine the anti-inflammatory effect of the plant extracts on PBMCs , $1×10^6$ cells/ml were seeded in a 24-well plate and either left untreated (culture in growth medium alone) or activated with 5 µg/ml mitogen concanavalin A (Con A) (Sigma, Vienna, Austria) for 30 min before the herbal treatment for all cytokines except for IL-12. Lipopo-lysaccharide (LPS; *Escherichia coli* O127:B8 [Sigma]) was used at a final concentration of 10 with 10 ng/ml of Interferon- gamma (IFN-γ) for the activation of IL-12 cytokine in PBMCs 30 min before herbal treatment. On the other hand, total splenocytes were plated at $5×10^6$cells/ml and one group was activated with 5 µg/ml Con A for 30 min before herbal treatment while the other was left without activation to determine their anti-inflammatory effect. Con A and LPs were used to induce inflammatory response. Both cell types with the two different groups for each were stimulated with one of the plant extracts at 100, 50 and 10 µg/ml concentrations separately in growth medium or left without extracts' stimulation (control). Cultures were incubated in a humidified atmosphere of 37°C and 5% CO2 overnight. Supernatants were then harvested for analysis by an enzyme-linked immunosorbent assay (ELISA). Untreated cells with or without Con A or LPs activation were used as negative control. .

Effect of the plant extracts on production of human IL-8, IL-1β, IL-12 and TNF-α and murine TNF-α

To examine the effect of the plant extracts on inflammation, $2×10^5$ cells/ml of the PBMCs from healthy immunocompetent subjects and mice splenocytes from each group (with and without mitogen activation) were split into triplicate wells in 96 well flat-bottomed microtitre plates, and left to grow either without or with one of the plant extracts at 100, 50 and 10 µg/ml concentrations separately in growth medium. Supernatants obtained from controls and stimulated cells were collected and assayed. The concentrations of human TNF-α, IL-1β, IL-8 and IL-12 (in 100 µl of PBMCs supernatant each) and murine TNF-α (in 100 µl of splenocytes supernatant) were determined by ELISA assay according to the manufacturer's protocol (eBioscience, San, Diego, CA). All incubation steps were performed at room temperature. The optical

Table 2. The antimicrobial activity of plant extracts against *P. acnes* and minimum inhibitory concentration (MIC) of the active extracts.

Plant extract	MIC (mg/ml)
A. Biebersteinii flowers	10
A. andrachne bark	15
A. andrachne leaves	5
A. andrachne flowers	ND
C. coronarium flowers	2.5
C. pumilum flowers	ND
Inula viscosa flowers	2.5
I. viscosa leaves	2.5
L. serriola leaves	ND
O.ficus-indica flowers	ND
O. syriacum leaves	1
P.granatum flowers	1.25
P. granatum peels	1.25
R. officinalis leaves	0.5
Mixtures	
Mixture 1: *R. officinalis* + *O. syriacum*	0.125 + 0.125
Mixture 2: *R. officinalis* + *O. syriacum* + *P. granatum*	0.03125 + 0.03125 + 0.078

ND, Not done, since no antimicrobial activity was detected using agar diffusion method.

density at 450 nm, corrected by the reference wavelength 570 nm, was measured with microplate reader (Biotek, USA). All cytokine assays were calibrated against the World Health Organization international standards by the kit manufacturer. The lower limit of detection for the individual assays of human IL-12, IL-8, IL-1β, TNF-α and murine TNF-α cytokines is 4 pg/ml. The two mixtures were prepared from equal ratios with 50 µg/ml concentrations for each extract and tested in the same way for their anti-inflammatory response.

Statistical analysis

The results are presented as means of three independent experiments. Data were analyzed by means of one way ANOVA to determine statistically significant variance between the groups for each plant extract. Statistical significance between groups was then calculated by using paired t test with GraphPad Prism 5 software (GraphPad Software, Inc, La Jolla, California). Data are expressed as means ± standard error of mean (S.E.M). Differences were considered significant at a *P* value of less than 0.01.

RESULTS

Evaluation of antimicrobial activity

The presence of antimicrobial activity of 14 methanolic plant extracts against *P. acne* was screened using agar diffusion method. Out of these extracts, 10 extracts showed antimicrobial activity (Table 2) and were further studied to determine their minimum inhibitory concen-

tration using agar dilution method (Table 2). *R. officinalis* showed highest activity (lowest MIC) with MIC value of 0.5 mg/ml, followed by *O. syriacum* (1 mg/ml) followed by *P. granatum* peel and flowers (1.25 mg/ml) for each. The least activity reported was in A. *andrachne* bark with MIC = 15 mg/ml.

For some plants, extracts from different parts of the plant showed similar activity against *P. acnes* others have variable activity. The flowers and leaves of *P. granatum* and the flowers and leaves of *I. viscosa* have shown similar MIC values (1.25 and 2.5 mg/ml respectively). On the other hand, different parts of *A.* andrachne have variable activity against *P. acnes*. The bark and leaves have MICs of 15 and 5 mg/ml respectively while the flowers showed no activity towards the tested microorganism.The MIC of the prepared mixtures of the extracts was measured. Mixture one MIC (*O. syriacum* and *R. officinalis* in 1:1 ratio) was found to be 0.125 mg/ml for both extracts (Table 2). On the other hand, the MIC value for mixture two (*P. granatum*, *R. officinalis* and *O. syriacum* in 2.5:1:1 ratio) was 0.078, 0.03125 and 0.03125 mg/ml respectively (Table 2).

Evaluation of the anti-inflammatory effect

The production of human IL-1β, IL-8, IL-12 and TNF-α *in vitro* with or without Con A or LPs pre-treatment of PBMCs or splenocytes was determined in presence or

absence of the six plant extracts that showed an antimicrobial activity. The two mixtures from the extracts that showed the highest antimicrobial activity were evaluated for their anti-inflammatory responses using 50 µg/ml concentrations from each extract with equal ratios.

The effect of the plant extracts on the production of human IL-1β, IL-8, IL-12 and TNF-α cytokines in PBMCs

Control PBMCs pretreated with Con A or LPs showed a significant increase in cytokine levels compared to non treated cells. The release of IL-1β from Con A treated PBMCs was significantly reduced with all plant extract treatments but almost all of them (except for *Chrysanthemum coronarium*) were significantly increased when PBMCs were not pretreated with Con A as indicated in Figure 1a and b. The best response was indicated when 50 µg/ml of the extract was used with Con A treated PBMCs. IL-8 level in Con A treated PBMCs and stimulated with plant extracts showed significant reduction in its level when 100 and 50 µg/ml of the extract was used except for *R. officinalis* which did not show any significant changes (Figure 1c). Whereas, 10 µg/ml of the plant extract significantly increased IL-8 level in Con A treated PBMCs when treated with *R. officinalis*, *P. granatum* or *A. andrachne* with no significant change was observed when stimulated with the other three herbs. TNF-α level was significantly reduced when plant extracts used in 100 µg/ml and 50 µg/ml concentrations (Figure 1e). On the other hand, 10 µg/ml of the *R. officinalis*, *P. granatum* or *A. andrachne* extracts significantly increased TNF-α level while being significantly decreased when *C. coronarium*, *O. syriacum* and *I. viscosa* extracts were used (Figure 1e).

   PBMCs stimulated with the plant extracts but without Con A treatment showed variable responses when tested for IL-8 and TNF-α level. Regarding this, IL-8 level was significantly reduced when all concentrations were used except for *A. andrachne* which did not show any changes at 10 µg/ml concentration (Figure 1d). On the other hand, TNF-α level was significantly reduced when 50 and 10 µg/ml but not 100 µg/ml of *R. officinalis*, *C. coronarium*, *O. syriacum* and *I. viscosa* extracts were used while for *P. granatum* and *A. andrachne* this was significant at 10 µg/ml concentration. On the other hand, 100 µg/ml of *P. granatum* significantly increased TNF-α level in non treated PBMCs whereas the rest did not show any significant effect (Figure 1f).

   The release of the IL-12 from LPs/ IFN-γ treated PBMCs was significantly inhibited by all extracts at 100 µg/ml. Furthermore, 50 µg/ml from all extracts except *R. officinalis* induced a significant reduction in IL-12 level whereas it led to a complete inhibition in its level when stimulated with 50 µg/ml of *R. officinalis*. Interestingly, 10 µg/ml of the plant extracts significantly increased IL-12

level compared to the control unstimulated cells (Figure 2). IL-12 release was not induced in LPs/ IFN-γ untreated PBMCs with or without extract stimulation (data not shown).

The effect of the plant extracts on the production of murine TNF-α cytokine

To determine the effect of the different plant extracts on murine splenocytes' inflammatory response, splenocytes were either Con A-pretreated or left untreated. Figure 3 illustrates the effect of the different plant extracts on the level of the murine TNF-α cytokine. Con A untreated splenocytes did not show an effect (data not shown). The release of mTNF-α in Con A-pretreated splenocytes was significantly reduced in all concentrations used from *R. officinalis*, *C. coronarium*, *O. syriacum* and *I. viscosa* extracts. On the other hand, *P. granatum* and *A. andrachne* did not show any significant changes in mTNF-α release in Con A-pretreated splenocytes compared to the control except for the 100 µg/ml of *P. granatum* which showed a slight reduction in its level (Figure 3).

   The potent activities for most of the plants that showed significant reduction on mTNF-α level were indicated when 100 µg/ml of the extracts been used followed by 50 µg/ml. Using 100 µg/ml of *I. viscosa* extracts completely inhibited mTNF-α release and 50 µg/ml of this extract significantly reduced its level.

The effect of the two mixtures from the plant extracts on the production of the human and murine cytokines

To determine the effect of a mixture from the most effective plant extracts on the inflammatory response, two mixtures were prepared. The potent activities that led to a significant reduction of all cytokines were when 50 µg/ml of the extracts have been used except for *P. granatum* and *A. andrachne* extracts as it was at 100 µg/ml. Table 3 and 4 shows the human and murine cytokine levels respectively when 50 µg/ml of the plant extracts were used. Therefore, 50 µg/ml of the plant extracts that showed the highest antimicrobial activity were used in combinations with equal ratios to evaluate their effect on the different cytokines when they are in a mixture. Table 5 summarizes the levels of each tested cytokine in the presence or absence (control) of the mixtures. Mixture one (*R. officinalis* and *O. syriacum,* 1:1 ratio) and mixture two (*R. officinalis*, *O. syriacum* and *P. granatum*, 1:1:1 ratio) significantly reduced human and murine cytokine levels in Con A or LPs pretreated and untreated cells except for IL-1β which did not show any significant change in the untreated PBMCs as indicated in Table 5 and Figure 4.
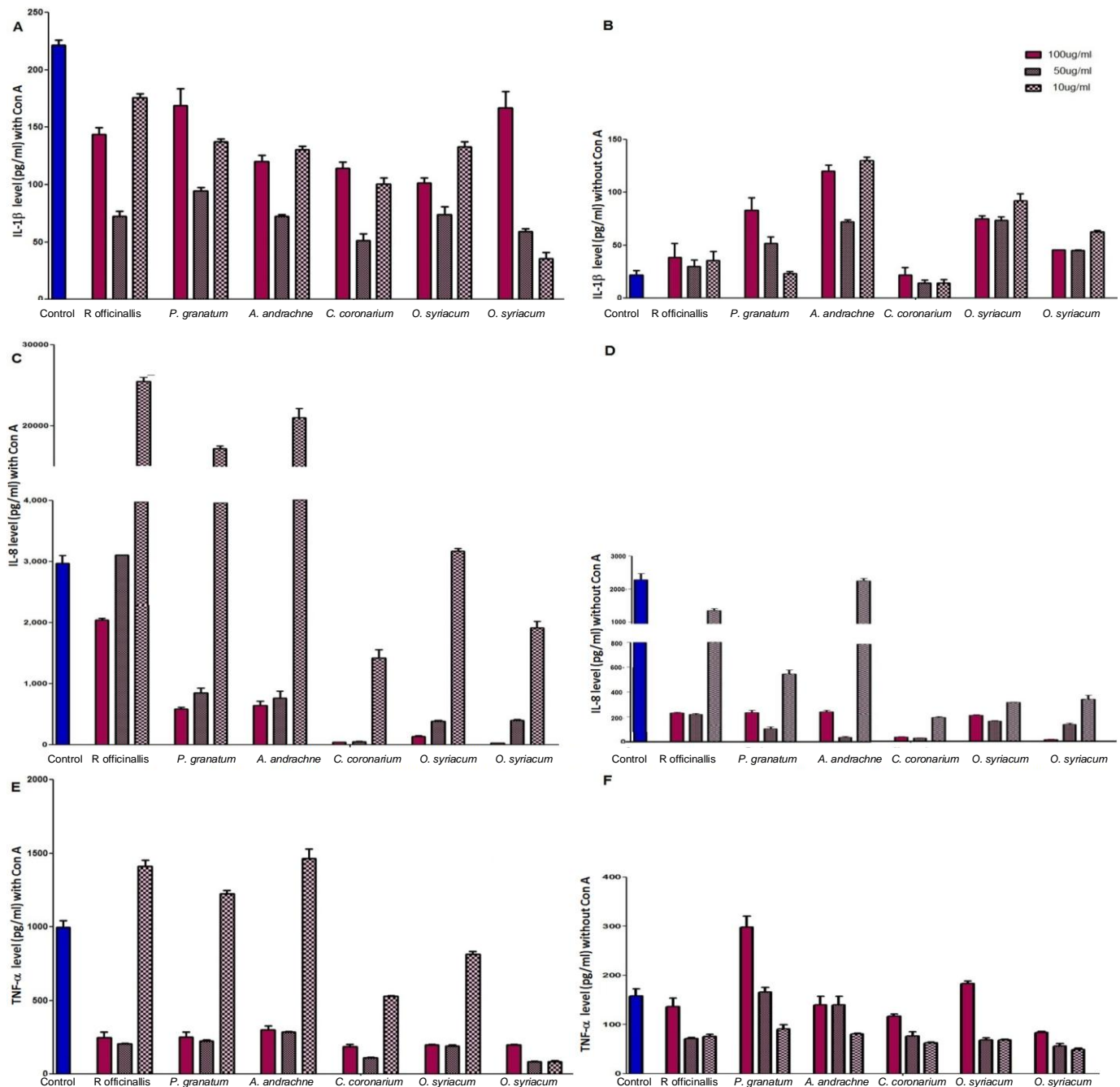
Figure 1. The anti-inflammatory effect of the plant extracts on PBMCs. Effects of the plant extracts (100, 50 and 10 µg/ml concentrations) on the release of the pro-inflammatory cytokines from untreated and 5 µg/ml Con A-pretreated PBMCs. (A) IL-1β with Con A, (B) IL-1β without Con A, (C) IL-8 with Con A, (D) IL-8 without Con A, (E) TNF-α with Con A and (F) TNF-α without Con A. Plant parts used were *Punica granatum* peels, *Chrysanthemum coronarium* flowers and *Rosmarinus officinalis*, *Arbutus andrachne*, Origanum *syriacum* and *Inula viscosa* leaves. Data represent the mean concentration pg/ml of triplicates ± S.E.M. Differences were considered significant at *P* < 0.01 vs. control.

DISCUSSION

In this study we were looking for medicinal plants that grow in Jordan where large portions of the population rely on them for health care, to investigate their antimicrobial and anti-inflammatory activities towards acne vulgaris. The majority of the plants evaluated in this study are known to have antimicrobial activity against different microorganisms and anti-inflammatory effect. However, few studies, if any, have evaluated their effect against *P.*
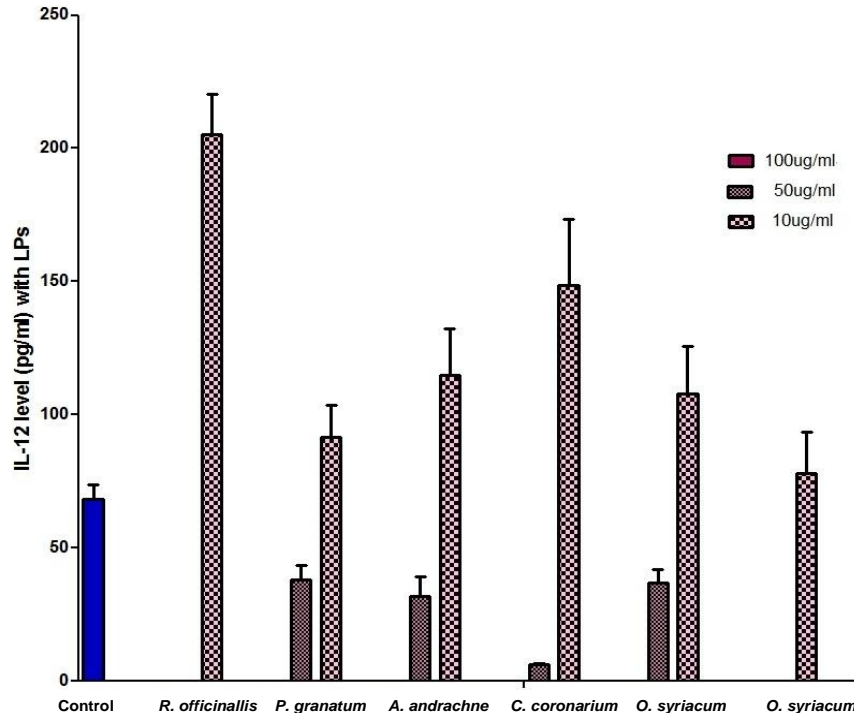
Figure 2. Effect of the plant extracts on the release of IL-12 in treated PBMCs. Effect of the plant extracts (100, 50 and 10 µg/ml concentrations) on the release of the pro-inflammatory cytokines, IL-12, from LPs/ IFN-γ -treated PBMCs (10 ng/ml LPs with 10 ng/ml of IFN-γ). Plant parts used were *P. granatum* peels, *C. coronarium* flowers and *R. officinalis*, *A. andrachne*, *O. syriacum* and *I. viscosa* leaves. Data represent the mean concentration pg/ml of triplicates ± S.E.M. Differences were considered significant at *P* < 0.01 vs. control.
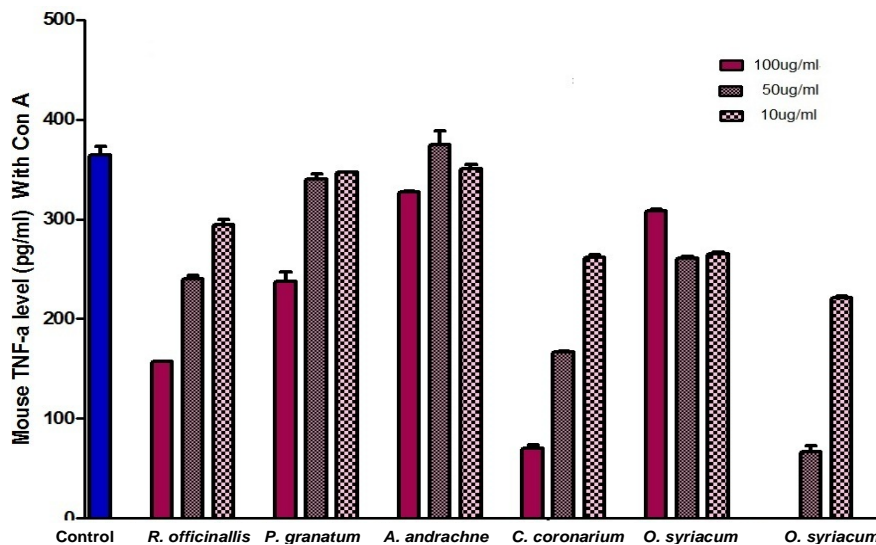

Figure 3. The anti-inflammatory effect of the plant extracts on the mouse splenocytes. Effect of the plant extracts (100, 50 and 10 µg/ml concentrations) on the release of the pro-inflammatory cytokines, murine TNF-α, from 5 µg/ml Con A pre-treated splenocytes. Plant parts used were *P. granatum* peels, *C. coronarium* flowers and *R. officinalis*, *A. andrachne*, *O. syriacum* and *I. viscosa* leaves. Data represent the mean concentration pg/ml of triplicates ± S.E.M. Differences were considered significant at *P* < 0.01 vs. control.

Table 3. The anti-inflammatory effect of the plant extracts on PBMCs.

Plant extract	IL-1β (Pg/ml)		IL-8 (pg/ml)		IL-12 (pg/ml)	TNF- α (Pg/ml)	
	Con A	None	Con A	None	LPs	Con A	None
Control	221.7±4.4	21.7±4.4	2969±132	2284±181.8	68.31±5.5	997.3±46.9	158±14
R. officinalis	72.67±4.3	30±5.77	3103±3.89	220±7.7	0.0±0.0	206±3.06	71.3 ±2.3
P. granatum	94.33±2.9	51.67±6	850±73.96	104.4±15.5	34.55±2.2	227.3±7.54	166±9.54
A. andrachne	72.33±1.5	51.67±6	765±108.8	35±6.74	34.91±4.3	287±2.65	140±17.2
C. coronarium	51.33±5.9	14±3.05	51.3±3.87	30.15±1.33	6.08±0.54	113.3±1.67	80±5.77
O. syriacum	73.67±7.3	73.3±3.3	385.4±11	166.3±4.22	36.8±5.03	191.7±7.3	68.3±4.4
I. viscose	59.33±2.3	45±0.58	398.9±16	139.9±9.62	0.0±0.0	86±3.055	56.7±4.4

Effects of the plant extracts at 50 µg/ml concentrations on the release of the pro-inflammatory cytokines, IL-1β, IL-8, IL-12 and TNF-α, from untreated and Con A (or 10 ng/ml LPs with 10 ng/ml of IFN-γ for IL-12) pretreated PBMCs. Plant parts used were *Punica granatum* peels, *Chrysanthemum coronarium* flowers and *Rosmarinus officinalis*, *Arbutus andrachne*, *Origanum syriacum* and *Inula viscosa* leaves. Data represent the mean concentration pg/ml ± S.E.M of triplicates. Differences were considered significant at $P < 0.01$ vs. control.

Table 4. The anti-inflammatory effect of the plant extracts on mouse splenocytes.

Plant extracts	Murine TNF- α (Pg/ml)
	Con A
Control	364.5±8.2
R. officinalis leaves	240.2±3.4
P. granatum peels	340±5.4
A. andrachne leaves	375.2±13.2
C. coronarium flowers	167.2±0.4
O. syriacum leaves	260.8±1.5
I. viscosa leaves	66.8±5.5

Effect of the plant extracts at 50 µg/ml concentrations on the release of the pro-inflammatory cytokines, murine TNF-α, from Con A-pretreated splenocytes. Data represent the mean concentration pg/ml ± S.E.M of triplicates. Differences were considered significant at $P < 0.01$ vs. control.

Table 5. The anti-inflammatory effects of the plant extract combinations on PBMCs and splenocytes.

Plant extract	IL-1β (Pg/ml)		IL-8 (pg/ml)		TNF- α (pg/ml)		IL-12 (pg/ml)	mTNF-α (pg/ml)
	Con A	None	Con A	None	Con A	None	LPs	Con A
Control	221.7±4.4	21.7±4	2969±132	2284±182	997.3±47	158±14	68±5.5	364.5±8
Mixture 1	108±14.8	15±2.9	633±52.9	131.1±16	280.7±7.3	69.7±12	0.0±0.0	0.0±0.0
Mixture 2	61.7±14.8	48.3±6	262±17.4	40±13.9	205.7±15	64±7.4	0.0±0.0	0.0±0.0

Mixture one (50 µg/ml *R. officinalis* leaves and 50 µg/ml *O. syriacum* leaves) and Mixture two (50 µg/ml *R. officinalis*, 50 µg/ml *O. syriacum* and 50 µg/ml *P. granatum* peels) on the release of the pro-inflammatory cytokines, IL-1β, IL-8, IL-12 and TNF-α or murine mTNF-α, from untreated and Con A (or 10 ng/ml LPs with 10 ng/ml of IFN-γ for IL-12) pretreated PBMCs or murine splenocytes (for mTNF-α). Data represent the mean concentration pg/ml ± S.E.M of triplicates. Differences were considered significant at $P < 0.01$ vs. control.

acnes, one of the known causative agents of acne vulgaris, or for their anti-inflammatory activity on the release of the cytokines. *Achillea biebersteinii* flowers have shown moderate activity against *P. acnes* with MIC 10 mg/ml. The antimicrobial activity of this plant had been studied earlier on aerobic microorganisms where its activity was variable from good activity against Gram-positive bacteria to moderate activity on Gram-negative bacteria to no effect against *Pseudomonas aeruginosa* (Kharma and Hassawi, 2006).
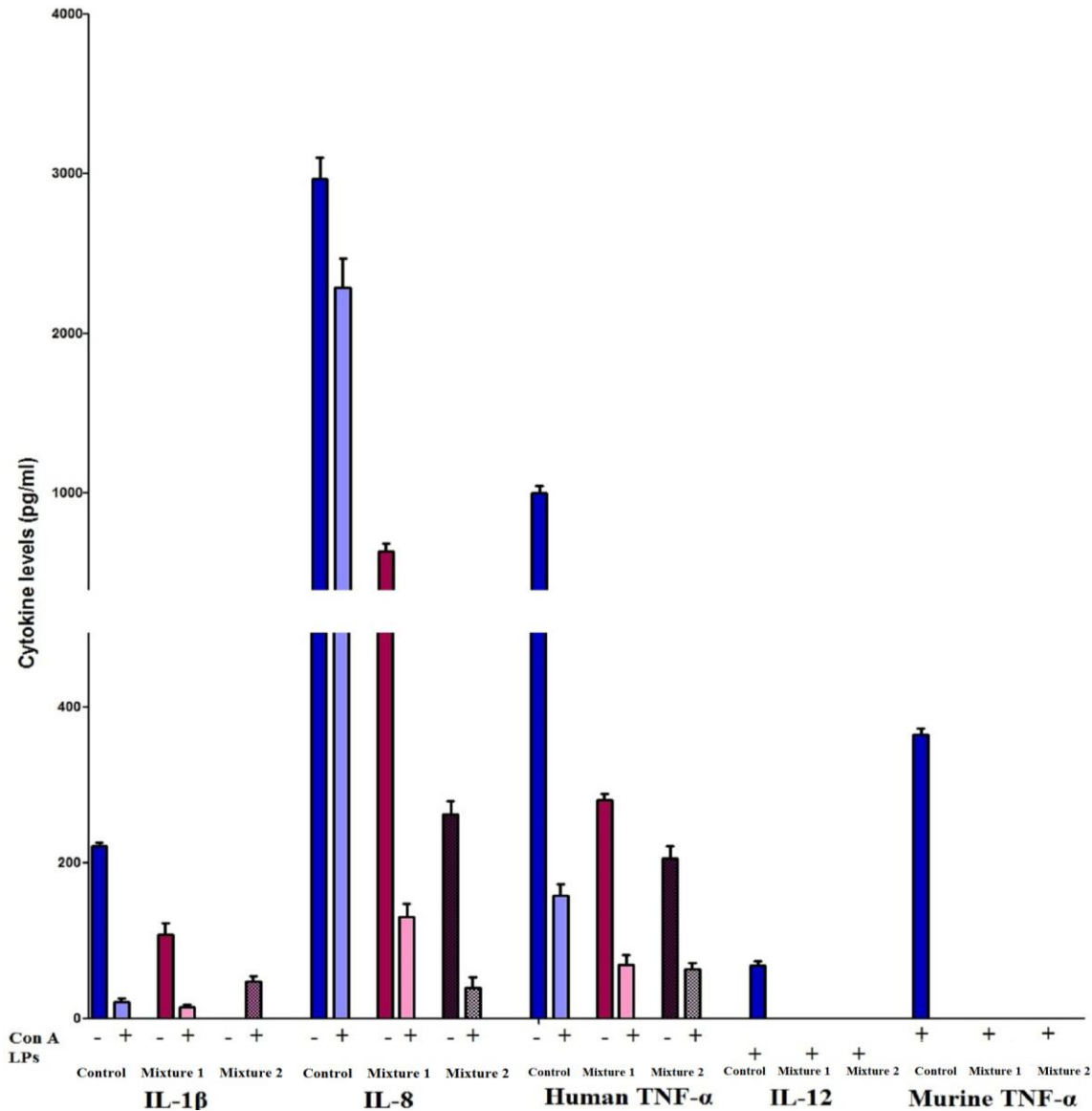
Figure 4. The anti-inflammatory effects of the plant extract combinations on PBMCs and splenocytes. Mixture one (50 µg/ml *Rosmarinus officinalis* leaves and 50 µg/ml *Origanum syriacum* leaves) and mixture two (50 µg/ml *R. officinalis*, 50 µg/ml *O. syriacum* and 50 µg/ml *Punica granatum* peels) on the release of the pro-inflammatory cytokines, IL-1β, IL-8, IL-12 and TNF-α or murine mTNF-α, from untreated and Con A (or 10 ng/ml LPs with 10 ng/ml of IFN-γ for IL-12) pretreated PBMCs or murine splenocytes (for mTNF-α). Data represent the mean concentration pg/ml of triplicates ± S.E.M. Differences were considered significant at $P < 0.01$ vs. control.

A. andrachne have exhibited variable inhibitory activity toward *P. acnes* depending on the part of the tree tested. The leaves have the highest activity followed by the bark which has weak activity (MIC 15 mg/ml). The flowers have shown no activity at the setting of this experiment. To our knowledge, no antimicrobial activity of the species *A. andrachne* is reported in literature against this microbe although many reports have revealed variable anti-microbial activities in other species such as Arbutus *unedo* and Arbutus *menziesii* (Kabadi and Hammarlund,

1963; Orak et al., 2011). The methanolic extract of the flowers of *C. coronarium* has exhibited noticeable activity with MIC 2.5 mg/ml, although a previous report by Urzua and Mendoza (2003) had shown good antimicrobial activity of methylene chloride extract against a group of aerobic Gram-positive and Gram-negative bacteria but no activity in methanolic extract. However, in their study they did not evaluate anaerobic bacteria nor *P. acnes*, which could explain the discrepancy in the two results. The methanolic extract of *I. viscosa* showed good activity

against *P. acnes*. The flowers and leaves have same MIC values (2.5 mg/ml) indicating similar inhibitory effect. In fact, several published work had shown that antimicrobial activity is exhibited by *I. viscosa* against a wide range of microorganisms. None of these studies had investigated the activity against *P. acnes*. A study by Ammar et al. (2012) showed that the antimicrobial activity of the flowers of *Opuntia ficus –indica* at the full flowering stage was only against *Staphylococcus aureus* but not against other Gram-positive or Gram-negative or yeast and fungi although another species of *Opunita* was active against them (Ammar et al., 2012). In our study, the methanolic extract of the flowers was inactive against *P. acnes*.

On the other hand, extracts from peels and flowers of *P. granatum* have exhibited good and similar activity against *P. acnes* with MIC of 1.25 mg/ml. This result is in line with other reports that had shown presence of antimicrobial activity in various parts of *P. granatum* fruit (seed, juice, peel) against a group of aerobic Gram-positive and Gram-negative bacteria and fungi (Dahham et al., 2010). *R. officinalis* has exhibited the highest activity amongst all the extracts tested (MIC 0.5mg/ml) followed by *O. syriacum* extract (MIC 1 mg/ml). Atomic force microscopy has revealed the effect of rosmarinus essential oil on *P. acnes* where it distorted the cell shape leading to cytoplasm leakage followed by cell wall damage (Fu et al., 2007). On the other hand, the essential oils of *O. syriacum* leaves had significant antimicrobial activity against a wide range of aerobic bacteria and fungi (Alma et al., 2003).

Two mixtures of the plants showing highest activity were prepared and tested to evaluate their antimicrobial activity and whether synergy or antagonism between the ingredients would occur. In order to classify the activity of any antimicrobial combination as synergy, antagonism or additive, the concept of isobologram was applied (Bayani and Azanza, 2005). The first mixture prepared and tested was *O. syriacum* and *R. officinalis* in 1:1 ratio. After plotting the isobologram (data not shown), if additive effect is expected, then 0.125 mg/ml *R. officinalis* would require the addition of 0.75mg/ml of *O. syriacum*. However, only 0.125 mg/ml *O. syriacum* was needed to be added to *R. officinalis* to inhibit *P. acnes* growth (Table 2), that is, there were six times reduction in the concentration of *O. syriacum* when combined with *R. officinalis*. Thus, *R. officinalis* has synergistic effect when combined with *O. syriacum*. In addition, the effect of adding *P. granatum* to (*R. officinalis* and *O. syriacum*) mixture (Table 2) was evaluated using the same concept of isobologram. Thus a mixture of these plants in a ratio of 2.5:1:1 respectively was prepared and evaluated. The addition of *P. granatum* to the first mixture (*R. officinalis/ O. syriacum)* resulted in twelve fold decrease in the MIC of *P. granatum* (data not shown) indicating synergistic effect. According to these results, the combinations of the two or three extracts are potential candidates to be formulated in acne preparations.

On the other hand, acne is an inflammatory disease where several cytokines play key roles in mediating acute inflammatory responses, such as IL-1β, IL-8 and TNF-α, which are extremely potent inflammatory molecules. *P. acnes* were found to stimulate monocytes to secrete pro-inflammatory cytokines including TNF-α, IL-1β and IL-8 (Vowels et al., 1995). It was also found to initiate T-helper cell-1 (Th-1)-mediated immune response by activating monocyte/macrophage release of IL-12 via Toll-Like receptors (TLR)-2 in acne (Kim et al., 2002). These biologically active molecules originate from white blood cells, keratinocytes, and sebocytes in and around the damaged follicle, as the host cross-talks with skin defense and immune cells to fight and protect against the infection (Vowels et al., 1995; Kim et al., 2002; Koreck et al., 2003; Graham et al., 2004). The inhibition of the overproduction of such mediators, especially pro-inflammatory cytokines, may prevent or suppress inflammation in acne patients. In preliminary experiments undertaken in our laboratory and 0.1 μg/ml of extract concentrations showed further induction in the cytokine levels compared to 10 μg/ml concentrations (data not shown). Since we were looking for the concentration that inhibits the cytokine release, the use of 1 and 0.1 μg/ml concentrations in later experiments was not needed.

In the current study, we used Con A and LPs models to study the inflammatory response in acne as it mimics many inflammatory effects of cytokines, such as TNF- α, IL-1β or IL-6. PBMCs pretreated with Con A or LPs showed a significant increase in cytokine levels compared to non treated cells indicating the induction of the inflammatory response in pretreated cells. Also, mouse splenocytes pretreated with Con A showed a significant production in mTNF-α level compared to non treated cells. The study has demonstrated that all the six plant extracts tested and showed an antimicrobial activity had an anti-inflammatory potential for the treatment of acne vulgaris, but relatively with variable responses and in a dose-dependent manner. The most effective extracts at inhibiting the release of the pro-inflammatory cytokines TNF-α, IL-1β, IL-8 and IL-12 from PBMCs and mTNF-α from splenocytes were *C. coronarium*, *O. syriacum* and *I. viscosa*. This effect was the best when 100 or 50 μg/ml of the extracts had been used. Moreover, 100 or 50 μg/ml of *P. granatum* and *A. andrachne* extracts significantly reduced the release of all the tested cytokines from PBMCs but not mTNF-α from splenocytes which only showed a significant effect when 100 μg/ml was used. Although *R. officinalis* has exhibited the highest antimicrobial activity amongst all the extracts tested it did not show a significant inhibition for the release of IL-8. However, 100 and 50 μg/ml of this extract showed a significant reduction on all other tested cytokines. Most of the plants showed a dose-dependent effect on the release of the cytokines.Different plants have different chemical components and when having them in a mixture, strong beneficial effects may result. Also, the

combination of plants may help the body to manage potentially undesirable effects that each plant might have when used alone and when in combination/formulation they might play a curative or pacifying role. It is therefore preferable to use plant combination instead of relying on a single plant. In this regard, two mixtures were made according to their highest antimicrobial activity and tested for their anti-inflammatory effect where they showed a significant reduction in all tested cytokines. The presence of 50 µg/ml *R. officinalis* in a mixture, but not alone, enhanced its anti-inflammatory activity towards the release of IL-8. The two mixtures had their most effective response by completely inhibiting IL-12 release from PBMCs and mTNF-α release from mouse splenocytes. In Con A untreated PBMCs, the two mixtures had a significant anti-inflammatory effect on the release of IL-8 and TNF-α but not IL-1β. Furthermore, the addition of *P. granatum* to mixture one (making mixture two) significantly increased the anti-inflammatory activity in mixture two even when tested for mTNF-α release from splenocytes which did not show any significant effect when used alone at 50 µg/ml concentration.

Literature review shows few studies regarding the six tested plant extracts anti-inflammatory effect on cytokine release. *R. officinalis,* a common plant that is broadly used around the world for different medicinal purposes, is a component of various established anti-inflammatory plant drug preparations (Darshan and Doreswamy, 2004). Various pharmacological studies have demon-strated the antimicrobial and anti-inflammatory properties of rosemary suggesting that it has a high therapeutic potential (Altinier et al., 2007; Takaki et al., 2008; Juhas et al., 2009). In a previous study (Peng et al., 2007), 1 µg/ml of supercritical fluid-CO_2 treatment of *R. officinalis* fresh leaves induced significant production of TNF-α in human hepatoma cells (Hep 3B) whereas no effect was found in Chang liver cells. In this study, 10 µg/ml of *R. officinalis* leaves extracts showed the same significant induction in TNF-α production in LPs-pretreated PMBCs whereas it showed a slight reduction in mTNF-α from mouse splenocytes.

Few studies have been made on the anti-inflammatory effect of *C. coronarium* which were consistent with our results regarding its effect on TNF-α release. Strzelecka et al. (2005) showed a very strong inhibition in the synthesis of TNF-α when 100 µg/ml *C. coronarium* was used, but on cells of monocytic origin (P388D1). In another study, the stimulation with the extract of *C. coronarium* caused dendritic cells maturation as well as induction of IL-12 production when 12.5 µg/ml was used (Tanaka et al., 2011). This was consistent with our results when using 10 µg/ml whereby a significant induction of IL-12 production was observed in PBMCs pretreated with LPs.

Pomegranate, *P. granatum*, is an ancient, unique fruit, long-living tree cultivated throughout the Mediterranean region has been valued through the ages for its medicinal

properties (Ahmed et al., 2005; Lansky and Newman, 2007; Jurenka, 2008). Jung and colleagues (2006) studied the anti-inflammatory effect of *P. granatum* in LPs treated and untreated BV2 microglial cells. Although they used different cells but their results were consistent with our results here indicating that LPS-stimulated *P. granatum* suppressed TNF-α production in BV2 microglial cells with a dose-dependent manner. Another study showed that the pomegranate peels have anti-inflammatory activity by directly inhibiting neutrophil myeloperoxidase activity *in vitro* (Bachoual et al., 2011).

O. syriacum is a popular aromatic plant in Middle Eastern cuisine found in the Mediterranean flora. It exhibits multiple biological activities including antibacterial and anti-inflammatory properties (Aslim et al., 2008; Ocana-Fuentus et al., 2010). In a previous study, supercritical oregano extracts showed a decrease in pro-inflammatory TNF-α, IL-1β and IL-6 cytokines synthesis, as well as an increase in the production of anti-inflammatory cytokine IL-10 in activated human THP-1 macrophages (Ocana-Fuentus et al., 2010). This was consistent with our results where *O. syriacum* significantly reduced TNF-α and IL-1β cytokine release in LPs- pretreated PBMCs and mTNF-α release in mouse splenocytes.

To our knowledge, no known anti-inflammatory studies of the species *A. andrachne* had been reported in literature while few studies detected the anti-inflammatory activities in other species such as *Arbutus unedo*. Aqueous extract of *A. unedo* was associated with reduction in TNF-α, IL-1β and IL-6 cytokines synthesis in pleural exudates (Mariotto et al., 2008) which is similar to our results when *A. andrachne* extracts stimulated LPs-pretreated PBMCs.

I. viscosa is a perennial weed that is found in most of the Mediterranean flora and has been used for years in folk medicine for its anti-inflammatory activity (Hernández et al., 2001; Hernández et al., 2007). Its anti-inflammatory properties have been tested both *in vitro* and *in vivo* by determining the activity of enzymes involved in the inflammatory response such as elastase and myeloperoxidase (MPO) (Hernández et al., 2007). To our knowledge, no reported literature showed its effect on cytokine release.

The innate and adaptive immune response of the host to *P. acne*s is important in the pathogenesis of acne. Keratinocytes were found to play an important role in the inflammatory reaction of the skin, synthesizing a number of cytokines, adhesion molecules and growth factors (Partridge et al., 1991; Koreck et., al 2003; Graham et al., 2004). Other studies showed that IL-1β, IL-8 and IL-12 as well as TNF-α, were significantly increased in peripheral blood mononuclear cells from acne patients (Dessinioti and Katsambas, 2010). Furthermore, Th-1 cells that recognize the *P. acne*s antigens are present in inflammatory acne lesions (Koreck et al., 2003). It is important, therefore, that antimicrobial agents are able to

down regulate T cell responses and facilitate protective Th-2 responses. Our data showing suppression of IL-1β, IL-8, IL-12 and TNF-α production by the tested extracts are promising in this regard.

In conclusion, this is the first study to determine the potential use of these methanolic extracts as antimicrobial agents towards *P. acne*s. Also, it is the first to evaluate the anti-inflammatory effect of these extracts on the release of IL-1β, IL-8, IL-12 and TNF-α from PBMCs and mTNF-α from mouse splenocytes. Moreover, this study shows a novel antimicrobial and anti-inflammatory effect when a mixture of plant extracts were used. The combinations of the medicinal plants in the two mixtures showed a synergic antimicrobial and anti-inflammatory activity, while a single constituent may or may not have a pharmacological effect. Also, the extracts inhibited the expression of inflammatory mediators with apparent dose-dependent responses. Nevertheless, topical application of potential anti-microbial and anti-inflammatory treatments can have a benefit to easily reach *P. acne*s, and also act on the keratinocytes. Naturally, they may also have an effect on the adaptive immune cells. Further study is required to assess the active constituents and possible mechanisms of action.

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DISCLOSURE STATEMENT

The authors declare that they have no competing interests.

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ABBREVIATIONS

MIC, Minimal inhibitory concentration; **ELISA**, enzyme-linked immunosorbent assay; **PBMC**s; Con A, concanavalin **A; LPs**, lipopolysaccharide**; IL,** interleukin; **TNF-α**, tumor necrosis factor-alpha. **IFN-γ,** interferon-gamma

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