JIDC | THE JOURNAL OF INFECTION IN DEVELOPING COUNTRIES

Original Article

Antimicrobial and anti-inflammatory potential therapy for opportunistic microorganisms

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Abstract

Introduction: Methanolic extracts of six plants (*Arbutus andrachne*, *Chrysanthemum coronarium*, *Inula viscosa*, *Origanum syriacum*, *Punica granatum*, and *Rosmarinus officinalis*) used in traditional medicine for the treatment of bacterial and fungal infections were evaluated. The present study was conducted to evaluate the antimicrobial and anti-inflammatory activity of some medicinal plants in lowering the risk of opportunistic infections of the oral cavity caused by *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Candida albicans*. Extracts were evaluated separately and in a mixture.

Methodology: The methanolic plant extracts were tested against three opportunistic microorganisms by determining the minimum inhibitory concentration (MIC). They were also evaluated for their ability to suppress the release of the pro-inflammatory cytokine IL-6 while not suppressing the release of the anti-inflammatory cytokine IL-10 from peripheral blood mononuclear cells using ELISA.

Results: All extracts showed both antimicrobial and anti-inflammatory activities. However, *O. syriacum* exhibited the highest antimicrobial activity for the three microorganisms among all of the tested extracts (MIC *S. aureus*: 1 mg/mL; *P. aeruginosa*: 2 mg/mL; and *C. albicans*: 1 mg/mL). The extracts inhibited the expression of the pro-inflammatory cytokine IL-6 with apparent dose-dependent responses while they attenuated the secretion of the anti-inflammatory cytokine IL-10. The mixture of *O. syriacum* and *R. officinalis* showed an anti-inflammatory effect, with a synergistic antimicrobial effect.

Conclusion: These findings support the idea that a diet rich in plants and herbs may contribute to the reduction of inflammation and microbial growth and may also be preventive against various infections, including those related to the oral cavity.

Key words: cytokines; MIC; ELISA; opportunistic microorganisms; plant extract; infection.

J Infect Dev Ctries 2016; 10(5):494-505. doi:10.3855/jidc.7610

(Received 31 August 2015 – Accepted 04 January 2016)

Introduction

Oral cavities can be considered as reservoirs for a variety of opportunistic species of microorganisms causing infections in individuals of poor health [1]. The most represented opportunistic microorganisms include *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Candida albicans* [2,3]. Unfortunately, due to the overuse and misuse of antibiotics, most of these organisms become drug resistant, leading to difficulties in curing related infectious diseases and thus reducing therapeutic options [3,4].

Exposure to microorganisms induces the immune system to locally produce pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, interferon-γ (IFN-γ) and IL-8, and anti-inflammatory cytokines (*e.g.*, IL-10), which would stimulate and regulate the immune response to limit the spread of infection [5]. Cytokines are known to play a critical role in protection against bacterial, fungal, and viral infections, and have been found to be involved in the pathogenesis and development of symptoms of infection [6]. Inflammatory responses are advantageous for eradicating bacteria, as long as they are under control, but when they are out of control, a massive production of pro-inflammatory cytokines (*e.g.*, IL-6) can cause tissue injury and even multiple organ failure. The inflammatory process is controlled by immune-suppression cytokines such as IL-10 and IL-4. Thus, the pathogenesis of most infectious diseases is related to the infection and the inflammation associated with it. Therefore, an efficient treatment for microbial infections would target both the microbe and its associated inflammatory response.

Medicinal plants have been used extensively as alternative agents for the treatment of various infections and diseases for thousands of years [1]. The compilation of *Materia medica* in the first century CE indicates the importance of such plants. The Middle East is rich in natural flora. Hundreds of its plants are used as traditional medicine to treat various diseases.

Jordan, as part of the Middle East, has plenty of useful medicinal plants, whether cultivated or growing wild. A large proportion of the population relies on them for health care, nutrition, flavoring, and beverages. In Jordan, herbal tea products are commonly consumed on a daily basis and applied in folk medicine. Za'ter (*Origanum syriacum*), most frequently used as a table condiment, and Haslban (*Rosmarinus officinalis*) are two very popular herbal teas used widely during wintertime as remedies for the common cold, abdominal pain, constipation, and as antiseptic agents [7]. They are usually used alone or mixed with other herbal teas to provide fragrance and a pleasant taste. Romman (*Punica granatum*) is a popular fruit in Jordan during summer, and is used as a skin moisturizer and for treatment of skin infections. Kaikab (*Arbutus andrachne*), Besbas (*Chrysanthemum coronarium*), and Taioon (*Inula viscosa*) herbs are commonly used in herbalists' preparations for medical or cosmetic uses.

The aim of this study is to determine the antimicrobial and anti-inflammatory activity of six plants (*Arbutus andrachne*, *Chrysanthemum coronarium*, *Inula viscosa*, *Origanum syriacum*, *Punica granatum*, and *Rosmarinus officinalis*) used in Jordanians' cuisine and used as traditional medicine for treating various ailments including infections. Some of the plants were tested for the effect of their different parts (flowers, bark, peels, or leaves). The plant extracts were evaluated for their ability to lower the risk of opportunistic infections of the oral cavity caused by *Staphylococcus aureus*, *Pseudomonas aeruginosa*, or *Candida albicans*. Thus, the herbal agent that inhibits the release of IL-6 pro-inflammatory cytokine while not suppressing the release of IL-10 will be considered an anti-inflammatory drug candidate. In addition, since herbalists prepare blends of plants for treating diseases, combinations of some plants were prepared and screened for the presence of synergistic activity.

Methodology

Plant material

The plants used in this study were collected either from the wild or from cultivated crops in Jordan. Table 1 shows a list of the plants evaluated in this study, with their common name and their traditional use as medicinal plants. The taxonomic identity of each plant was authenticated as mentioned in a previous study [8].

Plant extraction

The plant samples were dried carefully under shade at room temperature and then grinded to powder and stored in airtight bottles. Suitable amounts of the powdered plants were soaked in methanol for 72 hours at room temperature and were continuously stirred. The crude methanolic extracts were filtered using glass microfiber (grade GF/B 1 µm, Whatman, GE Healthcare, Little Chalfont, UK) and then the solvent was desiccated under reduced pressure using a rotary evaporator (Heidolph Laborota, Munich, Germany). The residues were further desiccated by being incubated for 8 days at 30°C. The crude extracts were either used directly or stored in an airtight container for further use.

Microbial strains

In this study, opportunistic microorganisms that grow in the oral cavity were selected to include Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923), Gram-negative bacteria (*Pseudomonas aeruginosa* ATCC 9027), and yeast (*Candida albicans*

Table 1. Plants used in the study, their common name, and traditional use in medicine

Plant extract	Part used	Family	Common Arabic traditional name	Uses in traditional medicine
Arbutus andrachne L.	Bark			
Arbutus andrachne L.	Flowers	Ericaceae	Kaikab	Urinary tract infections [9]
Arbutus andrachne L.	Leaves			
Chrysanthemum coronarium	Flowers	Asteraceae	Besbas	Dermal infections [10]
Inula viscosa L.	Leaves	Asteraceae	Taioon	Antihelminthic , lung cancer, muscle relaxant, cutaneous abscesses, wound healing, tuberculosis, bronchial infections, antiseptic [10-12]
Inula viscosa L.	Flowers			
Origanum syriacum	Leaves	Lamiaceae	Za'ter	Colon, upper respiratory tract infections, wound infection, vaginitis [7,13]
Punica granatum L.	Flowers	Punicaceae	Romman	Respiratory system infections, gastrointestinal disorders [14]
Punica granatum L.	Peels	Punicaceae	Romman	
Rosmarinus officinalis L.	Leaves	Lamiaceae	Haslban	Antiseptic, antispasmodic, diuretic [7,15]

ATCC 10231). All the strains were stored at -20°C in tryptic soya broth (TSB; Oxoid, Basingstoke, UK) with 20% glycerol.

Preparation of inocula

The inocula of microorganism strains were prepared from an overnight culture in TSB, and suspensions were adjusted to 0.5 McFarland standard turbidity (~10^8 colony-forming units [CFU]/mL).

Agar diffusion method

The antimicrobial activity of the methanolic extracts was initially evaluated against all microorganisms using the agar diffusion cup plate method. Inocula of different microorganisms were spread separately on 20 mL Mueller-Hinton agar plates (Oxoid, Basingstoke, UK). One-centimeter diameter wells were punched out with stainless steel cylinders. Equal volumes (150 µL) of saturated solutions of extracts in dimethylsulfoxide (DMSO) were used to fill the wells.

The plates were incubated overnight at 35°C for bacteria and for 48 hours for the yeast. 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 were chosen for further studies.

Minimum inhibitory concentration (MIC) by agar dilution test

Extracts showing antimicrobial activity against any microorganism tested were assayed for their MIC against all the selected microorganisms. Saturated stock solutions of the extracts were prepared in DMSO. Double serial dilutions were also prepared in the same solvent. Aliquots of the solutions (1 mL) were mixed with a fixed amount of molten Muller-Hinton agar at 45°C to get the final concentrations. When the agar solidified, the plates were inoculated with 10 µL microbial suspension (~10^6 CFU/mL). The inoculated plates were incubated for 24 hours at 35°C for bacteria and for 48 hours for the yeast. Positive controls for all microorganisms were prepared using 1 mL DMSO instead of an extract solution.

After incubation, the plates were inspected visually. The MIC was defined as the minimum concentration that resulted in no growth. The test was not considered valid unless the positive controls showed significant microbial growth. The same procedure was applied when the two-component extract mixture of *R. officinalis* and *O. syriacum* leaves was tested.

Anti-inflammatory effect

Plants that showed some antimicrobial activity were evaluated for their anti-inflammatory effect. Also, when extracts from different parts of the plant (*i.e.*, leaves, flower, or bark) showed an antimicrobial effect, the most active part was evaluated for anti-inflammatory activity. Accordingly, the peels were used from *P. granatum*, flowers from *C. coronarium*, and the leaves from *R. officinalis*, *A. andrachne*, *O. syriacum*, and *I. viscosa*. To determine their anti-inflammatory activity, the release of the pro-inflammatory cytokine IL-6 and the anti-inflammatory cytokine IL-10 was investigated in human peripheral blood mononuclear cells (PBMCs). A mixture of *R. officinalis* leaves and *O. syriacum* leaves in a 1:1 ratio was also evaluated for its anti-inflammatory activity.

PBMC isolation

Venous blood was collected from healthy volunteers into heparinized vacutainer tubes (Becton Dickinson, Mountain View, USA). The heparinized blood was diluted 1:1 with phosphate-buffered saline (PBS) (pH 7.2) and layered on a Ficoll-Hypaque gradient (Lymphoprep; Accurate Chemical, Westbury, USA). The gradient was centrifuged at 2,500 rpm for 15 minutes at room temperature, and the buffy coat containing the PBMCs was collected and washed twice in PBS.

Cell culture

PBMCs were cultured in RPMI 1640 medium (Lonza, Verviers, Belgium) containing 10% heat-inactivated fetal bovine serum (FBS) (Gibco,Waltham, USA), HEPES buffer (10 mM), L-glutamine (2 mM), gentamicin (50 µg/mL), penicillin (100U/mL), and streptomycin sulfate (100 mg/mL) (all Lonza, Verviers, Belgium). Cell count and viability were assessed by trypan blue dye exclusion.

Effects of the plant extracts on the production of human pro-inflammatory IL-6 and anti-inflammatory IL-10 cytokines

To determine the effect of the plant extracts *in vitro*, 1×10^6 cells/mL of PBMCs were seeded in a 24-well plate and either left without activation (cells in growth medium alone) or activated with 5 µg/mL mitogen, concanavalin A (ConA) (Sigma, Vienna, Austria), for 30 minutes before the plant extract treatment. PBMCs (2×10^5 cells/mL) of each group (ConA-pretreated and the cells left alone) were split into triplicate wells in 96-well flat-bottomed microtiter plates. Both groups were treated with one of the plant extracts at 100, 50, and 10

µg/mL concentrations separately in growth medium or left without extract treatment (control). Cultures were incubated in a humidified atmosphere of 37°C and 5% CO_2 overnight. Supernatants obtained from controls and treated cells were harvested for analysis by an enzyme-linked immunosorbent assay (ELISA). Non-treated cells with or without ConA pretreatment were used as negative controls.

The concentrations of IL-6 and IL-10 cytokines (in 100 µL of PBMCs supernatant) were determined by ELISA assay according to the manufacturer's protocol (eBioscience, San Diego, USA). All incubation steps were performed at room temperature. The optical density at 450 nm, corrected by the reference wavelength 570 nm, was measured with microplate reader (Biotek, Winooski, VT, USA). All cytokine assays were calibrated against the World Health Organization's international standards by the kit's manufacturer. The lower limit of detection for the individual assays of human IL-6 and IL-10 cytokines was 4 pg/mL for each.

Statistical analyses

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

Results

Agar diffusion method

The presence of antimicrobial activity of the 10 methanolic plant extracts shown in Table 1 was assessed using an agar diffusion method. The activity was screened against microbes, representing Gram-positive and Gram-negative bacteria and a yeast, which are known to cause oral infections. All of these extracts showed antimicrobial activity to at least one of the microbes tested (data not shown), and were further studied to determine their minimum inhibitory concentration using an agar dilution method .

Minimum inhibitory concentration

Minimum inhibitory concentrations for the extracts were determined using an agar dilution method (Table 2). *R. officinalis* and *O. syriacum* showed the highest activity (lowest MIC) against *S. aureus* (1 mg/mL for each) followed by *P. granatum* (flowers and peels) and *I. viscosa* leaves (2.5 mg/mL for each). *A. andrachne* flowers and leaves showed three times lower MIC values than the bark against *S. aureus* (2.5 mg/mL and 7.5 mg/mL, respectively). For most of the extracts, the concentrations needed to inhibit *P. aeruginosa* were higher than those needed to inhibit the other microbes. In fact, for the majority of the extracts, the determination of MICs against *P. aeruginosa* could not be achieved under the experimental conditions since precipitation of the extract occurred before achieving growth inhibition. However, among the determined MIC values, *O. syriacum* showed the lowest MIC value (2 mg/mL) followed by *P. granatum* and *A. andrachne* leaves (5 mg/mL). *O. syriacum* also showed the lowest MIC against the tested yeast *C. albicans* (1 mg/mL) followed by *I. viscosa* and *C. coronarium* (5 mg/mL) and *P. granatum* (10 mg/mL). *A. andrachne* and *R.*

Table 2. Minimum inhibitory concentration (MIC) values of different methanolic plant extracts against *S. aureus*, *P. aeruginosa*, and *C. albicans.*

	MIC mg/mL		
	S. aureus	***P. aeruginosa***	***C. albicans***
Arbutus andrachne L. bark	7.5	15	> 15
Arbutus andrachne L. flowers	2.5	> 5	> 5
Arbutus andrachne L. leaves	2.5	5	> 5
Chrysanthemum coronarium flowers	5	> 5	5
Inula viscosa flowers	5	> 5	5
*Inula viscos*a leaves	2.5	> 5	5
Origanum syriacum leaves	1	2	1
Punica granatum peels	2.5	5	10
Punica granatum flowers	2.5	5	10
Rosmarinus officinalis leaves	1	> 2	> 2
Mixture: *R. officinalis* + *O. syriacum*	0.25 + 0.25	1 + 1	0.5 + 0.5

officinalis did not show anti-candidal activity under experimental conditions.

For some plants, extracts from different parts of the plant showed variable activity against microorganisms. *Inula viscosa* flowers had better activity against *S. aureus* than did the leaves, whereas *A. andrachne* leaves exhibited the highest activity against *S. aureus* and *P. aeruginosa* when compared to the bark and flowers. On the other hand, *P. granatum* flowers and peels showed similar activity against the tested microorganisms.

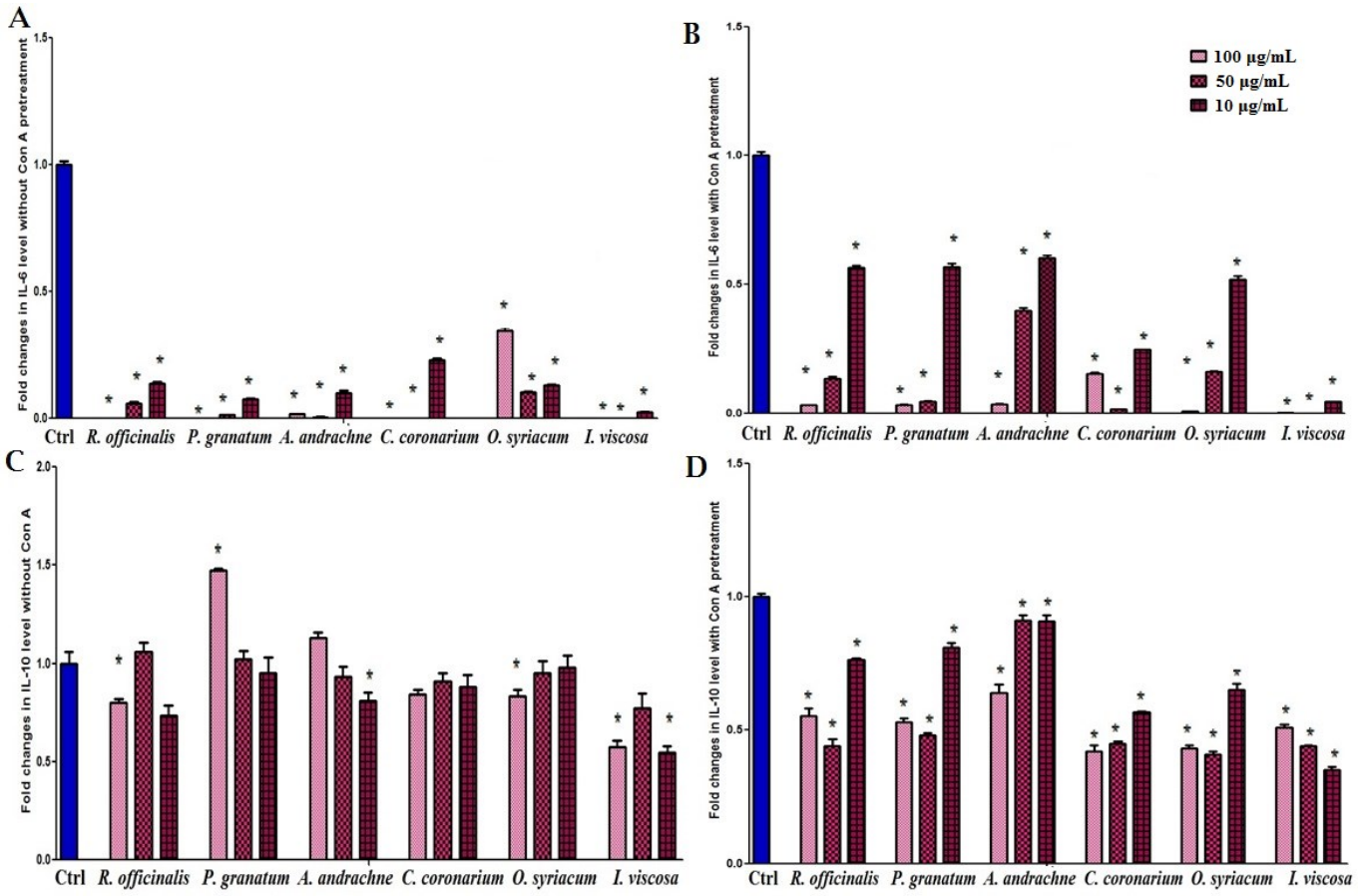
In a previous study [8], a two-component mixture of *R. officinalis* and *O. syriacum* had shown synergistic activity against the anaerobic microorganism *Propionibacterium acnes*. In this study, the presence of synergistic activity of this mixture towards *P. aeruginosa*, *S. aureus*, and *C. albicans* was also investigated.

The two-component mixture was prepared from the highest soluble concentrations of *R. officinalis* and *O. syriacum* that could be achieved, which resulted in 1:1 combination. MICs for the mixture against *S. aureus*, *P. aeruginosa*, and *C. albicans* were found to be 0.25, 1, and 0.5 mg/mL, respectively, for each component (Table 2).

Evaluation of the anti-inflammatory effect

The effect of the extracts that showed an antimicrobial activity in a model of inflammation was studied. Inflammation is mediated by cytokines released from PBMCs in response to a mitogen, ConA, a lectin which non-specifically activates T cells with little effect on B cells. In this study, PBMCs were pretreated with ConA or left without treatment before being stimulated with different concentrations of the extracts overnight to determine cytokine release by ELISA assay (Figure 1). An overnight incubation time

Figure 1. The anti-inflammatory effect of the plant extracts on peripheral blood mononuclear cells (PBMCs).



Effects of the plant extracts (100 µg/mL, 50 µg/mL, and 10 µg/mL concentrations) on the release of the cytokines from the untreated and from the 5 µg/mL ConA-pretreated PBMCs. (A) Pro-inflammatory cytokine IL-6 with ConA; (B) IL-6 without ConA; (C) Anti-inflammatory cytokine IL-10 with ConA; (D) IL-10 without ConA. Plants used were *P. granatum* peels, *C. coronarium* flowers, and *R. officinalis*, *A. andrachne*, *O. syriacum*, and *Inula viscosa* leaves. Data represent the mean concentration pg/mL of triplicates ± standard error of mean. Differences were considered significant at $p < 0.01$ versus control (Ctrl).

was chosen since it enables sufficient levels of secreted cytokines to accumulate in the cell medium. In addition, it was found to be the optimal length of time to obtain a significant effect in cytokine reduction compared to positive controls [8]. The *R. officinalis*/*O. syriacum* mixture was also evaluated for its anti-inflammatory responses, using 50 µg/mL concentration from each extract.

The effect of the plant extracts on the production of human IL-6 and IL-10 cytokines in PBMCs

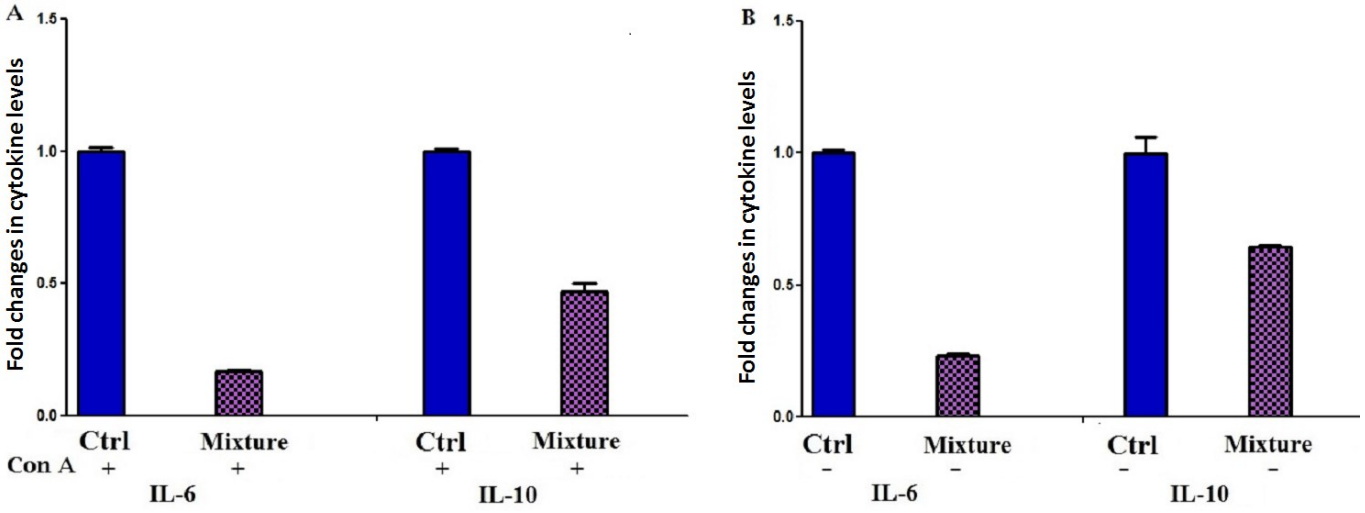
All tested plants showed some antimicrobial effect; the part of the plant that showed the best antimicrobial activity on the three tested microorganisms was tested for its anti-inflammatory activity. Control PBMCs pretreated with ConA showed a significant increase in cytokine levels compared to non-treated cells (Figure 1). The release of IL-6 from ConA-pretreated PBMCs (Figure 1B) was significantly reduced with all plant extract treatments when compared with the control (Figure 1A). More than 80% reduction in IL-6 levels was exhibited when 100 and 50 µg/mL of the extracts were used with ConA-pretreated PBMCs except for 50 µg/mL of the *A. andrachne* extract, which showed a 60% reduction. On the other hand, 10 µg/mL of most of the extracts showed almost 40% reduction except for *C. coronarium*, which showed 75% reduction, while *I. viscosa* showed 95% reduction. *I. viscosa* had the best response in all concentrations. In addition, at least 90% reduction was exhibited in IL-6 level in almost all extract treatments from PBMCs that were not pretreated with ConA except for 10 µg/mL of *C. coronarium* and 100 µg/mL of *I. viscosa* extracts, which showed around 70% reduction.

The extracts attenuated the production of IL-10 in ConA-pretreated PBMCs (Figure 1D), where an almost 50% reduction in IL-10 level was exhibited when 100 µg/mL and 50 µg/mL of the extracts were used compared to the control, except for *A. andrachne*, which showed 35% reduction at 100 µg/mL and only 9% reduction when 50 and 10 µg/mL of the extract were used. On the other hand, PBMCs that were not pretreated with ConA but were stimulated with the extracts (Figure 1C) showed less than 20% reduction in IL-10 in most of the treatments. Some of the treatments (50 µg/mL of *R. officinalis*, 100 and 50 µg/mL of *P. granatum*, and 10 µg/mL of *A. andrachne* extracts) showed a significant induction in IL-10 release compared to the control. PBMCs stimulated with 100 µg/mL or 10 µg/mL of *I. viscosa* extracts without ConA pretreatment (Figure 1C) showed almost 40% reduction in IL-10. When 50 µg/mL was used, only 20% reduction in IL-10 level was exhibited. Most of the plants showed a dose-dependent effect on the release of the cytokines.

The effect of the (R. officinalis/O. syriacum) extract mixture on the production of IL-6 and IL-10 cytokines

The effect of the extract mixture (*R. officinalis*/*O. syriacum*) on the production of IL-6 and IL-10 cytokines was studied at 50 µg/mL concentration of each plant. This concentration was selected since most

Figure 2. The anti-inflammatory effects of the plants extract combinations on PBMCs.



The effect of a mixture of 50 µg/ml *R. officinalis* leaves and 50 µg/ml *O. syriacum* leaves on the release of the pro-inflammatory cytokine, IL-6 and the anti-inflammatory cytokine, IL-10 from Con A pretreated and non-pretreated PBMCs. Data represent the mean concentration pg/ml of triplicates ± S.E.M. Differences were considered significant at $P < 0.01$ vs. control.

of the plant extracts showed significant reduction in IL-6 level and significant change in IL-10 level at 50 µg/mL concentration. Table 3 summarizes the levels of the two tested cytokines in the presence or absence (control) of the extracts at 50 µg/mL concentration. The mixture significantly reduced IL-6 (80% reduction) and attenuated the production of IL-10 level by about 50% in ConA-pretreated PBMCs compared to the non-pretreated cells as indicated in Table 3 and Figure 2. In general, the combination of *R. officinalis* and *O. syriacum*, did not show a significant change in the cytokine levels compared to each extract alone in PBMCs pretreated with ConA.

Discussion

Opportunistic infections are associated with individuals of poor health and are caused by several different microorganisms. Due to the rising prevalence of antibiotic-resistant opportunistic microorganisms and the severe damage from the inflammatory response such microorganisms can lead to, the demand for finding new alternative antimicrobial and anti-inflammatory agents is increasing. The use of medicinal plants in traditional medicine is very common in developing countries and Jordan in particular [7,14]. In the present study, the use of some plants in ethnomedicine in Jordan for the treatment of a variety of opportunistic microorganisms causing infections and inflammatory reactions in the oral cavity was investigated *in vitro.*

A. andrachne is an evergreen tree that grows in hilly areas in Jordan. Various parts of this plant (bark, flowers, and leaves) showed good activity against Gram-positive bacteria and Gram-negative bacteria, but no anti-*Candida* activity was found under the experimental setting (Table 2). Few studies have reported the antimicrobial activity of the species *A. andrachne* [8], although many reports indicated variable antimicrobial activities in other species such as *A. unedo* and *A. menziesii* [16,17].

Another plant studied was the flowers of *C. coronarium*. The flowers are yellow-orange and grow widely in valleys and along the roadsides in some parts of Jordan. The extract has shown a noticeable activity against *S. aureus* and *C. albicans.* A previous report [18] found antimicrobial activity of *C. coronarium* methylene chloride extract against a group of Gram-positive and Gram-negative bacteria, while methanolic extracts were inactive. Our results in this study and a previous [8] study contradict these methanolic extract results. This could be related to the concentrations of extracts used or the methodology applied. On the other hand, extracts of two parts of the wild plant *I. viscosa* were studied: the flowers and the leaves. Our results indicated that both parts exhibited good activity against *S. aureus* and *C. albicans*, although no activity was exhibited against *P. aeruginosa* at the experimental conditions. These results are in line with other studies that showed antimicrobial activity of this plant against a wide range of microorganisms [8,19]. The difference in MIC values found in our study compared with those of other researchers could be justified by variations in the extraction procedure, microbial strains, and the method used to determine MIC values.

O. syriacum is a culinary herb that is indispensable in many Mediterranean cuisines. Earlier studies had shown multiple biological activities of this plant, including antimicrobial and anti-inflammatory activities [8,10,21]. In this study, *O. syriacum* exhibited the highest antimicrobial activity among the plants tested. This was indicated by the lowest MIC values against all the microorganisms studied (Table 2). These results agree with the findings of other studies, in which *O. syriacum* essential oils showed a significant antimicrobial effect on *Staphylococcus aureus* and *Candida albicans* on both standard and wild-type strains isolated from human oral cavities [22]. Such

Table 3. The anti-inflammatory effect of the plant extracts on peripheral blood mononuclear cells (PBMCs).

Plant extract	IL-6 (pg/mL)		IL-10 (pg/mL)	
	ConA	None	ConA	None
Control	79.56 ± 1.04	39.14 ± 0.54	17.63 ± 0.203	3.367 ± 0.2028
Arbutus andrachne leaves	31.63 ± 0.79	0.17 ± 0.067	16.03 ± 0.376	3.13 ± 0.186
Chrysanthemum coronarium flowers	1.17 ± 0.033	0.0 ± 0.0	7.90 ± 0.153	3.07 ± 0.133
Inula viscosa leaves	0.0 ± 0.0	0.0 ± 0.0	7.77 ± 0.067	2.60 ± 0.252
Origanum syriacum leaves	12.77 ± 0.33	4.07 ± 0.088	7.20.4 ± 0.208	3.20 ± 0.2082
Punica granatum peels	3.62 ± 0.127	0.51 ± 0.05	8.50 ± 0.153	3.43 ± 0.145
Rosmarinus officinalis leaves	10.63 ± 0.63	2.33 ± 0.299	7.77 ± 0.4702	3.57 ± 0.167
Mixture (R. officinalis, O. syriacum)	13.33 ± 0.306	9.14 ± 0.31	8.27 ± 0.57	2.17 ± 0.033

Effects of the plant extracts, at 50 µg/mL concentrations, on the release of the pro-inflammatory cytokine IL-6 and anti-inflammatory cytokine IL-10 from non-activated PBMCs and Con A-pretreated PBMCs. Data represent the mean concentration pg/mL ± standard error of mean of triplicates. Differences were considered significant at $p < 0.01$ vs. control.

activity of *O. syriacum* against a wide range of microbes rationalizes its use as an antiseptic and for oral cavity infections (Table 1).

P. granatum is widely cultivated in the Mediterranean region and used in folkloric medicine to treat various diseases [23]. The presence of antimicrobial activity in the peels, seeds, and juice of *P. granatum* had been shown previously [8,24]. Our results showed a substantial antimicrobial activity of not only the peels but also the flowers against the three microorganisms tested. To our knowledge, no previous studies have reported such activity for the flowers against these microoganisms. These results are consistent with the use of this plant in folkloric medicine to treat many infectious diseases (Table 2).

R. officinalis is an evergreen shrub that is widely cultivated in Jordan and is very commonly seen as hedges in public and private gardens. *R. officinalis* has exhibited noticeable activity against the Gram-positive bacteria with the lowest MIC of the plants tested. However, no activity against *P. aeruginosa* or *C. albicans* was detected in this study (Table 2). Previous studies had detected variable antimicrobial activity of *R. officinalis* against different microorganisms, where it was highly active against some microorganisms [8,25] and moderately active to inactive against others [25]. A previous study demonstrated the activity of *R. officinalis* ethanolic extract against *S. aureus* and *C. albicans* (but not against *P. aeruginosa*) using the disk diffusion method [25]. These results are consistent with the results from this study except for *C. albicans*, where no inhibition to *C. albicans* at the highest applicable concentration (2 mg/mL) was found. This variation in results could be attributed to the differences in the organic solvents used for the extraction or the method of the antimicrobial assays between the two studies.

In many instances, combinations of chemotherapeutic agents are administered to patients in order to widen their spectrum of activity or to increase their effectiveness by producing a synergistic effect. The same applies to medicinal plants where mixtures are prepared and used. In a previous study [8], a 1:1 mixture of the two herb extracts *O. syriacum* and *R. officinalis* showed a remarkable synergistic anti-inflammatory and antimicrobial activity against *Propionibacterium acnes*. A mixture of these herbs is widely used in cooking and seasoning of many Mediterranean cuisines. In the current study, the antimicrobial and anti-inflammatory activity of this mixture was evaluated against other microorganisms known to cause various infections in order to investigate the effect of their interaction.

In order to classify the activity of any antimicrobial combination as synergy, antagonism or additive (no effect) interactions, the concept of isobologram was applied [26,27]. Testing of this mixture indicated that 0.25 mg/mL *R. officinalis* required 0.25 mg/mL *O. syriacum* as a minimum concentration to inhibit *S. aureus* growth (Table 4). If these extracts had no effect on each other (*i.e,*. if an additive effect was exhibited), then the 0.25 mg/mL *R. officinalis* would require 0.75 mg/mL of *O. syriacum* to produce the same effect. This means that presence of *R. officinalis* increased the activity of *O. syriacum* against *S. aureus* threefold.

For *C. albicans*, 1 mg/mL of *O. syriacum* was needed to inhibit its growth; due to precipitation of the extract at concentrations above 2 mg/mL, the concentration of *R. officinalis* extract required to inhibit *C. albicans* was not obtainable at the experimental setting (Table 2). When the mixture was tested, only 1 mg/mL of both *O. syriacum* and *R. officinalis* were needed to inhibit the yeast. This indicates that a 1.5-fold increase in activity of *O. syriacum* was achieved when mixed with *R. officinalis* (Table 4). For *P. aeruginosa*, the increase in activity of *O. syriacum* was more than one fold when mixed with *R. officinalis*. This means that in the worst case, if the effect is not synergistic, it is additive (Table 4).

On the other hand, studies showed that those opportunistic microorganisms and their products are capable of strongly inducing various cytokines that could lead to increased inflammatory infiltrate into the target organs [28,29]. The pro-inflammatory cytokine

Table 4. The change in minimum inhibitory concentrations (MICs) of *O. syriacum* when *R. officinalis* was added to it in a 1:1 ratio.

Microorganisms tested	MIC of *R. Officinalis* (mg/mL)	Concentration of *O. syriacum* needed if additive effect happened (mg/mL)	Actual concentration of *O. syriacum* needed	Increase in *O. syriacum* activity
S. aureus	0.25	0.75	0.25	3 times
P. aeruginosa	1	* > 1	1	> 1*
C. albicans	0.5	* > 0.75	0.5	> 1.5*

*Could not be determined exactly since isobologram was not plotted because MIC of *R. officinalis* was not obtained at highest concentration achievable at the test conditions.

IL-6 and the anti-inflammatory cytokine IL-10 were found to be increased during infections.

Although IL-6 is a multifunctional cytokine that is most often classified as a pro-inflammatory cytokine, data consistently demonstrate that it also has an anti-inflammatory effect. In general, IL-6 stimulates the inflammatory processes in many diseases. In acute infections, it is produced commonly at local tissue sites and released into circulation. It also has a critical role in the generation of immunity against chronic intracellular infections and induction of acute phase reactions needed to maintain homeostasis. Previous studies showed that the endogenous IL-6 cytokine is essential for limitation of bacterial growth in certain areas. On the other hand, IL-10 hampers antimicrobial defenses in such models [30,31]. When such responses are under control, they can manage microbial action. But whenever they are out of control, an enormous production of the pro-inflammatory cytokine IL-6 will occur, which can cause tissue injury and multiple organ failure. IL-10 remains one of the most important anti-inflammatory cytokines that plays a key role in many infections such as sepsis and acute lung injury by modulating the inflammatory response. In various model systems, IL-10 has been shown to inhibit production of several pro-inflammatory cytokines (*e.g.*, TNF-α, IL-1β, IL-6) that are known to be involved in the development of acute inflammatory states.

IL-10 was found to be involved in host resistance to *S. aureus* infection through the regulation of IFN- γ [32]. Also, a recent study [33] showed that infections with *S. aureus* induced high levels of IL-10 as well as IL-6. The exact role of induction of IL-6 responses during *S. aureus* infection is still not clear. On the other hand, *S. aureus* has the potential to disable host immunity at the time of infection through the induction of IL-10. Wang *et al*. [33] indicated that IL-10 might inhibit the innate immune response by decreasing the release of inflammatory cytokines. Hence, controlling induction of IL-6 and IL-10 may have profound clinical value, especially during vaccinations. Another study [34] showed that both *S. aureus* and *P. aeruginosa* were found to potently activate the pro-inflammatory cytokine IL-6 expression immediately upon contact with airway epithelial cells. In severe bacterial pneumonia, excessive pro-inflammatory signaling results in airway obstruction and respiratory failure.

A very recent study [35] showed that 1,000 ng/mL lipopolysaccharide (LPS) from *P. aeruginosa* led to significant secretion of TNF-α, IL-1β, IL-6, IL-8, and IL-10 in whole blood. Another study showed that IL-10 overexpression resulted in enhanced *P. aeruginosa*-induced lethality but with a time-dependent effect [36]. The researchers indicated that IL-10 is necessary to attenuate inflammation early in sepsis. At a later stage, blocking IL-10 helps to improve secondary bacterial clearance.

Similar to the previously mentioned opportunistic bacteria, *Candida albicans*, an opportunistic fungal microorganism, was also found to cause tissue damage, releasing the inflammatory mediators that initiate and sustain local inflammation. A recent study showed that *C. albicans* would induce different immune responses depending on whether the morphology was yeast or hyphae type when macrophages were treated with them [37]. The yeast form stimulated the synthesis of IL-6 cytokine and thus induced an inflammatory reaction, but with no changes in IL-10 level. As *C. albicans* morphology switch from yeast to hyphae, the expression of the pro-inflammatory cytokine IL-6 decreased and the expression of IL-10 increased, allowing the hyphae to evade the immune response of the host cells. Another study done by Cheng *et al*. [38] indicated that live *C. albicans* cells can release soluble factors to actively potentiate IL-6 production induced in human mononuclear cells by heat-killed *C. albicans* cells.

Plant extracts that showed some antimicrobial action were tested to determine if they had immunomodulating effects depending on the type and time of infection. As *S. aureus* and *P. aeruginosa* infections lead to the induction in IL-6 production, we were looking for the extract that inhibits its production. On the other hand, the yeast form of *C. albicans* would stimulate the synthesis of IL-6 and induce an inflammatory reaction, while the hyphae form leads to the reduction in its release. To prevent the inflammatory response released from live *C. albicans* cells, we looked for extracts that would inhibit IL-6 release. In our study, all the six plant extracts tested showed an inhibitory effect on IL-6 production.

The role of IL-10 was relatively variable among the three microorganisms. *S. aureus* has the potential to disable host immunity at the time of infection through the induction of IL-10 [33]. Another study showed that IL-10 plays a beneficial role in host resistance to *S. aureus* infection [32]. In this regard, we wanted a plant extract that inhibits the overproduction without complete inhibition of IL-10 while keeping its induction to suppress the inflammatory response. In *P. aeruginosa* infections, IL-10 was overexpressed but with a time-dependent effect, resulting in enhanced *P. aeruginosa*-induced lethality. As a result, IL-10 overproduction should be inhibited depending on the

stage of the infection, but without affecting its anti-inflammatory function. The infection with the hyphae of *C. albicans* is known to increase the expression of IL-10, allowing the hyphae to evade the immune response of the host cells, while the yeast form will not show an effect on IL-10 release. Thus extracts that would inhibit the overproduction of IL-10 while not affecting its anti-inflammatory function may have therapeutic value for various infectious and inflammatory disorders independent of its antimicrobial properties.

There are few reports in the literature regarding the anti-inflammatory properties of the six tested plant extracts on cytokine release; all these studies reported anti-inflammatory responses [8,20,21,39-43]. Our results were consistent with previous studies demonstrating that all of the six plant extracts tested showed an inhibitory effect on IL-6 production while most of them attenuated the release of IL-10, but relatively with variable responses and in a dose-dependent manner. The most effective extract showing immunomodulating effects was 50 µg/mL of *Arbutus andrachne*. It inhibited the release of the pro-inflammatory cytokine IL-6 while slightly attenuating the production of the anti-inflammatory cytokine IL-10 from ConA-pretreated PBMCs. The mixture of 50 µg/mL *Rosmarinus officinalis* leaves and 50 µg/mL *Origanum syriacum* leaves significantly reduced IL-6 release and attenuated the release of IL-10 in ConA-pretreated PBMCs. On the other hand, the effect of each extract alone showed no significant difference when compared to the mixture, indicating that the mixture did not have a synergistic effect.

To our knowledge, this is the first study to investigate the effect of these extracts on IL-6 and IL-10 release from PBMCs pretreated with ConA or left without treatment. Abrham *et al*. [44] evaluated the anti-inflammatory effect of purified compounds of *I. viscosa* leaves on the secretion of pro-inflammatory cytokines from PBMCs upon stimulation with LPS or phorbol myristate acetate (PMA). Their results showed no effect on the secretion of IL-6. In another study, Mueller *et al*. [45] used LPS-stimulated macrophage as a model for testing plant extracts for pro- or anti-inflammatory activity. Secretion of IL-6 was significantly reduced when 0.5 mg/mL of oregano (*Origanum onites*) leaves were used, while 0.2 mg/mL of oregano enhanced the secretion of IL-10, but not when 0.5 mg/mL of oregano was incubated with the stimulated macrophages. On the other hand, *R. officinalis* leaves and *P. granatum* fruit did not reduce IL-6 release, while rosemary reduced IL-10 secretion and pomegranate enhanced its secretion. No study could be found that showed the effect of *A. andrachne* and *C. coronarium* on the release of IL-6 and IL-10.

Conclusions

A diet rich in antimicrobial and anti-inflammatory compounds derived from fruits, vegetables, and/or herbs may lower the risk of opportunistic infections of the oral cavity caused by *Staphylococcus aureus*, *Pseudomonas aeruginosa*, or *Candida albicans*. The results of our study demonstrate enhanced antimicrobial and anti-inflammatory response when the methanolic extract of *O. syriacum* was used. Also, to the best of our knowledge, this is the first study to evaluate the anti-inflammatory effect of these extracts on the release of IL-6 and IL-10 from PBMCs pretreated with ConA. The use of a *R. officinalis* and *O. syriacum* mixture showed a synergistic antimicrobial effect and a good anti-inflammatory effect. The anti-inflammatory effect was additive, not synergistic, when compared with the effect of each extract alone. Moreover, the extracts inhibited the expression of the pro-inflammatory cytokine IL-6 with apparent dose-dependent responses, while they attenuated the secretion of the anti-inflammatory cytokine IL-10. These findings support the idea that a diet rich in plants and herbs may contribute to the reduction of inflammation and microbial growth and be preventive against related diseases. Although our results demonstrate promising antimicrobial and anti-inflammatory properties for the treatment of opportunistic microorganisms, further studies are required to confirm the pharmacological relevance of the findings.

Acknowledgements

The authors would like to thank the Deanship of the Scientific Research in the University of Jordan for the financial support, Miss Rula Al Selawi, research assistant at the Faculty of Pharmacy, for her work in the microbiology part, and Mr. Mashhour T. Assaf for his valuable contribution to the writing and editing of the manuscript.

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Conflict of interests: No conflict of interests is declared.