Plants of the genus *Eremurus* have been used in Kurdish medicine since ancient times. Particularly, the “*Eremurus roots*” sold at the local market (presently known as *Eremurus* spp.) are used as both topical and oral remedies for treating inflammatory disorders. In the present paper, the root ethanolic extracts (EE) of *Eremurus persicus* and *Eremurus spectabilis*, the most common *Eremurus* species in the region, were prepared and their phytochemical profiles drawn with the aim of identifying the plant material sold at the local market. Comparison of the high performance liquid chromatography-ultraviolet/photodiode-circular dichroism (HPLC-UV/PAD-CD) and HPLC-electrospray ionisation-tandem mass spectrometry (HPLC-ESI-MS/MS) fingerprints of both species with that of *Eremurus* spp. showed that the main component of *Eremurus* spp. is *E. spectabilis*, with a minor presence of *E. persicus*. All EEs were then subjected to an exhaustive in vitro investigation of anti-inflammatory activity. The assay with cultured human peripheral blood mononuclear cells (hPBMC) activated by phytohaemagglutinin A revealed that *E. persicus* was more effective in inhibiting T-cell proliferation and in reducing the tumor necrosis factor alpha (TNFα) levels in vitro than *E. spectabilis* and *Eremurus* spp. Regarding the *Eremurus* spp. extract, its anti-inflammatory effect appeared to be intermediate compared to the other two extracts examined when considering both anti-proliferative and anti-TNF-α results and in accordance with the analytical results, thus validating the ethnomedical use of *Eremurus* roots in traditional Kurdish medicine. Moreover, results herein reported clearly showed the anti-inflammatory potential of *E. persicus* and its capacity to inhibit both in vitro hPBMC proliferation and cytokine secretion at non-toxic doses. *E. persicus* root ethanolic extract will undergo further investigations in the near future in order to identify the metabolites responsible for the activity.

Key words: *Eremurus*, Kurdish ethnomedicine, chromatographic profile, anti-inflammatory activity, TNF-α blocker.
INTRODUCTION

In the last two decades, an increasing use of herbal drugs was recorded, probably due to the media contributing to the mass spread of traditional medicine (Mati and de Bore, 2011). In particular, Kurdish folkloric herbal medicine still represents the first choice for primary healthcare in towns, villages and rural areas. Although sometimes, plant remedies could be directly collected in the wild by the final users, only a small number of people retain the knowledge about the correct identification of the plants and their proper use. For these reasons, people usually rely on local markets in order to get rapid insight into the use of plants in traditional medicine and to purchase them (Cunningham, 2001). Nevertheless plants sold in local markets are often not correctly labelled or identified. This is the case of “Eremurus roots”, which is sold in many kurdish local markets without any indication of the species on the label (from this point on this will be referred to as Eremurus spp.) and is used for treating skin inflammatory disorders, both as a topical and an oral remedy (folk medicine).

The plants of the genus Eremurus (Xanthorrhoeaceae) comprise nearly 50 species and are mainly restricted to the mountains of central and western Asia (Li et al., 2000). Among them, Eremurus persicus (Jaub and Spach) Boiss is widely distributed in the south, east and west of Iran, where it is called “Sarish” (Karl, 1982; Wendelibo, 1982; Safar et al., 2009; Vala et al., 2011) and Eremurus spectabilis M.Bieb. is mainly distributed in Central Asia (Kurdistan, Turkey, Iran), where it is called “Ciris” (Karl, 1982; Ozturk and Olcucu, 2011). Both species have been traditionally used in Kurdish ethnomedicine to cure diseases having a common pathophysiological factor related to inflammation. It has been reported that the aerial parts of the plants, along with the rhizome and root nodules are eaten by native people for treating inflamed eyes, diabetes and eczema (Karaman et al., 2011; Yesil and Akalin, 2009), while their boiled roots are described to be efficacious in relieving rheumatism, gastrointestinal disorders (Ozturk and Olcucu, 2011), scabies (Karaman and Kocabas, 2001) and inflammatory skin conditions (Mamedov and Gardner, 2004) (Table 1).

In this context, the present work was focused on the identification of the species of Eremurus possibly present in “Eremurus roots” sold at the Sulaymaniyah market (north-western Iraq/Kurdistan) and locally known as Eremurus spp. With this aim, since E. persicus and E. spectabilis are the most common species of Eremurus in the kurdish area, both were collected in the Iranian/Iraqi mountains, “Eremurus roots” were purchased and a preliminary phytochemical investigation of all drugs was performed. Firstly, different extracts of Eremurus spp., E. persicus and E. spectabilis roots, were prepared and their phytochemical fingerprints were drawn by using high performance liquid chromatography-ultraviolet-photodiode array detector coupled on-line to a circular dichroism detector (HPLC-UV/PAD-CD) as well as HPLC-tandem mass spectrometry (HPLC-ESI-MS/MS) and compared. To the best of our knowledge, no study has been carried out to draw the phytochemical profiles of root extracts obtained from different Eremurus species. Indeed, polysaccharides are considered to be the chemical constituents targeting the species, and fructans in the case of E. spectabilis (Pourfarzad et al., 2014, 2015), and no scientific investigations into the phytochemical contents of E. persicus or E. spectabilis root extracts have been reported so far.

MATERIALS AND METHODS

Chemicals

All solvents used were of analytical and high performance liquid chromatography (HPLC) grade, and purchased from Carlo Erba (Milano, Italy). The 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH), polyvinylpyrrolidone (PVP), and gallic acid were obtained from Sigma Aldrich (Milan, Italy). Folin-Ciocalteau reagent (FCR) was purchased from Carlo Erba (Milano, Italy). Green tea extract (Green Select®) used as an antioxidant standard was obtained from Indena (Milano, Italy). RPMI-1640 medium was purchased from Gibco, Life Technologies (Monza, Italy), foetal bovine serum (FBS) from Euroclones S.p.A (Milan, Italy) and Phytohaemagglutinin A (PHA) from Roche (Mannheim, Germany). The 3H-thymidine (3HtdR) was obtained from Amersham Pharmacia Biotec (Milan, Italy). MTS reagent was bought from Promega (Milan, Italy).

Plant material

Powdered “Eremurus spp.” drug was purchased from Sulaymaniyah market, located in north-western Iraq/Kurdistan. E. persicus Boiss was collected in an area of the Gulestan Kuh mountain probe Golpayegan at an altitude of 3000 to 3200 m located 120 km from Isfahan/Iran, in August, 2011. This is a spontaneous and ornamental plant, it can reach 30 to 70 cm in height, seeds 8 to 10 mm long, broadly winged. The leaves can be as broad as 1 cm on the base of the stem. Its tuberous roots have a very strange shape; thick fleshy roots fan out in all directions from a central hub. These roots must be handled very carefully. E. spectabilis Bieb was collected from the KaniMeran village (KaniMeran mountain), which belongs to the Penjwen-Sulaymaniyah/Kurdistan region, north of Iraq, in July, 2011. This is an uncultivated perennial herbaceous vigorous plant shrub that can reach a height 75 to 200 cm, with shortened rhizome and radically divergent fusiform thickened roots. The leaves can broad 4.5 cm glabrous, margin scabrid or smooth, the roots are short, fleshy and descending. The collected plant materials were identified and classified by Dr. Abdulla Sa’ad at the

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Education Science Department, Faculty of Biology, Salahaddin University, Hawler, Iraq. The voucher specimens (no. 6856) and (no.6873), respectively were deposited in the ESUH (Education Salahaddin University Herbarium), Hawler, Iraq. Freshly cut roots were stored, dried in a drying room with active ventilation at room temperature (about 20 to 22°C) until they reached a constant weight. Roots were cut into a small size and ground with a blade mill to obtain a homogeneous fine powder. The drug was stored in the dark.

**Extraction**

_Eremurus_ spp. purchased from the market, as well as the dried powdered roots of _E. persicus_ and _E. spectabilis_ were defatted with petroleum ether 10% (v/v) for 1 h at room temperature under mechanical stirring (Gaggeri et al., 2012), before the extraction was performed. Each dried drug (50 g) was macerated with ethanol (1 L) for 1 h (EE) at room temperature under mechanical stirring. The extraction procedure was repeated three times (each drug), and the filtrates obtained were combined and dried under reduced pressure (Laborota 4000, Heidolph Instruments, Schwabach, Germany). All crude extracts were treated with: 1) charcoal, and 2) polyvinylpyrrolidone (PVP). First, charcoal (0.5 g) was added to crude extract (1 g), dissolved in methanol (500 ml solution) under mechanical stirring for 15 min at room temperature. The solvent was then evaporated under reducing pressure. Second, treatment with PVP was used, as reported by Makkar et al. (1993). Briefly, 1 g extract was suspended in acetone 70% (50 ml) and ethanol (50 ml) before adding 1 g of PVP. After 15 min of mechanical stirring in an ice bath, the solvent was filtered and evaporated at reduced pressure. All obtained dried samples were kept at room temperature in dark conditions.

**Phytochemical profile**

The ethanolic extracts (EE) were analysed by thin layer chromatography (TLC) on silica gel with a mobile phase of ethyl acetate-methanol-water (100:13.5:10 v/v/v) and revealed with cerium reagent, using UV-365 nm light.

**Total phenolics content (TPC)**

The TPC of each extract was determined by the method described by Singleton et al. (1999), with some modifications. Briefly, 1 ml Folin-Ciocâlteu reagent (FCR) was added to 9 ml of deionized water and kept in a dark bottle. The reaction mixture consisted of 1 ml of sample solubilized in 10% EtOH, 6 ml of deionized water and 500 µl of FCR. The mixture was stirred at room temperature for 3 min, before 1.5 ml of sodium carbonate solution (20% w/v) was added. The mixture was then diluted in water to 10 ml and stored in the dark for 2 h at room temperature. Absorbance of mixtures was measured at 760 nm, against a blank containing 1 ml of EtOH 10%. TPC of each individual sample was expressed as % (w/w) of gallic acid. The analyses were conducted in triplicate and results expressed as mean ± standard error (SE).

**HPLC-PAD/UV-CD and HPLC-ESI-MS/MS analysis**

EE were analysed using both a high performance liquid chromatography-diode array system coupled on-line to a circular dichroism detector, and high performance liquid chromatography-electrospray-tandem mass spectrometry. Each sample was dissolved in methanol (3 mg/ml) and filtered with a 0.45 µm GH polypro (GHP) membrane before injection into the HPLC-system. The samples were separated on a Chromolith SpeedROD RP-18 endcapped column (50 mm × 4.6 mm, ID 3 mm, macropore size 2 µm, mesopore size 13 nm, Merck, Darmstadt, Germany). The mobile phase consisted of water containing 0.1% (v/v) formic acid (A) and acetonitrile (B), in gradient elution: 2% of B for 5 min, from 2 to 5% B in 5 min, from 5 to 40% B in 20 min, from 40 to 90% B in 10 min, from 10 to 98% B in 10 min, followed by a re-equilibration step of 5 min. The flow rate was set at 1 ml/min and detection was fixed at 297 nm for both UV and CD detectors.

HPLC-UV/PAD-CD analyses were performed on a Jasco system (Japan) equipped with a Jasco AS-2055 plus autosampler, a PU-2089 plus pump and a MD-2010 plus multi-wavelength detector coupled to a CD-2095 plus circular dichroism detector. The HPLC-ESI-MS/MS analyses were carried out on Finnigan LCQ fleet ion trap system, controlled by Xcalibur software 1.4 (ThermoFinnigan, San Jose CA, USA). Mass spectra were generated both in positive and in negative ion mode under constant instrumental conditions. For positive ion mode: ion spray voltage 5 kV, capillary voltage 46 V, capillary temperature 220°C, and tube lens voltage 120 V. For negative ion mode: ion spray voltage 5 kV, capillary voltage -35 V, capillary temperature 220°C, and tube lens voltage -100 V.

**Free radical scavenging activity**

The free radical scavenging activity (FRS) of the extracts was determined by using a 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay (Gaggeri et al., 2012). A commercially available standardized green tea extract (Green Select®) was used as a standard. Briefly, both dried extracts and the standard were dissolved in MeOH at a concentration of 10 mg/ml; stock solutions were then serially diluted in MeOH two-fold. The reaction mixture was prepared by adding 100 µl of each extract solution (or standard solution) to 3.9 ml of DPPH solution, freshly prepared by dissolving DPPH in methanol/KH₂PO₄ and NaOH buffer (50/50 v/v) at a concentration of 6 × 10⁻² M, giving test solutions with final concentrations of 250, 125, 62.5, 31.25, 15.62, 7.81 µg/ml. After 30 min of incubation at room temperature, the absorbance was measured at 515 nm by a UV-Visible spectrophotometer (Lambda 25 UV/VIS spectrometer, Perkin Elmer instruments, Massachusetts, USA). FRS was expressed as a percent comparison with the control, consisting of 3.9 ml of DPPH solution and 100 µl of methanol. The percent inhibition of the DPPH radical by the test solution was calculated using the following formula:

\[ \text{FRS}\% = \left( \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{Sample}}}{\text{Abs}_{\text{control}}} \right) \times 100 \]

The analyses were carried out in triplicate and results expressed as mean ± SE. IC₅₀ values were calculated by using Graph Pad Prism 4.0.

**Anti-inflammatory activity**

An in vitro proliferation assay on human peripheral blood mononuclear cell (hPBMC) was carried out as previously described (Gaggeri et al., 2013b). Briefly, hPBMC were obtained from healthy donor peripheral blood and grown in RPMI 1640 medium supplemented with 10% foetal bovine serum (FBS), with or without 4 µg/ml of phytohaemagglutinin A (PHA). hPBMC were cultured in the presence of increasing doses of test solutions [dimethyl sulphoxide (DMSO)] solutions of _Eremurus_ spp., _E. persicus_ and _E. spectabilis_ extracts diluted in a 1:2 (v/v) with medium; final concentrations: 800 to 12 µg/ml] or vehicle (control cells). After a
**Table 1. Eremurus** drugs investigated.

<table>
<thead>
<tr>
<th>Species</th>
<th>Part used in this study</th>
<th>Origin</th>
<th>Folk medicine</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. persicus</em> Boiss.</td>
<td>Roots</td>
<td>Isfahan region (Iran)</td>
<td>Inflammation and skin disorder (folkloric); source of natural glue (Vala et al., 2011)</td>
</tr>
<tr>
<td><em>E. spectabilis</em> Bieb.</td>
<td>Roots</td>
<td>Kurdistan region (Iraq)</td>
<td>Scabies (Karaman and Kocabas, 2001); rheumatism and gastrointestinal disorders (Ozturk and Olcucu, 2011); treatment of inflammatory skin conditions (Mamedov and Gardner, 2004); rheumatism (Cakicioglu et al., 2011)</td>
</tr>
<tr>
<td><em>Eremurus</em> spp.</td>
<td>Roots</td>
<td>Sulaymaniyah market (Iraqi Kurdistan)</td>
<td>Inflammation and skin disorders (folkloric) (Mamedov and Gardner, 2004)</td>
</tr>
</tbody>
</table>

3-day incubation, 18 h before harvesting, 25 µCi/well of 3H-thymidine (3H-TdR) was added to each well. Radioactivity was measured (TopCount, Packard Instrument) and results were expressed as stimulation index (SI = cpm of simulated cultures/cpm of unstimulated cultures). Cell cultures were carried out in the presence of DMSO alone (at concentrations used in the test solutions) and the effect of DMSO was subtracted. The viability of cultured cells, under the same experimental conditions, was evaluated by the trypan blue exclusion method.

**Measurements of cytokines in supernatants**

Under the same experimental conditions cytokine measurement was performed. The concentrations of TNF-α, IL-6 and IFN-γ in supernatants were quantified by ELISA using monoclonal antibody pairs (Pierce Endogen, Rockford, IL, USA). Plates were read at 450 nm (Titertek Plus MS 212M).

**Cell viability assay**

A cell viability test was performed to assess the effect of the three extracts on cell growth. Three tumour cell lines were used: A549 (lung cancer), MCF-7 (breast cancer) and CaCo-2 (colon cancer). The tumour cell lines were grown in RPMI 1640 supplemented with L-glutamine, penicillin, streptomycin and 10% foetal bovine serum in a 5% CO2 incubator at 37°C. After a proper dilution, separated cells were plated in 96-well flat-bottom microplates at a density of 3 × 10^3 cells in 100 µl of growth medium. After 12 h, growth medium was replaced with 100 µl of test medium (growth medium plus DMSO extracts). The following concentrations were used: 600, 60, 6, 0.6, and 0.06 µg/ml. DMSO extracts were diluted to a final DMSO concentration of 2.5%. After 72 h incubation, the medium was replaced and 20 µl of MTS reagent was added to each well. After a 2 h incubation, the absorbance was measured at 490 nm wavelength using a plate reader. Five wells for each experimental point were used and each experiment was performed at least twice.

**Statistical analysis**

Two way analysis of variance considering concentrations and different extracts was performed. Post hoc comparisons between extracts were performed with the Wald test, applying the Bonferroni correction for multiple comparisons. Results where p < 0.05 were considered significant.

**RESULTS AND DISCUSSION**

Extracts of all three drugs (*Eremurus* spp, *E. persicus* and *E. spectabilis*) were prepared and their biological properties evaluated. First, extractions were performed using water and a water/methanol mixture, according to the literature (Karaman et al., 2011). In both cases, the formation of highly viscous solutions was observed, probably due to the presence of glucomannan, a water-soluble polysaccharide (Smirnova et al., 2001). Therefore, we modified the extraction protocol, avoided the water addition and used ethanol instead of methanol because of its lesser toxicity and more environmentally friendly. This choice is in accordance with the principles of green chemistry and in line with the European directives for products for human use. The ethanolic extracts (EE) were prepared by dynamic maceration (ME) overnight and treated with vegetal charcoal, as previously reported in the literature (Iqbal et al., 2005; Martino et al., 2008; Uddin et al., 2012). After the removal of solvent, this procedure lead to well-dried yellow solid. Finally, to remove tannins, which may interfere with some biological assays, a treatment with polivinylpirrolidone (PVP) was performed (Makkar et al., 1993). The extraction yields are reported in Table 2.

The TLC analyses of all EE and the revelation with different reagents showed the presence of polyphenols, flavonoids and naphthoquinones, while coumarins, alkaloids and saponins were not detected. The total phenolic content of the EE of the three samples of *Eremurus*, determined using the Folin-Ciocalteau assay, ranged from a mean (± standard deviation) of 44.93% (±0.28) to 49.84% (±0.54) compared to the standard. These data are very similar to each other.

In order to obtain the phytochemical fingerprint...
Table 2. Extraction procedures.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Extraction method</th>
<th>Time</th>
<th>Yield (%, w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. persicus</em></td>
<td>ME</td>
<td>Overnight</td>
<td>10.0</td>
</tr>
<tr>
<td><em>E. spectabilis</em></td>
<td>ME</td>
<td>Overnight</td>
<td>3.3</td>
</tr>
<tr>
<td><em>Eremurus spp.</em></td>
<td>ME</td>
<td>Overnight</td>
<td>3.7</td>
</tr>
</tbody>
</table>

of *Eremurus* species here studied, a reliable chromatographic method was developed by using HPLC-UV/PAD-CD, a powerful tool for a rapid detection of chiral compounds naturally occurring in crude extracts. By comparing the HPLC-UV/PAD chromatograms of the three extracts acquired at different wavelengths of each main peak, it was found that 297 nm best represented the profile of the major constituents. Representative HPLC-UV/PAD-CD chromatograms (297 nm) for each ethanolic extract are reported in Figure 1. The developed chromatographic method was also applied to HPLC-ESI-MS/MS experiments and the MS experimental parameters were optimized for both positive and negative ion modes. The two early peaks with retention time (RT) ranging from 12.4 to 13.5 min (peak 1 and 2) were present in all chromatograms, with highest intensity in *Eremurus* spp. and *E. spectabilis* extracts (Figure 1). The nearly identical UV spectra profiles (maximum at 220, 295 and 320 nm, Figure 2) of these two peaks suggested that they may belong to the same phytochemical class and their CD traces showed that they are not optically active (Figure 1). ESI-MS spectrum in negative-ion mode of peak 1 showed a quasi-molecular ion \([M+H]^+\) at 179 m/z and an MS\(^2\) spectrum with a prominent 135 m/z. Regarding peak 2, an intense ion at 393 m/z in the negative ion mode and 395 m/z in the positive ion mode allowed us to predict a molecular weight of 394 Da. The ESI-MS and UV spectra of compound 1 (negative ion) and 2 (positive ion) are reported in Figure 2.

Interestingly, both *"Eremurus spp" and E. spectabilis* HPLC-UV/PAD profiles showed a prominent peak at 34.4 min (peak 4), which was undetectable in *E. persicus* (Figure 1). The analysis of UV, MS and MS\(^2\) spectra unambiguously showed that the peaks referred to the same compound. This compound (still unknown) has a molecular weight of 540 Da, given that the corresponding MS spectrum clearly display the quasi-molecular ion \([M+H]^+\) at 541 m/z (Figure 3). *E. persicus* chromatographic profile showed an intense peak at 27.4 min (peak 3), also detected in *"Eremurus spp" with a very low peak area percent This compound was present in *E. persicus* extract and, as suggested by the positive signal in the CD profile, it was optically active (Figure 1B). The corresponding MS spectrum showed the \([M+H]^+\) at 273 m/z and a dimeric ion \([2M+Na]^+\) at 567 corresponding to a molecular weight of 272 Da (Figure 3).

The overall analytical results clearly showed that: i) all three samples analysed possessed the same initial peaks, peak 1 (r.t. 12.4) and peak 2 (13.5); ii) *Eremurus* spp. and *E. spectabilis* EE showed another common peak eluted at 34.4 min (peak 4); iii) the *E. persicus* HPLC-MS/MS profile differed from the other two samples, due to the absence of the compound eluted at 34.4 r.t. (peak 4) as well as the presence of a prominent peak at 27.4 min (peak 3). Peak 3 had to be considered the characteristic peak of *E. persicus*. In the *Eremurus* spp. Chromatogram, it had a very low intensity. Since *Eremurus* spp. and *E. spectabilis* ethanolic extracts possessed a very similar qualitative fingerprint, and were characterized by the same main peaks (1, 2 and 4), it may be concluded that *Eremurus* spp is predominantly *E. spectabilis* with a very small amount of *E. persicus* as evidenced by the presence in *Eremurus* spp. extract chromatogram of a limited amount of peak 3 (Figure 1).

Regarding the biological activity, the free radical scavenging effect, the anti-inflammatory activity and the cytotoxicity were evaluated. Keeping in mind that reactive oxygen species (ROS) are involved in TNF\(_\alpha\)-induced inflammation (Young et al., 2008) and that some plants of *Eremurus* genus are used in Kurdish folk medicine as treatment of inflammatory states, the antioxidant potential of all extracts was firstly investigated determining their free radical scavenging (FRS) properties through the DPPH assay. The antioxidant potential of extracts was initially evaluated at a stock concentration of 250 µg/ml. Successively, stock solutions were serially diluted into a range of 187.5 to 7.8 µg/ml (in methanol) and their corresponding FRS activity was determined as percentage values. Generally, an interesting FRS activity % was shown for all three plants (Figure 5), since they reached a maximum effect (E\(_{\text{max}}\)) at a range of 65 to 68% at 250 µg/ml. Over this concentration, the activity did not increase, confirming that the maximum effect had already been reached.

As shown in Figure 4, the three extracts exert a comparable dose-response antiradical effect, since the curves are very similar to each other. Analogously, the IC\(_{50}\) values were very close for *Eremurus* spp. (64.03 µg/ml), *E. persicus* (62.12 µg/ml) and *E. spectabilis* (65.52 µg/ml), confirming that these extracts showed similar FRS activities. Moreover, as a part of our ongoing research for novel anti-inflammatory agents, the effect of...
the three drugs was evaluated using in vitro cultured human peripheral blood mononuclear cells (hPBMC). The root EE of *E. persicus* was recently studied by our group, demonstrating an interesting in vitro anti-inflammatory effect, inhibiting the PHA-induced hPBMC proliferation as well as TNF-α release (Gaggeri et al., 2013a). The investigation was then extended to the ethanolic root extract of *Eremurus* spp. and *E. spectabilis*. Briefly, hPBMC from three healthy donors were stimulated with phytohaemagglutinin A (PHA) in the presence of increasing doses (50 to 800 µg/ml) of the EE and the cell proliferation was evaluated by monitoring the thymidine incorporation. Since PHA response, expressed as stimulation index (SI), is different among subjects, in order to normalize the data, the results were expressed as the percentage of the corresponding PHA response. As shown in Figure 5, *E. persicus* and *Eremurus* spp extracts exerted a similar effect in the range of 800 to 25 µg/ml, while no inhibition was observed for *E. spectabilis*.

To confirm that the inhibitory effect was specific and to exclude that it was due to cell death, the cell viability was evaluated for PHA stimulated hPBMC by Tripot blue exclusion (Gaggeri et al., 2013a), under the same experimental conditions. Cell viability was 98%, confirming the absence of toxicity (data not shown). Moreover, none of the samples induced any proliferative effect on unstimulated hPBMC, indicating that they are devoid of any adjuvant activity. Additionally, an MTS assay was performed on three different cancer cell lines (A549, MCF-7 and CaCo-2) to evaluate any possible nonspecific toxic effect or effects on cell growth. No difference in cell viability was observed between control cells and cells treated with the extracts, even at the highest concentration; these data show that the cell number did not vary in treated cells, compared to controls, thus excluding cytotoxic effects exerted by the extracts.

Given that stimulated hPBMC release cytokines, the observed inhibitory effect could be confirmed by a decrease of the cytokine amount in the supernatants. Hence, the quantification of cytokines (TNF-α, IL-6, and IFN-γ) in the supernatants obtained under the same culture conditions as described earlier, both with or without PHA induction was carried out.

In PHA induced cells, a significant effect (p < 0.05) was observed on TNF-α release while no effect was detected on IL-6 and IFN-γ release (data not shown). As shown in Figure 6, TNF-α release was reduced by the three
extracts at 800 and 400 µg/ml, showing a comparable TNF-α blocker effect, while at 200 µg/ml the inhibitory effect on TNF-α release was only determined by *E. persicus*. Moreover, to confirm that the inhibitory effect was specific in parallel experiments where three cancer cell lines were exposed to increasing concentrations of each extract was performed. Obtained data confirmed that the observed inhibition of lymphocyte proliferation cannot be ascribed to a toxic effect leading to cell death but is in fact entirely due to the anti-inflammatory properties of the extracts.

**Conclusion**

The characterization of *Eremurus* spp. commonly used by native Kurdish people for their anti-inflammatory properties was performed, comparing the phytochemical profiling of *Eremurus* spp ethanolic extract with those of *E. persicus* and *E. spectabilis*. The analytical work demonstrated that the drug commonly sold at the local market is predominantly composed of *E. spectabilis*, since both the HPLC-UV/PAD-CD and the HPLC-ESI-MS/MS profiles of the two extracts are nearly identical. Regarding the biological activity, although *Eremurus* spp. extract exerted an *in vitro* anti-inflammatory effect comparable to that of *E. persicus* extracts in inhibiting *in vitro* PHA-induced lymphocyte proliferation, *E. persicus* EE showed the best TNF-α blocker activity, being the only one effective at 200 µg/ml.

In conclusion, a rapid methodology useful for identifying the *Eremurus* species in the plant material was herein developed. Moreover, taken together, the results support the use of the *Eremurus* plant as anti-inflammatory remedy.
Figure 3. ESI-MS (positive ion mode, full scan) and UV spectra of compound 1(A) and 2(B).

Figure 4. Free radical scavenging activity (FRS) % of *Eremurus* spp., *E. persicus* and *E. spectabilis* extracts.
Figure 5. Proliferation of PHA-activated hPBMC in the presence of *Eremurus* spp., *E. persicus* and *E. spectabilis* extracts expressed as percentage of the corresponding PHA response. *P = 0.002 vs. PHA.

Figure 6. TNF-α levels in culture supernatants of PHA-activated hPBMC after addition of different concentrations of *Eremurus* spp., *E. persicus* and *E. spectabilis*. *P<0.05 vs. PHA.

In Kurdish folk medicine, which exerts its activity through the inhibition of TNF-α. Moreover, and of particular interest, the *E. persicus* roots ethanolic extract, has been shown to be worthy of further investigation to identify the
metabolite(s) responsible for the biological activity and in particular of the anti-inflammatory activity through both in vitro and in vivo (murine model of endotoxaemia) experiments. Indeed, the inhibition of hPBMC proliferation suggests a possible immunosuppressive action that should be evaluated in future studies.

Conflict of Interests

The authors have not declared any conflict of interest.

ACKNOWLEDGMENTS

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Abbreviations

DPPH, 2, 2-diphenyl-1-picrylhydrazyl; EE, ethanolic extract; FCR, Folin-Ciocalteau reagent; FRS, free radical scavenging; Hpbmc, human peripheral blood mononuclear cell; IL-6, interleukin-6; IFN-γ, interferon-gamma; PHA, phytohemagglutinin A; PVP, polyvinylpyrrolidone; TLC, thin layer chromatography; TNF-α, tumor necrosis factor alpha; TPC, total phenolics content.

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