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Anti-inflammatory properties of ethanolic root extract of *Eremurus persicus* (Jaub and Spach) Boiss, a Kurdish herbal remedy

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This study reports for the first time a preliminary phytochemical and biological screening of ethanolic extract (EE) from roots of *Eremurus persicus* (Jaub and Spach) Boiss, a Kurdish herbal remedy for treating inflammatory disorders. The phytochemical analysis was performed by thin layer chromatography and it revealed the presence of flavonoids and antracenones. The total phenolic compounds were also determined. Free radical scavenging (FRS) effect was evaluated by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay and the anti-inflammatory activity investigated by evaluating the inhibition of *in vitro* lymphocyte proliferation. To exclude citotoxic effect of EE, cell viability tests were also performed. Overall, biological investigation showed that the *E. persicus* (Jaub and Spach) Boiss root EE possess an interesting anti-inflammatory activity without cytotoxic effects.

Key words: Anti-inflammatory activity, Kurdish ethnomedicine, *Eremurus persicus* (Jaub and Spach) Boiss, ethanolic root extract.

INTRODUCTION

The plants of genus *Eremurus* (Liliaceae), which comprises nearly fifty species, are mainly restricted to the mountains of Central and Western Asia (Li et al., 2000) and are used in folk medicine to cure several diseases. Seven species of *Eremurus* have been recorded in Iran and 24 in Central Asia (Safar et al., 2009). These species have got several commercial usages (food, source of natural glue and ornamental flowers) and have been employed as herbal remedies since ancient times (Naghibi et al., 2005; Safar et al., 2009).

Eremurus persicus (Jaub and Spach) Boiss is widely distributed in South, East and West of Iran, where it is called "*Sarish*" (Karl, 1982; Wendelibo, 1982; Safar et al., 2009; Vala et al., 2011). It is used by native people of Kurdistan as food and also to cure diseases having a common physiopathological factor related to inflammation (Mati et al., 2011). In details, the aerial parts possess antiglycation, antibacterial and cytotoxic properties

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(Asgarpanah et al., 2011; Vala et al., 2011) and are used to relieve liver and stomach disorder, constipation and diabetes and as regards the roots, they are described as a source of natural glue (Vala et al., 2011), while root decoction is used in Kurdish folk medicine as anti-inflammatory remedy. In this paper, our efforts to assess the folk claim of *E. persicus* (J. and S.) Boiss root extract were addressed. To this aim, the plant was collected in Iran/Iraq area and a preliminary phytochemical investigation was performed. The free radical scavenging (FRS) and the anti-inflammatory *in vitro* activities were then evaluated. To the best of our knowledge, this is the first study on phytochemical and biological investigation of root extracts obtained from *E. persicus* (Jaub and Spach) Boiss. Indeed, although polysaccharides are considered as chemical constituents targeting the species, no scientific investigations on the phytochemical content of *E. persicus* (Jaub and Spach) Boiss roots extracts have been reported till date.

MATERIALS AND METHODS

Plant

E. persicus (Jaub and Spach) Boiss was collected in the area of the Gulestan Kuh mountain Golpayegan at an altitude of 3000 to 3200 m, located at 120 km from Isfahan/Iran, in August 2011. The collected plant material was identified and classified by Dr. Abdulla Sa'ad at the Education Science Department, Faculty of Biology, Salahaddin University, Hawler/Iraq. The voucher specimen (No. 6856) was deposited in the Education Salahaddin University Herbarium (ESUH), Hawler/Iraq. Freshly cut roots were stored out, dried in a drying room with active ventilation at room temperature (about 20 to 22°C) until they reached constant weight. Roots were cut in small size and grounded with a blade mill to obtain a homogeneous fine powder. The drug was stored in dark conditions.

Chemicals and reagents

All solvents used were of analytical grade and HPLC grade was purchased from Carlo Erba (Milano, Italy). 2, 2-diphenyl-1-picrylhydrazyl (DPPH), polyvinylpyrrolidone (PVP) and gallic acid were purchased from Sigma Aldrich (Milan, Italy). Folin-Ciocalteau reagent (FCR) was purchased from Carlo Erba (Milano, Italy). Green tea extract (Green Select®) was obtained from Indena (Milano, Italy). RPMI 1640 medium was purchased from Gibco, Life Technologies (Monza, Italy), foetal bovine serum (FBS) from Euroclone S.p.A. (Milan, Italy) and phytohemagglutinin (PHA) from Roche (Mannheim, Germany). ^{3}H-thymidine (^{3}HTdR) was purchased from Amershampharmacia Biotec (Milan, Italy). 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reagent was bought from Promega (Milan, Italy). Analytical thin-layer-chromatography (TLC) was carried out on silica gel precoated glass-backed plates (Fluka Kieselgel 60 F254, Merck).

Preparation of ethanolic extract (EE)

The dried powdered roots of *E. persicus* (Jaub and Spach) Boiss (100 g) were pre-treated with petroleum ether 10% (w/v) for 1 h at room temperature under mechanical stirring (Gaggeri et al., 2011) and then extracted with ethanol (1 L) by overnight dynamic maceration at room temperature. The mixture was filtrated and solvent evaporated to dryness (Laborota 4000, Heidolph Instruments, Schwabach, Germany), yielding 3.8 g of a brown oil. The crude extract was dissolved in methanol (500 ml) and added of charcoal (0.5 g); the mixture was stirred at room temperature for 15 min, filtered and the solvent evaporated to dryness. The residue (3.5 g, yellow solid) was then treated with PVP, according to Makkar et al. (1993).

Briefly, the extract was suspended in acetone 70% (150 ml) and ethanol (150 ml), with addition of PVP (3 g) and was stirred at 0°C for 15 min. The mixture was then filtered and the solvent evaporated, yielding 3.3 g of a yellow solid. The dried EE was kept at room temperature in the dark.

Phytochemical profile

The EE was analyzed by TLC, eluting with different mobile phases as described by Wagner and Bladt (1996), seeking to highlight the main groups of secondary metabolism.

Determination of total phenolics content (TPC)

The TPC of EE was determined according to Singleton et al. (1999) with some modifications. Briefly, EE solubilized in 10% EtOH (1 ml) was added of deionized water (6 ml) and FCR (freshly diluted ten-fold with deionized water, 500 µl). The mixture was stirred at room temperature for 3 min, then 20% w/v sodium carbonate solution (1.5 ml) was added. The mixture was finally diluted in water to 10 ml and stored in the dark for 2 h at room temperature. A blank was also made by mixing 10% EtOH and reagents. Absorbance was measured at 760 nm (Lambda 25 UV/VIS spectrometer, Perkin Elmer instruments, Massachusetts, USA). TPC of EE was expressed as % (w/w) of gallic acid. The analyses were conducted in triplicate and results are expressed as mean ± standard error (SE).

Determination of FRS activity

FRS activity of EE was determined by using DPPH assay (Gaggeri et al., 2010). A commercially available standardized green tea extract (Green Select®) was used as standard. Briefly, both EE and standard were dissolved in MeOH, to prepare stock solutions (c= 10 mg/ml, which were then serially diluted in MeOH by two folds. Reaction mixture was prepared by adding 100 µl of sample solution (or standard solution) to 3.9 ml of DPPH solution, freshly prepared dissolving DPPH in methanol/KH_2PO_4 and NaOH buffer (50/50 v/v) at a concentration of 6×10^{-5} M. In this way test solutions with the final concentration of 250, 187.5, 125, 62.5, 31.25, 15.62, and 7.81 µg/ml were obtained. 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 percentage compared 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:

$$FRS\ (\%) = [(Abs_{control} - Abs_{sample}) / Abs_{control}] \times 100.$$

The analyses were carried out in triplicate and results are expressed as mean ± SE. $EC_{50,}$ values were calculated using Graph Pad Prism 4.0. The commercially available standardized green tea extract (Green Select®) was used as standard (EC_{50} = 4.6 µg/ml).

Evaluation of anti-inflammatory activity

In vitro inhibitory activity

In vitro proliferation assay on human peripheral blood mononuclear cell (hPBMC) was carried-out as previously described by Bernardo et al. (2009). Briefly, hPBMC were obtained from healthy donor peripheral blood and grown in RPMI 1640 medium supplemented with 10% of FBS, with or without 4 µg/ml of PHA. hPBMC were cultured in the presence of increasing doses of test solutions [EE DMSO solutions diluted 1:2 (v/v) with medium; final concentrations: 800 to 12.5 µg/ml] or vehicle (control cells). After 3-day incubation, 18 h before harvesting, 25 µCi/well of ^{3}HTdR were 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). Cells were cultured 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 cytochines in supernatants

Experiments are conducted under the experimental conditions described earlier for the inhibitory activity assay. Tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6) and interferon-gamma (IFN-γ) were quantified in supernatants by ELISA using monoclonal antibody pairs (Pierce Endogen, Rockford, IL, USA). Plates were read at 450 nm (Titertek Plus MS 212 M).

Cell viability assay

A cell viability test was performed to assess the effect of EE 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% FBS in a 5% CO_2 incubator at 37°C. After a proper dilution, separated cells were plated in a 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 final concentrations were used: 800, 400, 200, 100, 50, 25, and 12.5 µg/ml. EE was diluted to have a final DMSO concentration of 2.5%. After 72 h' incubation, the medium was replaced and 20 µL of MTS reagent were added to each well. After a 2 hours' 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

A two way-analysis of variance considering concentrations was performed. Post hoc comparison between extracts was performed with Wald test applying Bonferroni correction for multiple comparison. $P < 0.05$ were considered significant.

RESULTS AND DISCUSSION

EE preparation and phytochemical profile

The *E. persicus* (Jaub and Spach) Boiss root extract studied in the present work was prepared by dynamic maceration in ethanol. The use of this solvent is in accordance with the principles of green chemistry and in line with the European directives for products for human use. Since a hygroscopic dark coloured (brown to black) residue was observed in the crude extract, a purification using vegetal charcoal (Iqbal et al., 2005) was applied, with the aim to improve the physical characteristics of the extract. In this way a well-dried yellow solid (loss of weight around 7.7%) was obtained. A treatment with PVP was then performed to remove tannins, since they are mostly ubiquitous in vegetal drugs and may interfere with some biological assays (Makkar et al., 1992) (loss of weight: 6.5%).

The TLC analysis of the obtained EE revealed the presence of polyphenols, flavonoids and naphthoquinones, while coumarins, alkaloids, and saponins were not detected. The TPC of the EE was also determined using the Folin-Ciocalteau assay, which is a simple and straightforward method for determining the polyphenols in complex mixture. The percentage of TPC was 49.84% (±0.54).

FRS activity

Since *E. persicus* (Jaub and Spach) Boiss is mainly used in Kurdish folk medicine for treating inflammation-related diseases, and based on the evidence that reactive oxygen species (ROS) are involved in TNFα-induced inflammation (Young et al., 2008), the biological evaluation of the EE by determining its FRS activity using the DPPH assay were approached.

The FRS potential was initially evaluated at a stock concentration of 250 µg/ml. Successively, stock solutions were serially diluted and their corresponding FRS activity was determined into the concentration range of 187.5 to 7.8 µg/ml. Results are expressed as percentage values. As clearly reported in Figure 1, EE was proved to be endowed with an interesting FRS percent activity, showing a maximum effect of 68% at 250 µg/ml and an EC_{50} value of 62.1 µg/ml. Accordingly, EE is worthy of being deeply investigated.

Anti-inflammatory activity

EE was subjected to an extensive biological evaluation. Firstly, the EE inhibitory effect on the *in vitro* lymphocyte proliferation was investigated using the hPBMC *in vitro* proliferation assay in bulk culture (Bernardo et *a*l., 2009), which is a useful method to evaluate lymphocyte proliferation after mitogen or antigen *in vitro* stimulation. In this work, hPBMC from three healthy donors were stimulated with PHA in the presence of increasing doses (12.5 to 800 µg/ml) of EE and cells proliferation was evaluated by ^{3}HTdR incorporation. Results, expressed as the percentage of the corresponding PHA response,

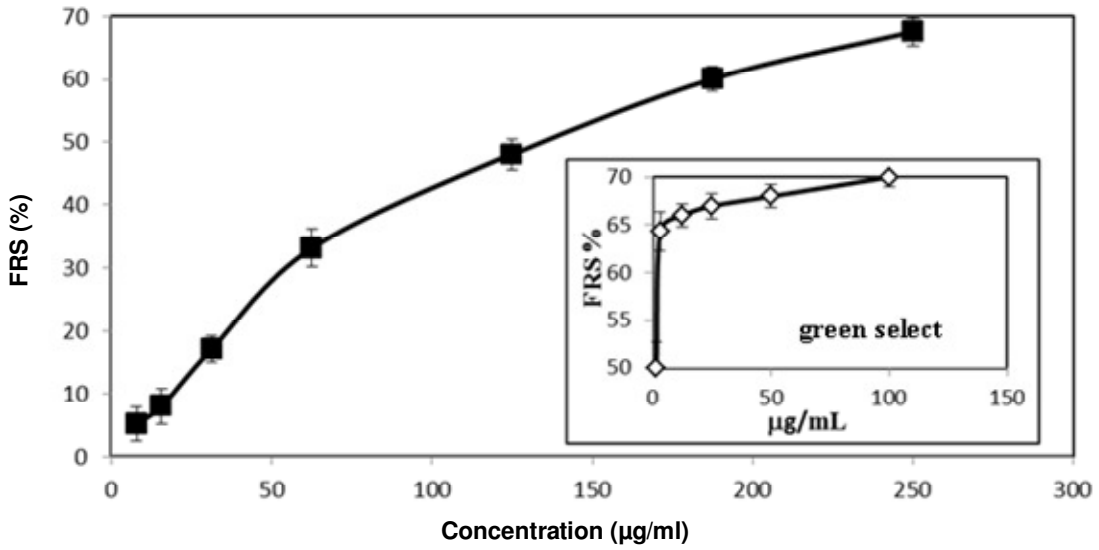

Figure 1. Free radical scavenging activity (FRS, %) of *E. persicus* ethanolic extract.

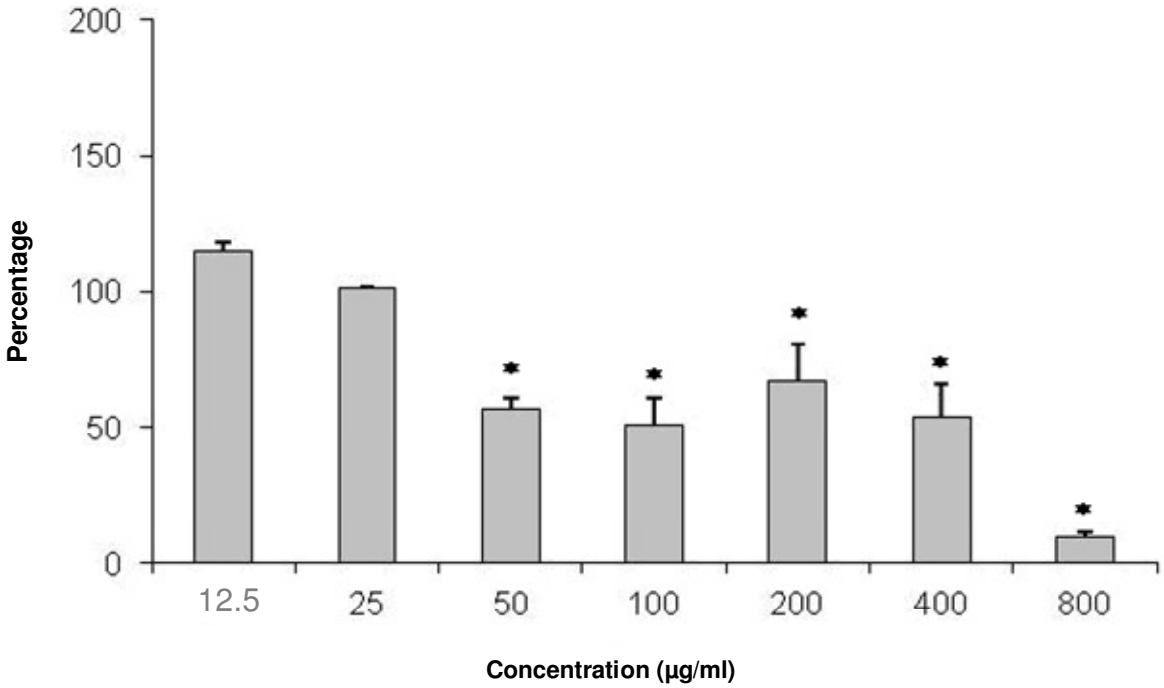

Figure 2. Proliferation of PHA-activated hPBMC in the presence of *E. persicus* (J. and S.) Boiss ethanolic extract expressed as percentage of the corresponding PHA response. *P = 0.0002 vs. PHA.

clearly evidenced that EE is able to inhibit PHA stimulated hPBMC proliferation (Figure 2). Particularly, EE significantly reduced the PHA-stimulated hPBMC proliferation in the concentration range 800 to 50 µg/ml (P = 0.0002).

The effect of EE on the release of cytokines (TNF-α, IL-6 and IFN-γ) from PHA-stimulated hPBMC was also evaluated. Interestingly, EE did not affect the release of IL-6 and IFN-γ, while a significant effect ($P < 0.05$) was observed on TNF-α release.

Particularly, as shown in Figure 3, PHA-induced TNF-α release was significantly reduced by the extract in the concentration range 200 to 800 µg/ml ($P < 0.05$). These results are in agreement with data obtained in the anti-proliferative assay.

To confirm that the inhibitory effect was specific and to

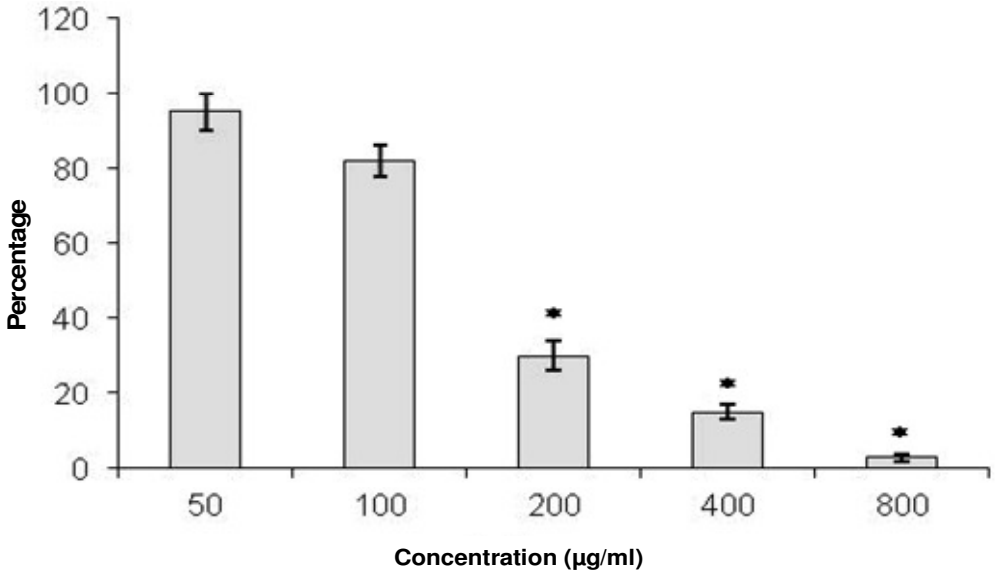

Figure 3. TNF-α levels, expressed as percentage of the corresponding PHA response, in culture supernatants of PHA-activated hPBMC after addition of different concentrations of *E. persicus* (J. and S.) Boiss ethanolic extract. *P < 0.05 vs. PHA.

exclude that it was due to cell death, the cell viability was evaluated for PHA stimulated hPBMC by Tripan blue exclusion, under the same experimental conditions. Cell viability was 98%, confirming the absence of toxicity. Moreover, EE did not induce any proliferative effect on unstimulated hPBMC, therefore it can be stated that it is 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 non-specific toxic effect or effects on cell growth. No difference in cell viability was observed between control cells and cells treated with the extract, 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 extract.

Conclusion

In this paper, the ethnomedical use of *E. persicus* (Jaub and Spach) Boiss roots was assessed. The root EE showed an interesting *in vitro* anti-inflammatory effect, being effective in inhibiting the PHA-induced hPBMC proliferation as well as TNF-α release. Moreover, the observed inhibition of hPBMC proliferation is entirely due to anti-inflammatory properties of the extract and not to cytotoxic effect, as proved by cell viability experiments.

In general, based on the overall biological results, it can be stated that the ethnomedical use of *E. persicus* (Jaub and Spach) Boiss roots in traditional Kurdish medicine is mainly based on its anti-inflammatory properties and we suggest that this use could be supported by the observed inhibitory effect on TNF-α release. Our current efforts are directed towards the isolation and structural characterization of the main compounds of *E. persicus* (Jaub and Spach) Boiss with the final aim to elucidate the mechanism underlaying TNF-α release inhibition as well as to study its anti-inflammatory activity *in vivo* (murine model of endotoxemia).

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ABBREVIATIONS

DPPH, 2, 2-diphenyl-1-picrylhydrazyl; **EE,** ethanolic extract; **FBS,** foetal bovine serum; **FCR,** Folin-Ciocalteau reagent; **FRS,** free radical scavenging; **hPBMC,** human peripheral blood mononuclear cell; **^{3}HTdR,** ^{3}H-thymidine; **IL-6,** interleukin-6; **IFN-γ,** interferon-gamma; **MTS,** 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; **PHA,** phytohemagglutinin A; **PVP,** polyvinylpyrrolidone; **RT,** room temperature; **TLC,** thin layer chromatography; **TNF-α,** tumor necrosis factor alpha; **TPC,** total phenolic content.

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