



Anti-inflammatory effects of the *Portulaca oleracea* hydroalcoholic extract on human peripheral blood mononuclear cells

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Received: 23 Apr 2017

Published: 3 Sep 2018

Abstract

Background: *Portulaca oleracea*, known as *Purslane*, is an annual growing herb with wide distribution around the world and traditionally used to manage several diseases. Different therapeutic properties as an anti-fever agent as well as anti-inflammatory and analgesic effects have been attributed to *P. oleracea*. The aim of this study was to investigate the effects of *P. oleracea* aerial extract on production of pro- and anti-inflammatory cytokines by human peripheral blood mononuclear cells (PBMCs).

Methods: Aerial parts of *P. oleracea* (stems and leaves) were collected and extracted by percolation using methanol. The optimal and non-cytotoxic dose of hydro-alcoholic extract for cell culture analysis was determined by MTT assay. To assess the anti-inflammatory effects of *P. oleracea*, PBMCs obtained from 12 normal volunteers were cultured in RPMI complete medium and co-treated with *E. coli* lipopolysaccharide (LPS) and *P. oleracea* hydro-alcoholic extract. Following 18-hour incubation, culture supernatants were harvested for measurement of secreted TNF- α , IL-6 and IL-10 by ELISA. Statistical analyses were performed using the SPSS v.20, and data analyzed by Kolmogorov-Smirnov, Mann-Whitney U, Kruskal-Wallis and post Hoc tests. P-values<0.05 were considered significant.

Results: The optimal non-cytotoxic concentration of *P. oleracea* aerial extract was defined as 100 μ g/ml based on MTT viability assay. *P. oleracea* hydro-alcoholic extract significantly decreased the concentration of both pro-inflammatory cytokines TNF- α and IL-6 in LPS-stimulated PBMCs ($p<0.001$ and $p<0.001$, respectively). However, the concentration of IL-10 as an anti-inflammatory cytokine, did not show any statistically significant change ($p=0.390$).

Conclusion: Our findings highlighted the potential anti-inflammatory properties of *P. oleracea* in herbal medicine. Future analysis on different constituents of total extract may confirm its therapeutic effects as a promising anti-inflammatory compound.

Keywords: *Portulaca oleracea*, Anti-inflammatory agent, Tumor necrosis factor-alpha, Interleukin-6, Interleukin-10

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Cite this article as: Allahmoradi E, Taghiloo S, Omrani-Nava V, Shobeiri SS, Tehrani M, Ebrahimzadeh MA, Asgarian-Omran H. Anti-inflammatory effects of the *Portulaca oleracea* hydroalcoholic extract on human peripheral blood mononuclear cells. *Med J Islam Repub Iran.* 2018 (3 Sep);32:80. <https://doi.org/10.14196/mjiri.32.80>

Introduction

Inflammation is a biological protective response to traumatic stimuli, pathogens and damaged cells (1). During inflammation, regular physiological and immunological processes coordinate by soluble signaling molecules of the immune system. Subsequently, the corresponding cells

are transferred to inflamed sites to resolve the atypical state and finally cause the healing process (2). Following removal of stimuli agents, inflammatory response must be restricted to prevent any uncontrolled damage and further possible autoimmune disorders such as rheumatoid arthri-

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↑What is “already known” in this topic:

Different therapeutic properties as an anti-fever agent as well as anti-inflammatory and analgesic effects have been attributed to *P. oleracea*. The anti-inflammatory effects of *P. oleracea* were also investigated in several studies but no data are available regarding its direct anti-inflammatory effects on human peripheral blood mononuclear cells (PBMCs).

→What this article adds:

Our preliminary experiments demonstrated for the first time that *P. oleracea* exhibit the immunomodulatory effects by suppression of pro-inflammatory cytokines such as TNF- α and IL-6 on LPS-stimulated human PBMCs.

tis, Crohn's disease, psoriatic arthritis, systemic lupus erythematosus and multiple sclerosis (3,4). Therapeutic interventions would then be necessary to prevent the progression of inflammation without any side effects on the normal host immune response (1,5). Recently, application of traditional herbs as potent anti-inflammatory agents has been dramatically increased as treatment or prevention strategies (4,6). Nevertheless, numerous traditional herbs do not have any scientific documentations and approval to be administered for human which it calls more investigations in this area.

Portulaca oleracea (*P. oleracea*, Family *Portulacaceae*), also known as *Purslane*, is an annual growing herb listed in the World Health Organization as one of the most used therapeutic plants (7,8). *P. oleracea* is an edible herb and widely distributed plant and commonly used as a traditional medicine in the Middle East, Mediterranean, United States, Central European and Asian countries (9, 10). They possess green color, slightly acidic with branched and cylindrical shape, stems up to 15–30 cm height and 3mm in diameter (10,11). According to the photochemical analysis, *Purslane* extract is a rich source of fatty acids particularly omega-3, glutathione, glutamic acid, aspartic acid and vitamins including A, B, C and etc. (2,9,12). Several pharmacological properties of *P. oleracea* like anti-oxidant and anti-microbial (7,13), skeletal muscle relaxant (14), wound-healing (10), as well as a traditional medicine for diuretic, febrifuge, sepsis, spasms, headache, scurvy, asthma and fever have been addressed in previous studies (7,8,11,15,16). In addition, other pharmacological actions such as neuroprotectivity, bronchodilator, hepatoprotectivity, anti-fatigue and anti-hypertensive activities have also been attributed to *P. oleracea* (13,17,18). The anti-oxidant and anti-proliferative effects of *P. oleracea* have been demonstrated on several cancer cell lines (15,19). The anti-inflammatory effects of Oleracimine, a novel carbon skeleton alkaloid from *P. oleracea*, has been studied on LPS-induced RAW 264.7 cell line by inhibiting the production of pro-inflammatory cytokines such as IL-6, TNF- α as well as nitric oxide (NO) and prostaglandin-E2 (20). Similar anti-inflammatory properties were also reported for Oleracone which constitute other skeleton alkaloid constituent of *P. oleracea* (21). Taking these considerations into accounts, although the anti-inflammatory effects of *P. oleracea* were documented in several studies (22,23), there is no data regarding its direct anti-inflammatory effects on human peripheral blood mononuclear cells (PBMCs).

In the present study, the potential anti-inflammatory properties of *P. oleracea* on human PBMCs were explored to more clarify its pharmacological mechanisms and possible effects on host immune responses.

Methods

Preparation of plant extract

P. oleracea was collected from Qeidar Mountains (Khodabandeh, Zanjan province, Iran) in May 2015. The sample was authenticated by Dr. Bahman Eslami (Plant systematic specialist, Islamic Azad University of

Qhaemshahr, Iran) and the voucher specimen was deposited (No. HS181) in the Sari School of Pharmacy Herbarium. Aerial parts (leaves and stems) of the fresh plant were dried at room temperature and coarsely ground before extraction. Dried materials were powdered using blender and then extracted at room temperature by maceration using methanol for 24 hours. The extract was then separated from the sample residue by filtration through filter paper. This procedure was repeated three times. The resultant extracts were concentrated under vacuum (Rotary Evaporator) until a crude solid extract obtained and then freeze-dried for complete solvent removal (16.5%). The dried extract was dissolved in phosphate buffer saline (PBS 0.15M pH: 7.4) and stored in 4°C as plant extract stock.

Study subjects

A total of 12 healthy normal volunteers including 6 males and 6 females with the mean age of 39.2 ± 16.65 years were incorporated in this study. All subjects were clinically evaluated to exclude any underlying diseases or consumption of anti-inflammatory or immunosuppressive drugs. Heparinized peripheral blood samples were taken from all subjects and subsequently used for PBMC isolation. The current study was confirmed by Ethical Committee of Mazandaran University of Medical Sciences and written informed consents were obtained from all participants.

Separation of peripheral blood mononuclear cells

PBMCs were isolated from fresh peripheral blood of all subjects using Ficoll-Histopaque (Biosera, Nuaille, France). Heparinized whole blood was diluted in equivalent volume of RPMI-1640 culture medium (Biosera, Nuaille, France), then carefully layered over a ficoll density gradient medium and centrifuged at 400g for 20 min. Isolated PBMCs were washed twice with RPMI-1640 and applied for further experiments. The viability of isolated cells was 98% as assessed by trypan blue staining.

Determination of the optimum and non-cytotoxic dose of *P. oleracea* hydro-alcoholic extract for cell culture analysis

To determine the non-cytotoxic dose of *P. oleracea* hydro-alcoholic extract, 2×10^5 PBMCs were seeded at flat-bottomed 96-well microplates (SPL, South Korea) in duplicate and treated with different concentrations of extract from 12.5 to 500 $\mu\text{g/ml}$ for 24 hours at 37 °C in 5% CO₂. Phytohemagglutinin (PHA) was also used as a positive control of cell proliferation at 2 $\mu\text{g/ml}$. Cells viability was measured by MTT colorimetric assay (Sigma-Aldrich, Missouri, USA). Following incubation, MTT reagent was added to each well at final concentration of 0.5 mg/ml and cells were incubated at 37 °C for 4 hours until purple precipitate was visible. Culture plates were centrifuged at 300g for 10 min and supernatants were removed. Finally, MTT crystals were dissolved in 150 μl of DMSO (Biosera, Nuaille, France) and shook at room temperature for 20 min. Optical densities were recorded at 570 nm with multi scan plate reader (Synergy H1 BioTek, Winooski, USA).

Cell culture and stimulation

To assess the effects of *P. oleracea* hydroalcoholic extract on cytokine production from PBMCs, 2×10^5 cells were cultured in 200 μ l RPMI-1640 medium in flat-bottomed 96-well microplates supplemented with penicillin (100 IU/ml), streptomycin (100 μ g/ml) and 10% heat inactivated fetal bovine serum (Biosera, Nuaille, France). The cells were treated with *E. coli* Lipopolysaccharide (LPS-100 pg/ml), as a potent stimulator of pro-inflammatory cytokines production, together with 100 μ g/ml of *P. oleracea* extract. Non-stimulated and LPS-stimulated PBMCs were applied as control wells to compare the results. The effects of *P. oleracea* extract on production of pro- and anti-inflammatory cytokines by PBMCs were studied in two conditions. At initial experiments, PBMCs were cultured and treated with *P. oleracea* extract without LPS in a non-inflammatory condition and then, the potential anti-inflammatory properties of *P. oleracea* extract evaluated on LPS-stimulated PBMCs. All cultures were run in duplicate and incubated at 37°C in 5% CO₂ for 18 hours. Culture supernatants were harvested and stored at -20°C for cytokines measurement.

Cytokines measurement by ELISA

Following an 18-hour incubation, culture supernatants were collected and the concentrations of TNF- α and IL-6 as pro-inflammatory cytokines and IL-10 as an anti-inflammatory cytokine were measured by ELISA kits using the manufacturer's protocol (Sanquin, Amsterdam, Netherlands). All samples were measured in duplicate. All ELISA kits were based on a sandwich assay following by detection with streptavidin-HRP conjugate with the sensitivity of 2pg/ml. The absorbance of all plates was recorded at 450nm as well as 630nm as reference wavelength with microplate spectrophotometer.

Statistical Analysis

Statistical analyses were performed using the SPSS statistical package version 20 (SPSS, Chicago, USA). Data are represented as mean \pm standard error of the mean (SEM). Normality distribution of the obtained data was determined by Kolmogorov-Smirnov test, and the Mann-Whitney U test used to calculate the mean difference between two groups. For comparison between three groups, the Kruskal-Wallis and post Hoc tests were applied. P-values < 0.05 were considered to be significant.

Results

To determine the optimum and non-cytotoxic dose of *P. oleracea* hydro-alcoholic extract for cell culture analysis, PBMCs were treated with different concentrations of crude extract and cells viability was determined by MTT assay. PBMCs were proliferated in response to PHA as a polyclonal activator and mitogen for peripheral blood lymphocytes. Based on OD values obtained from MTT assay, a slight proliferation of PBMCs was observed following treatment with lower concentrations of *Portulaca oleracea* extract compared to control wells (Fig. 1). But, this proliferative response was not statistically significant

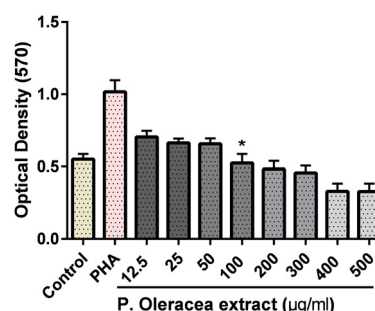


Fig. 1. The non-cytotoxic dose of *P. oleracea* hydro-alcoholic extract for cell culture analysis determined by MTT assay. Human PBMCs were isolated and treated with serial concentrations of *P. oleracea* crude extract for 24 hours and cell viability was determined by MTT assay. PHA was used as a positive control and polyclonal mitogen. *Concentration of 100 μ g/ml was selected as non-cytotoxic dose for all experiments.

for any of the extract doses. *P. oleracea* extract did not show any cytotoxic effects up to 100 μ g/ml based on cells viability result. Thus, this concentration was selected for further experimental procedures (Fig. 1).

As represented in Fig. 2, the levels of all measured pro- and anti-inflammatory cytokines including TNF- α , IL-6 and IL-10 were statistically similar in PBMCs stimulated with *P. oleracea* extract compared to untreated cells ($p = 0.112$, $p = 0.083$ and $p = 0.862$, respectively). In the next experiments, the potential anti-inflammatory properties of *P. oleracea* extract were evaluated on LPS-stimulated PBMCs in the inflammatory condition. To address this issue, PBMCs from all volunteers were co-treated with *P. oleracea* and LPS as well as with LPS alone. While the production of TNF- α ($p=0.009$) and IL-6 ($p=0.003$) was significantly elevated by PBMCs in response to LPS, the concentration of IL-10 did not show any difference after stimulation with LPS (Fig. 3A-3C). Interestingly, *P. oleracea* dramatically suppressed the production of TNF- α ($p=0.042$) and IL-6 ($p=0.010$) in LPS-stimulated PBMCs (Fig. 3A and 3B) which indicate its potential anti-inflammatory effects. However, LPS-induced PBMCs were similarly produced IL-10 in the presence or absence of extract (Fig. 3C).

Discussion

Inflammation is associated with increasing many inflammatory mediators such as cytokines, chemokines and nitric oxide produced during inflammatory response to stimuli (1, 2). TNF- α , IL-1 β and IL-6 are the major pro-inflammatory cytokines playing essential roles in the inflammatory response. Besides their protective effector functions in acute inflammatory conditions, extra production of these cytokines in autoimmune and inflammatory diseases could be harmful and pathologic (2, 24). Recent studies have been reported that immunomodulatory agents like medicinal plants provide an alternative therapy for a variety of inflammation-mediated disorders like rheumatoid arthritis and atherosclerosis (6). *Portulaca oleracea* is a well-defined plant in traditional medicine and has been addressed in previous studies for its various therapeutic

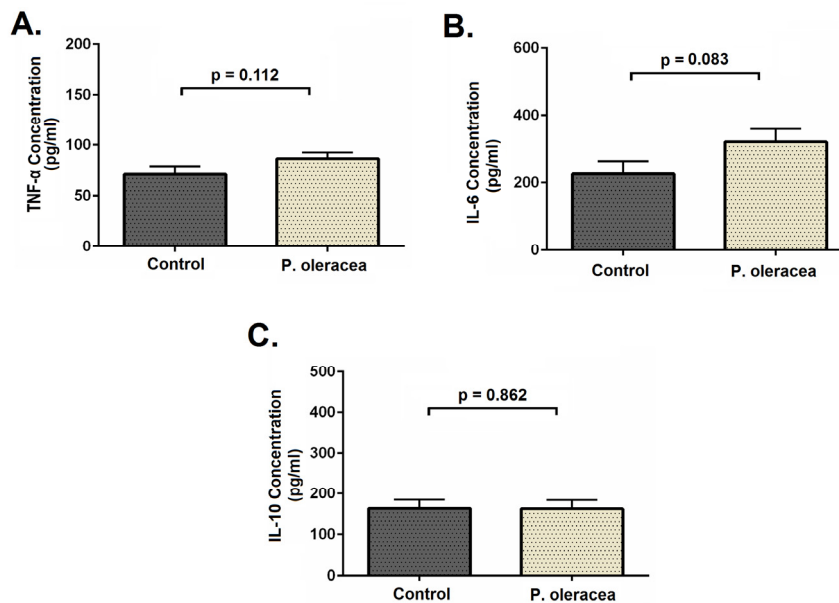


Fig. 2. *P. oleracea* effects on production of pro- and anti-inflammatory cytokines by human PBMCs measured by ELISA. Isolated PBMCs from normal volunteers (n=12) were treated with 100µg/ml of *P. oleracea* extract for 18 hours and the production of TNF-α (A), IL-6 (B) and IL-10 (C) were measured in culture supernatants by ELISA. The results are represented as mean ± SEM. P-values < 0.05 were considered significant.

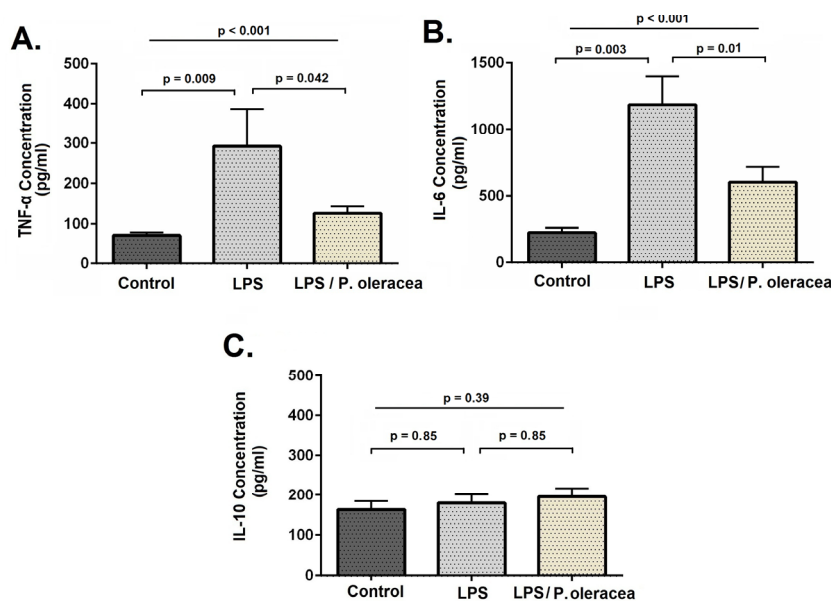


Fig. 3. *P. oleracea* extract has anti-inflammatory effects on LPS-stimulated human PBMCs. Human PBMCs were isolated from normal volunteers (n=12) and co-stimulated with LPS (100pg/ml) and *P. oleracea* extract (100µg/ml) for 18 hours as well as LPS alone. Culture supernatants were collected and used for measurement of TNF-α (A), IL-6 (B) and IL-10 (C) by ELISA. The results are represented as mean ± SEM. P-values < 0.05 were considered significant.

applications (9). Our results for the first time demonstrated the anti-inflammatory effects of *P. oleracea* on human PBMCs by down-regulation of TNF-α and IL-6 production.

TNF-α is mainly produced by mononuclear phagocytes in response to bacterial, viral and parasitic infections. However, aberrant and over-production of TNF-α may stimulate the secretion of other inflammatory mediators resulting DNA and cell damage (3,25). Previous studies have reported that blocking of TNF-α signaling pathway decrease the amounts of inflammatory mediators and amelioration of inflammatory diseases like Crohn's disease,

rheumatoid arthritis, ankylosing spondylitis and inflammatory bowel disease (IBD) (25). Inhibition of TNF-α can be achieved by various approaches such as monoclonal antibodies and fusion proteins which some of them are now clinically approved for treatment of TNF-α mediated complications. Together with TNF-α, IL-6 has also critical roles in immune responses to infections during inflammation and over-production of IL-6 has been observed in some pathological conditions including rheumatoid arthritis, osteoporosis, IBD, Crohn's disease and psoriasis (1,3, 26). Our initial experiments demonstrated that optimal and selected dose of *P. oleracea* extract (100µg/ml) was not

cytotoxic for PBMCs based on the viability assay and also had no effects on production of pro- and anti-inflammatory cytokines by PBMCs. To create an inflammatory environment, PBMCs were stimulated with LPS, as an strong inducer of cytokines production by inflammatory cells such as monocytes and macrophages (3,27). Over-production of TNF- α and IL-6 by LPS-induced PBMCs was remarkably suppressed by co-treatment with *P. oleracea* extract in our experiments. Our results are in line with the previous studies demonstrating the anti-inflammatory effects of novel alkaloid constituents from *P. oleracea* named as Oleracone and Oleracimine by inhibition of IL-6 and TNF- α in LPS-induced RAW264.7 macrophage cell line (21). In addition, it was shown that polysaccharide extract from *P. oleracea* could significantly decrease TNF- α and IL-6 levels in diabetic rats (28) as well as exhibit effective protection for Ulcerative colitis in mice by down-regulation of IL-6, IL-1 β and TNF- α (29). In other study by Ramadan et al., it has been reported that pretreatment with the aqueous extract of *P. oleracea* induced significant reduction of TNF- α and IL-6 in diabetic rats (30). The present results demonstrate that the anti-inflammatory properties of *P. oleracea* on human PBMCs could be more extended and confirm the previous findings with different approaches.

IL-10 is mainly known as an anti-inflammatory cytokine which suppress the production of pro-inflammatory cytokines including TNF- α , IL-6 and regulate immune responses (31). Blocking of IL-10 signaling pathway during acute infections increases the pathology and susceptibility to inflammatory and autoimmune disorders. Conversely, stimulation of IL-10 production by anti-inflammatory agents has therapeutic effects (32-34). In the present study, the concentration of IL-10 did not change in the supernatants of PBMCs treated with *P. oleracea* or LPS alone and also co-treated with LPS and *P. oleracea* compared to untreated control cells. Since, there are no published reports regarding the effects of *P. oleracea* on IL-10 production, our results in regard to lack of effect of *P. oleracea* extract on IL-10 production call on more studies with different approaches. Taken together, our data indicates that in non-inflammatory conditions, *P. oleracea* has not any cytotoxic and pro-inflammatory effects. But together with the initiation of inflammation like by LPS, *P. oleracea* restrict the inflammatory conditions by suppression of the pro-inflammatory cytokines production. Since, prolonged clinical use of conventional anti-inflammatory drugs such as non-steroidal anti-inflammatory drugs (NSAIDs) and slow acting anti-inflammatory drugs (SAARDs) have many unwanted and harmful side effect (6), our preliminary study showed that *P. oleracea* could be applied as an alternative candidate for routine anti-inflammatory conditions with minimal unwanted side effects. However, future works are required to determine the effective constituents of the crude extract of *P. oleracea*, and its potential therapeutic effects on inflammatory diseases. The current study was financially limited to investigate the immunomodulatory therapeutic effects of *P. oleracea* on animal model for inflammatory diseases and also on PBMCs from patients with inflama-

tory diseases.

Conclusion

Our promising preliminary experiments have demonstrated the immunomodulatory effects of *P. oleracea* aerial extract by suppression of pro-inflammatory cytokines on LPS-stimulated human PBMCs. These findings could suggest *P. oleracea* potential application as a useful therapeutic candidate for the treatment of inflammation mediated diseases.

Acknowledgements

This study was financially supported by a grant (No: 184-7) from the Student Research Committee of Mazandaran University of Medical Sciences.

Conflict of Interests

The authors declare that they have no competing interests.

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