

Original Research Article

***Olea europaea* Linn (Oleaceae) Fruit Pulp Extract Exhibits Potent Antioxidant Activity and Attenuates Neuroinflammatory Responses in Lipopolysaccharide-Stimulated Microglial Cells**

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Received: 26 March 2013

Revised accepted: 12 April 2013

Abstract

Purpose: To investigate the antioxidant and anti-neuroinflammatory potentials of *Olea europaea* Linn. fruit pulp (OFP-EA) extract in LPS-stimulated BV-2 microglial cells.

Methods: Cell viabilities were measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. Antioxidant properties were evaluated using 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging activity. Lipopolysaccharide (LPS) was used to stimulate BV-2 microglia. Nitric oxide (NO) production was measured using Griess assay. Inducible NO synthase (iNOS) expression and tumor necrosis factor-alpha (TNF- α) production were measured using enzyme-linked immunosorbent assay (ELISA) and Western blot analysis.

Results: OFP-EA extract significantly ($p < 0.001$ at 20-200 $\mu\text{g/ml}$, respectively) scavenged the free radicals in a dose-dependent fashion. The increased levels of NO stimulated by LPS (34 ± 2.41) were also inhibited by OFP-EA extract significantly and concentration dependently (27 ± 2.32 , 21 ± 2.54 , 17 ± 1.92 and 11 ± 1.94 at 10, 20, 40 and 80 $\mu\text{g/ml}$, respectively). Further, OFP-EA suppressed the elevated levels iNOS expression and TNF- α production ($p < 0.001$ at 20, 40 and 80 $\mu\text{g/ml}$) in LPS-stimulated BV-2 cells.

Conclusion: Results indicate that OFP-EA extract exhibited strong antioxidant properties and inhibited the excessive production of pro-inflammatory mediators such as NO, iNOS and TNF- α in LPS-stimulated BV-2 cells. The antioxidant activity exhibited by OFP-EA extract might play a critical role in ameliorating the inflammatory processes in LPS-stimulated BV-2 microglial cells.

Key words: Olive fruit pulp, antioxidant, neuroinflammation, microglia, TNF- α , iNOS.

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, International Pharmaceutical Abstract, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Microglial activation plays a pivotal role in neuro-inflammation and is involved in the neuro-pathological changes seen in several central

nervous system (CNS) diseases such as multiple sclerosis (MS), Parkinson's disease (PD), Alzheimer's disease (AD) and Huntington's disease [1, 2]. Activation of microglia results in release of different pro-inflammatory mediators

and free radicals [3]. It is well documented that reduction of pro-inflammatory mediators such as nitric oxide (NO), inducible NO synthase (iNOS), tumor necrosis factor- α (TNF- α) and other inflammatory cytokines in activated microglia could attenuate the severity of these disorders [4, 5]. Lipopolysaccharide (LPS), found in the outer membrane of Gram-negative bacteria can stimulate microglia both *in vitro* and *in vivo* to release various pro-inflammatory and neurotoxic factors [2]. Therefore LPS-stimulated microglia is a useful *in vitro* model for rapid screening of several anti-neuroinflammatory agents [6]. *Olea europaea* Linn. (family, Oleaceae), commonly known as olive is among the oldest known cultivated trees in the world with immense medicinal values. Olives and its associated products have been used widely as folk medicines in Spain, Italy, France, Greece, Israel, Morocco, Tunisia, Turkey and the Mediterranean islands for centuries [7]. Today, the olive plant is most well known for its fruit crop and oil. As a folk remedy, olives have been used to reduce the incidence of heart diseases [8]. Experimental studies on the fruits and leaf extracts from olives show that they possess anti-thrombotic, anti-hypertensive, anti-cancer, hypoglycemic, anti-inflammatory, anti-microbial and anti-atherogenic properties [9, 10]. The major active components of olives are phenolic constituents including oleuropein, hydroxytyrosol, tyrosol, 4-hydroxyphenyl acetic acid, protocatechuic acid, caffeic acid and p-coumaric acid. However, several other biologically active constituents are also present [8, 11].

Although the health beneficial effects of olive fruit and leaf oils have been confirmed *in vitro* using different cell lines [10], studies on the olive fruit pulp extracts and their protective effect on neuroinflammatory conditions using microglial cells have not been demonstrated. In the present study the ethyl acetate fraction obtained from olive fruit pulp extract (OFP-EA) was investigated for its anti-neuroinflammatory effects in LPS-stimulated BV-2 microglial cells and the antioxidant property was also evaluated to substantiate its anti-neuroinflammatory effects.

EXPERIMENTAL

Preparation of the OFP-EA extract

Green olive fruits collected at the end of September to about the middle of November were obtained from the local market, Seoul, South Korea. The collected fruit material was authenticated by Prof. Jong-Bo Kim, a taxonomist at Konkuk University, Korea and a voucher specimen (OL-KU2012) has been kept in our laboratory herbarium, Konkuk University,

Korea for future reference. To obtain the olive fruit pulp extract, 500 g of the fruit were ground in a mixer and defatted three times with three volumes of 80% ethanol. The residue (fruit pulp) was extracted with absolute ethanol (EtOH) at 1:10 ratio (w/v) for 2 h in a heating mantle at 70-80 °C. The supernatant was filtered and concentrated in a rotatory evaporator at 50°C. The ethanol extract of olive fruit pulp obtained (180 g) was re-suspended in water:EtOH (9:1, v/v) and partitioned successively with n-hexane, ethyl acetate (EA) and n-butanol to obtain a final yield of 19.4, 52 and 27.27 %, respectively. Since EA fraction of olive fruit pulp extract showed potent antioxidant effect in our preliminary evaluation, further studies on anti-neuroinflammatory effects in LPS-stimulated BV-2 microglial cells was investigated using OFP-EA extract. The extract was dissolved in sterile distilled water and filtered on 0.22 μ m filters before use. All reagents used in this study were of highest grade available commercially.

DPPH radical scavenging activity

The anti-oxidant activity of the OFP-EA extract was determined using the stable radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH, Sigma-Aldrich, St. Louis, MO, USA). The radical scavenging capacity was evaluated by employing a reaction mixture constituted by aliquots of the OFP-EA extract and a DPPH methanolic solution as described previously [12]. Briefly, a sample solution of 60 μ l of each OFP-EA extract, was added to 60 μ l of DPPH (60 μ M) in methanol. After mixing vigorously for 10 s, the mixture was then transferred into a 100 μ l Teflon capillary tube and the scavenging activity of each sample on DPPH radical was measured using a JES-FA ESR spectrometer (Jeol Ltd, Tokyo, Japan). A spin adduct was measured on an ESR spectrometer exactly after 2 min. Experimental conditions were as follows: central field, 3,475 G; modulation frequency, 100 kHz; modulation amplitude, 2 G; microwave power, 5 mW; gain, 6.3×10^5 , and temperature, 298°K.

Cell culture and viability assay

BV-2 microglia cells were cultured at 37 °C in 5 % CO₂ in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 5% FBS (Hyclone, Logan, UT, USA) and antibiotics (Invitrogen). In all experiments, cells were pre-treated with OFP-EA (10, 20, 40 and 80 μ g/ml) for 1 h before the addition of LPS (1 μ g/ml, Sigma-Aldrich, St Louis, MO, USA) in serum free DMEM. An equal volume of sterile water was added to all control treatments.

For viability assay, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT, Sigma-Aldrich, St. Louis, MO, USA) assay was used as described previously [13]. Briefly, BV-2 cells were plated onto 96 well plates and exposed to EA-OFP extract. MTT was added to each well then incubated for additional 2 h in dark at 37°C. The medium was then aspirated from the wells and the blue formazan product obtained was dissolved in DMSO. The plates were analyzed at 570 nm using a microplate reader (Tecan Trading AG, Switzerland). Each experiment was conducted in triplicate. Percentage of the cell viability was calculated as (O.D. of extract treated sample/O.D. of non-treated sample) x 100 %.

Immunoblot analysis and antibodies

Cells were washed in cold PBS three times and lysed in a buffer containing 50 mM Tris-HCl, pH 7.4, 1 % (v/v) NP-40, 0.25 % sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 25 mM NaF, 2 mM Na₃VO₄ and protease inhibitor cocktail (Complete Mini™, Roche, Mannheim, Germany) at 4°C. The lysate was clarified by centrifugation at 10,000 g for 20 min at 4 °C to remove insoluble components. Cell lysates were normalized for protein content using BCA reagent (Pierce, Rockford, IL, USA). Equal amounts of protein were loaded onto 10% PAGE gels and separated by standard SDS-PAGE procedure. Proteins were transferred to an NC membrane (S&S, Dassel, Germany) and blocked with 5% non-fat dry milk in TBS. To detect protein expression, the blots were probed with the specific antibodies against iNOS and TNF-α followed by the secondary antibodies coupled to horseradish peroxidase (Bio-Rad, Hercules, CA, USA). The detection of β-actin with a specific antibody was used for an internal control. The immunoreactive proteins on the membrane were detected by chemiluminescence using the West-Save substrate (Lab-Frontier, Seoul, Korea) on X-ray film. The antibodies against iNOS, TNF-α and β-actin were purchased from Cell Signaling Technology INC. (Beverly, MA, USA).

NO assay

Production of NO was assayed by measuring the levels of nitrite in the culture supernatant using colorimetric assay with Griess reagent [14]. Briefly, BV-2 cells (2 x 10⁵ cells/ml) were seeded in 6-well plates in 500 μl complete culture medium and treated with the OFP-EA extract at indicated concentrations for 1 h prior stimulation with LPS (1 μg/ml) for 2 h. Culture supernatant (50 μl) was reacted with an equal volume of

Griess reagent (0.1 % naphthylethylenediamine and 1% sulfanilamide in 5 % H₃PO₄) in 96-well plates at room temperature in the dark. Nitrite concentrations were determined by using standard solutions of sodium nitrite prepared in the culture medium. The absorbance was determined at 540 nm using a microplate reader (Tecan).

TNF-α assay

BV-2 microglia cells (1 x 10⁵ cells/well) were cultured on 96 well plates and treated with the OFP-EA extract at indicated concentrations for 1 hr and stimulated with LPS (1 μg/ml). At 4 hr post LPS treatment, the cells were collected and the supernatants were evaluated for TNF-α levels using a murine TNF-α ELISA kit from BD Biosciences (San Jose, CA, USA) according to the manufacturer's instructions.

RESULTS

Effect of OFP-EA extract on DPPH radical scavenging activity

As shown in Fig. 1A, OFP-EA exhibited significant DPPH radical scavenging activity in a concentration-dependent manner showing a maximum effect at 200 μg/ml of concentration ($p < 0.001$). The ESR spectroscopy data is represented in Fig 1B.

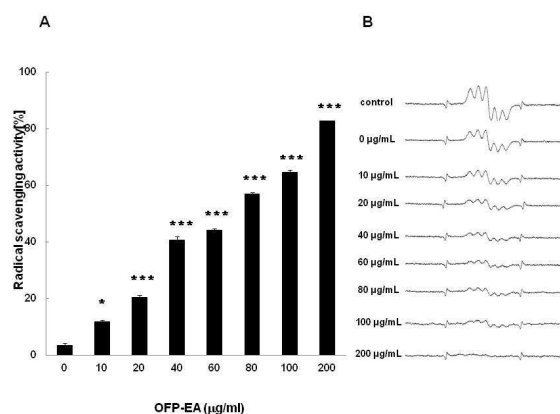


Fig 1: Effect of OFP-EA extract on DPPH radical scavenging activity. The capacities to scavenge the free radical DPPH by different concentrations of OFP-EA extract (A) and ESR spectra (B). BV-2 cells were treated with or without OFP-EA extract at the various concentrations (10, 20, 40, 60, 80, 100 and 200 μg/ml). The scavenging activity of each sample on DPPH radical was measured using a JES-FA ESR spectrometer. A spin adduct was measured on an ESR spectrometer exactly 2 min later. Data are presented as the mean ± S.E.M. (n = 3) for three independent experiments. * $p < 0.05$ and *** $p < 0.001$, compared with control group by Student t-test. (OFP-EA=olive fruit pulp-ethyl acetate).

Effect of OFP-EA on BV-2 cell viability

Treatment with OFP-EA at various concentrations ranging from 0.1 $\mu\text{g/ml}$ to 100 $\mu\text{g/ml}$ did not affect the overall cell viability nor did they exhibit any cytotoxicity on BV2 microglia cells (Fig. 2).

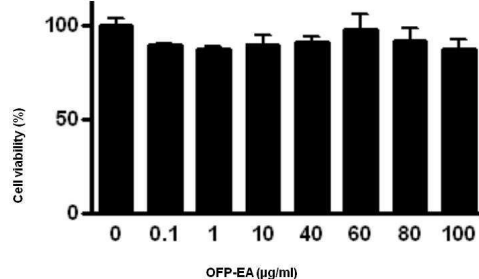


Fig 2: Effects of OFP-EA extract on the viability of BV-2 microglial cells. Viability in OFP-EA extract-treated cells was determined using MTT assay in the presence or absence of LPS (1 $\mu\text{g/ml}$). The results are depicted as percentage of control samples. Data are presented as the mean \pm S.E.M. ($n = 3$) for three independent experiments. (OFP-EA=olive fruit pulp-ethyl acetate.)

OFP-EA extract attenuates NO production in LPS-stimulated BV-2 cells

Cells treated with LPS alone significantly increased in NO levels ($p < 0.001$) Fig.3. Pre-treatment with OFP-EA extract significantly suppressed the LPS-stimulated increased NO release in BV-2 cells in a dose-dependent manner compared to LPS-treated cells. The maximum effect was observed at a concentration of 80 $\mu\text{g/ml}$ ($p < 0.001$). OFP-EA extract at 10, 20 and 40 $\mu\text{g/ml}$ also significantly and concentration-dependently inhibited the release of NO in LPS-stimulated BV-2 cells.

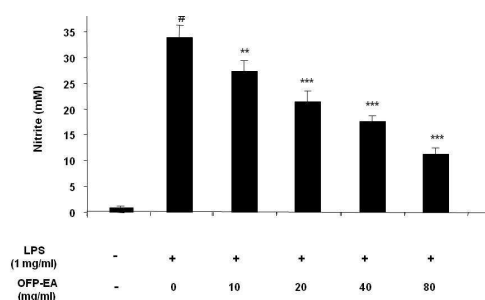


Fig 3: Effect of OFP-EA extract on NO Production in LPS-stimulated BV-2 microglial cells. BV-2 cells were treated with OFP-EA extract at various concentrations (10, 20, 40 and 80 $\mu\text{g/ml}$) with or without LPS (1 $\mu\text{g/ml}$) for 4 hr. The nitrite in the culture supernatant was evaluated using Griess reagent. Data are presented as the mean \pm S.E.M. ($n = 3$) for three independent experiments. [#] $p < 0.001$, when compared with control group. ^{**} $p < 0.01$ and ^{***} $p < 0.001$, when compared with LPS alone treated group by Student t-test. OFP-EA=olive fruit pulp-ethyl acetate.

OFP-EA extract attenuates iNOS expression in LPS-stimulated BV-2 cells

Western blot analysis showed that the protein expression of iNOS in the LPS-stimulated BV-2 cells was also suppressed in a concentration-dependent manner (Fig 4). Although LPS-stimulated BV-2 cells are associated with the increased iNOS expression, pre-treatment with OFP-EA extract suppressed the increased iNOS expression levels.

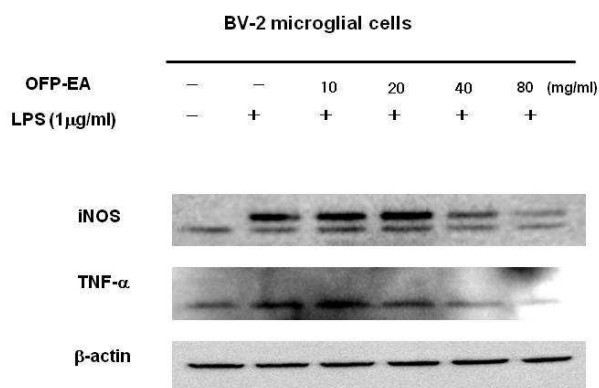


Fig 4: Effect of OFP-EA extract on iNOS and TNF- α expressional levels in LPS-stimulated BV-2 microglial cells. The expression levels of iNOS and TNF- α production in the LPS-stimulated BV-2 cells by various concentration of the OFP-EA extract was monitored by immunoblot analyses with the specific antibodies against iNOS and TNF- α . The internal control used was β -actin. (OFP-EA=olive fruit pulp-ethyl acetate.)

Effect of OFP-EA extract on TNF- α production in LPS-stimulated BV-2 cells

As shown in Fig. 5, TNF- α levels increased significantly after LPS treatment when compared to those in untreated cells ($p < 0.001$). However, OFP-EA extract significantly inhibited TNF- α production significantly in a concentration-dependent manner ($p < 0.01$ at 10 $\mu\text{g/ml}$ and $p < 0.001$ at 20, 40 and 80 $\mu\text{g/ml}$, respectively).

DISCUSSION

In the present study we report that the ethyl acetate fraction of olive fruit pulp extract significantly inhibited production of NO, suppressed the expression of iNOS protein level and attenuated the increased TNF- α production and protein expression in LPS-stimulated BV-2 microglial cells. Further the OFP-EA extract exhibited significant antioxidant activity evaluated by DPPH free radical scavenging method.

It is widely believed that free radicals and reactive oxygen species (ROS) are important causative factors in the development of age-

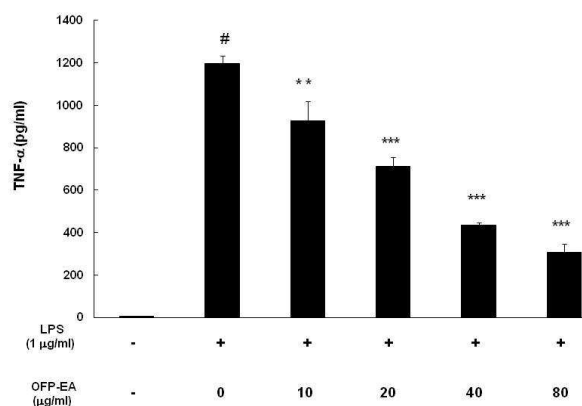


Fig 5: Effect of OFP-EA extract on TNF- α production in LPS-stimulated BV-2 microglial cells. Suppression of pro-inflammatory cytokine TNF- α expression by OFP-EA extract was measured with ELISA test. BV-2 cells were treated with OFP-EA extract at 10, 20, 40 and 80 μ g/ml with or without LPS (1 μ g/ml) for 4 h. The TNF- α in the culture supernatant was evaluated using a murine TNF- α ELISA kit. Data are presented as the mean \pm S.E.M. (n = 3) for three independent experiments. [#] p < 0.001, when compared with control group. ^{**} p < 0.01 and ^{***} p < 0.001, when compared with LPS alone group by Student t-test. (OFP-EA=olive fruit pulp-ethyl acetate.)

related neuro-inflammatory and neuro-degenerative diseases by stimulating release of cytokines which are responsible for the recruitment of additional neutrophils and macrophages [15]. Thus free radicals are important mediators that provoke or sustain inflammatory responses and their neutralization by antioxidants and radical scavengers can reduce neuro-inflammation. It was well known that DPPH radical assay as one of the widely used methods for evaluating the free radical scavenging activities of several antioxidants in a relatively short period of time [12].

Earlier studies revealed that olive oil phenols possessed strong antioxidant compounds [16]. In our present study, the ethyl acetate fraction obtained from the olive fruit pulp extract also exhibited significant free radical scavenging effect indicating that the fruit pulp extract might contain potential antioxidant agents.

Previous studies on tumor cell lines *in vitro* and animal models *in vivo* revealed that the olive oil and leaf extracts might be helpful in suppressing inflammatory conditions [9, 10]. The phenolic antioxidant compounds present in Olive oil extracts were also reported to show anti-inflammatory properties [17]. In light of such reports that antioxidants can reduce neuroinflammation, we used OFP-EA extract to

evaluate for its anti-neuroinflammatory activity in LPS-stimulated BV-2 microglial cells.

A significant number of reports have established that inflammatory mediators, including NO and iNOS are responsible for the symptoms of many neuro-inflammatory diseases [18,19]. Neuro-inflammatory response is indicated by activated microglia producing elevated levels of pro-inflammatory cytokines [1,2]. Thus, inhibition of cytokine production or function serves as a key mechanism in the control of inflammatory responses in neuro-degeneration. The present results from our study suggest that the OFP-EA extract significantly inhibited the increased NO production and iNOS protein expression in LPS-stimulated BV-2 cells.

TNF- α , an inflammatory cytokine, mainly produced by activated macrophages is involved in systemic inflammation that stimulate the acute phase reaction. TNF- α , not only amplifies the inflammatory cascade, but also causes the inflammatory injury [20,21]. TNF- α expression are up-regulated in the brains of patients with neuro-degenerative diseases such as AD and PD suggesting a causative role in neuro-degenerative disorders [22,23]. Microglial cell activation by LPS produces various cytokines, such as IL-6, and TNF- α , leading to the attraction of neutrophils and the accumulation of neutrophil-secreted proteases and reactive oxygen species (ROS) at the site of inflammation. From our results, it appears that OFP-EA may act by suppressing the expression of TNF- α at transcriptional (data not shown) and protein levels and thereby inhibiting NO production and iNOS expression levels in LPS-stimulated BV-2 cells.

CONCLUSION

This study demonstrates that OFP-EA plays a role in mitigating neuro-inflammatory responses in LPS-stimulated BV-2 microglial cells. The OFP-EA extract also showed potent antioxidant effects. Earlier reports on the pharmacological benefits of olives including anti-inflammatory, bacteriocidal and bacteriostatic activities were attributed mainly to their polyphenolic compounds such as hydroxytyrosol and tyrosol. In particular, hydroxytyrosol was found in great quantities in the remains from fruit oil processing, such as pomace olive oil, olive-mill waste water and rinse waters [24, 25]. The phenolic compounds present in olive fruit pulps might be responsible in exhibiting such potent antioxidant and anti-neuroinflammatory effects.

COMPETING INTEREST

The authors declare that there are no conflicts of interest.

ACKNOWLEDGEMENT

This research was supported by a grant from the Regional Strategic Planning Technology Development Program funded by the Ministry of Knowledge Economy of the Korean government and by a grant from Macrocare Tech., Ltd. The authors thank Chungbuk Technopark for excellent administrative assistance.

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