Assessment of the anti-inflammatory, analgesic and sedative effects of oleuropein from *Olea europaea* L.

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**Abstract:** More and more studies show that inflammation, pain and insomnia have become the main common diseases. Effective treatments of inflammation, pain and insomnia have become an issue of primary concern in clinical practice. Oleuropein (OLE), the main phenolic component of Mediterranean extra virgin olive oil, has shown many pharmacological properties. In the present study, the anti-inflammatory effect of OLE was firstly evaluated using RAW264.7 macrophages subjected to stimulation with lipopolysaccharide (LPS). The results obtained revealed that OLE caused significant and dose-dependent downregulation of nitric (NO), COX-2, inducible NO synthase iNOS, and the inflammation-associated cytokines IL-6 and TNF-α. From the mechanism, the expression of COX-2, cytokines IL-6 and TNF-α OLE is closely related to analgesic and sedation effect. Further evaluations showed significant analgesic and sedative effects of OLE in tail-flick test and sedation test conducted in SD rats in vivo. All these results indicate that OLE has anti-inflammatory, analgesic and sedative effects both in vitro and in vivo.

**Key words:** Anti-inflammatory; Analgesic and sedative effects; Olea europaea L.

**Introduction**

Oleuropein (OLE, Figure 1), is major secoiridoid derived from the olive tree, and the major component of Mediterranean extra virgin olive oil. Studies have revealed that OLE exerts many pharmacological properties such as antibacterial, antimalarial, anti-cancer and anti-cardiovascular disease (11-13).

Actually, inflammation, pain and insomnia have become the main common diseases. Especially the unpleasant experience associated with pain makes the search for its effective treatments a primary issue in clinical practice (1). Diverse efforts have been devoted to developing pain-relieving drugs, including opioids drugs and non-steroidal anti-inflammatory drugs (NSAIDs) (2). However, due to the adverse effects and ineffectiveness of these drugs in many types of pain, the development of pain therapy constitutes a serious medical issue (3). Over the years, emphasis has shifted to herbal sources as alternative remedy for pain. Plant-derived medications have several advantages over orthodox drugs: they have greater effectiveness, are less toxic and can easily serve as candidates for developing new drugs (4, 5).

Insomnia or sleeplessness is a common medical condition associated with impairment of sleep (6, 7). The most popular treatment for insomnia is taking drugs, although medical counselling is sometimes applied (8, 9). However, the use of sleeping pills is associated with unwanted side effects such as addiction, rebound sleeplessness and drug tolerance (10). These side effects are absent in plant-based remedies which may thus offer better alternatives to pharmaceutical formulations.

Therefore, the present study was aimed at investigating the anti-inflammatory, analgesic and sedative effects of OLE. In this study, RAW264.7 macrophages were selected for evaluation of anti-inflammatory activity of OLE, while Sprague Dawley rats were used for assessing its analgesic and sedative effects.

**Materials and Methods**

Stock solution of oleuropein was product of J&K Scientific (Beijing, China). Ibuprofen, MTT reagent, diazepam and ketamine were purchased from Sigma Chemical Co. (St. Louis, USA). Santa Cruz Biotech (Santa Cruz, US) supplied the IL-6, COX-2, TNF-α, and β-actin antibodies.
RAW264.7 macrophage culture

RAW264.7 macrophage cell line was supplied by ATCC (Manassas, USA). The cells (5 × 10^6 cells/mL) were cultured at 37 °C in RPMI medium containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and penicillin G (100 U/mL) in an incubator with 5% CO₂.

Determination of cell viability

The effect of OLE on the viability of RAW264.7 cells was assessed using MTT assay as described earlier (14).

Measurement of NO production from RAW264.7

RAW264.7 cells seeded at a concentration of 1×10⁵/ml were cultured for 24 h at 37°C in 96-well plates. Thereafter, they were incubated for another 2 h with different doses of OLE before exposure to 2 µg/ml LPS for 24 h. The culture medium was then assayed for NO with assay kits for nitrate/nitrite. In this assay, the color complex formed between NO and Greiss reagent is subject to spectrophotometric analysis at 540 nm.

Determination of pro-inflammatory cytokines TNF-α and IL-6

The levels of TNF-α and IL-6 were assayed for with ELISA kits from BD Biosciences in line with the instructions in the kit manual.

Experimental animals

Sprague Dawley rats obtained from the Animal Center of Yangzhou University were used in this study. They were maintained in a standard animal house at 25 °C with unrestricted availability of feed and drinking water prior to the commencement of the experiments. The study was approved by the ethical committee of our University.

Tail-flick test

The rats were subjected to tail flick test in accordance with the protocol described earlier (15). Before and after drug exposure, the distal end of the rat tail was placed in a 52 °C water bath, and the time lags prior to tail flip (latency periods) were recorded and compared. The maximum exposure period was fixed at 12 sec to prevent heat-induced damage to the rat tissues. The pain-relieving effect was estimated in terms of percentage of maximum possible effect (% MPE) viz:

\[ \text{MPE} = \frac{D - B}{B} \times 100 \]

where \( D \) and \( B \) are drug response time and basal response time, respectively.

Brt2.8 Sedative test

Hypnosis was induced using ketamine according to the method of Mimura et al (16). Five groups of rats were used (7 rats/group). These were vehicle group (5% DMSO), positive diazepam group (2mg/kg), and three OLE groups (10, 25 and 50 mg/kg). Rats in each group were treated for 60 min before being injected with ketamine. The indices determined were total sleeping time and sleeping latency.

Statistical analysis

Results were presented mean ± standard deviation (SD), and were statistically analyzed using ANOVA, prior to Students-Newman-Keuls test (SNK) for post-hoc comparisons. The results of the sedative test were compared using χ² test. Values of \( p < 0.05 \) were assumed significant.

Results

Effects of OLE on RAW264.7 cell viability

The cytotoxic effect of OLE was assessed with MTT assay after 48h-treatment at diverse doses. The results showed that OLE did not interfere with their viability at concentrations of 5, 10, 20, 50, 100 and 200 µM (Figure 2A). Therefore, these concentrations of OLE were considered suitable for use in subsequent experiments.

Effect of OLE on LPS-induced NO production

The RAW264.7 macrophages were exposed to OLE (0, 5, 10 and 20 µM) for 2 h before being LPS-simulated for 2 h at a dose of 2 µg/ml. Cells untreated with OLE served as control. The LPS stimulation of RAW264.7 macrophages resulted in significant elevation in NO levels, relative to untreated cells, while pretreatment with different concentrations of OLE dose-dependently reversed the LPS-induced increases in NO (Figure 2B).

Effect of OLE on the expressions of LPS-induced iNOS and COX-2

The expressions of iNOS and COX-2 proteins were significantly upregulated by LPS stimulation. However, prior treatment with OLE reversed the upregulation in iNOS and COX-2 protein expressions dose-dependently (Figure 3).

Figure 2. Influence of OLE on viability and NO levels in RAW264.7 cell line. (A) RAW264.7 cells (1×10⁵ cells/mL) were treated with 5, 10, 20, 50, 100 and 200 µM OLE for 48 h, and viability was assessed using MTT method; (B) RAW264.7 cells were treated with 5, 10 and 20 µM OLE for 2 h, prior to exposure to LPS for 24 h. The NO production in medium was determined using Griess reaction. Results are shown as mean ± SD. * \( p < 0.05, ** \( p < 0.01, *** \( p < 0.001, \) compared to LPS only.

Figure 3. Effect of OLE on LPS-induced iNOS and COX-2 protein levels in RAW264.7 cells. (A) Following treatments, the RAW264.7 cells were subjected to lysis and assay of COX-2 and iNOS using western blot. Results are presented as mean ± SD. * \( p < 0.05, ** \( p < 0.01, *** \( p < 0.001, \) compared to LPS only.
Effect of OLE on LPS-induced TNF-α and IL-6 production

Figure 4 showed that LPS upregulated the expressions of TNF-α and IL-6, relative to \( p < 0.01 \). However, the LPS-induced upregulations were dose-dependently inhibited by OLE, relative to cells treated with LPS only.

Analgesic effect of OLE on rats through tail-flick test

The analgesic effects of ibuprofen and OLE were significantly high, when compared with control group. The application of OLE resulted in dose-dependent increases in analgesic effect, with OLE at doses of 10 and 25 mg/kg producing % MPE comparable to that of ibuprofen, while its % MPE at 50 mg/kg was superior to that of ibuprofen (Figure 5).

Sedative effect of OLE in ketamine-treated rats

The results shown in Figure 6 indicate that diazepam (DEM, 2 mg/kg positive control) significantly reduced sleep latency and significantly enhanced total sleeping time in rats \( p < 0.05 \). Pretreatment with OLE at 25 and 50 mg/kg also significantly decreased sleep latency and extended total sleeping time due to ketamine, when compared to the vehicle, which was also comparable to DEM.

Discussion

Host defense against infections requires the participation of macrophages which produce inflammatory compounds such as TNF-α, NO and IL-6 (17). The pathogenesis of cancer and many inflammatory conditions has been attributed to excessive expression of these mediators (18). This informed the use of RAW264.7 cell line (macrophages) to investigate the suppressive and curative effects of OLE on the expressions of inflammatory mediators. Results from MTT assay revealed that OLE was not toxic to the RAW264.7 cell line. It has been established that three forms of iNOS are involved in the production of NO: endothelial NOS (eNOS), iNOS and neuronal NOS (nNOS) (19). Among these, iNOS produces far more amount of NO on a sustained basis than the other forms on NOS, and its activity is enhanced by microbial metabolites and cytokines. It has been shown that excessive levels of NO exhibit pro-inflammatory potency, resulting in inflammatory diseases (20, 21). Prostaglandins and COX-2 have been shown to enhance inflammation-related pain (22). Thus, it has been suggested that inflammation-associated disorders can be mitigated by reducing NO production and downregulating COX-2 and iNOS (23). In this study, OLE suppressed NO liberation by decreasing the expression of iNOS in RAW264.7 macrophages under LPS stimulation. Besides, OLE dose-dependently inhibited the expression of COX-2. These findings suggest that OLE can be considered as a promising agent for treating inflammatory responses and diseases.

Inflammation and pain are always associated with liberation of the pro-inflammatory cytokines IL-6 and TNF-α (24). The latter (TNF-α) is an important inflammatory protein involved in LPS-induced production of NO in macrophages (25). It also participates in immune responses, host defense and acute-phase reactions (26).

Increased expressions of TNF-α and IL-6 have been linked to several diseases (27). In the present study, results obtained from ELISA assay demonstrated that OLE suppressed the production of IL-6 and TNF-α in RAW264.7 cells subjected to stimulation with LPS. In addition, based on the downregulation of COX-2 which is closely related to the analgesic mechanism, the analgesic effects of OLE was investigated in vivo using the tail-flick test, and the results showed fast and dose-dependent decreases in analgesic influence of OLE. Furthermore, evaluating the sedative and analgesic effects simultaneously is logical. For further studies on OLE, its sedative effect was determined on rats treated with ketamine. The results showed that OLE at doses of 25 and 50 mg/kg significantly reduced sleep latency and extended total sleep time. This indicates that OLE possess sedative activity. Thus, in addition to analgesic and anti-inflammatory effects, OLE has sedative activities, indicating it may be a potent and effective agent for...
managing pain and related conditions. The present study appears to be the first to report the anti-inflammatory, analgesic and sedative effect of OLE both in vitro and in vivo. However, further investigations are required to unravel the precise mechanism that underlie these effects.

Acknowledgments
None.

Conflict of Interest
There are no conflicts of interest in this study.

Author’s contribution
All work was done by the author named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Zhiming Zhou; Bing Xia, Mingzhi Zheng collected and analysed the data; Xincheng Mao wrote the text and all authors have read and approved the text prior to publication.

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