

Protective Effect of Cichorium Intybus Root Extract and Esculetin on Lipopolysaccharide-Induced Pain and Inflammation in Male Mice: A Potential Role for Tumor Necrosis Factor- α and Interleukin-1 β

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Abstract

Lipopolysaccharide (LPS) has been widely used in animal modeling of inflammation-induced hyperalgesia. Proinflammatory cytokines (TNF- α , IL-1 β) are involved in the pathogenesis of pain. *Cichorium intybus* (CI) contains many compounds which inhibit inflammatory mediators. The present study was designed to evaluate the effect of CI root hydro-alcoholic extract (CE) and Esculetin (ESC) - a bioactive coumarin compound of CI- on LPS-induced pain and inflammation in male mice. CE constituents were evaluated by GC-MS. Two different concentration of CE (300 and 500 mg/kg/p.o.) and ESC (40 mg/kg/i.p.,o.) were used for experiments. Drugs were administered once daily for 7 days. On day 7, LPS (0.83 mg/kg/i.p.) was injected 30 min after the last drug administration. Blood samples were collected 90 min after LPS injection. Hot plate and formalin tests were performed 24 hours after LPS injection. Serum TNF- α and IL-1 β levels were measured using ELISA. LPS significantly increased the response time of animals in hot plate and both phases of formalin tests. CE (300 and 500 mg/kg/p.o.) significantly reduced the response time in comparison with the LPS group in hot plate and both phases of formalin tests. CE (500 mg/kg/p.o.) reduced serum TNF- α and IL-1 β levels. Esculetin increased the response time in hot plate and the second phase of formalin test compared to the LPS group and also decreased IL-1 β level, significantly. Our results demonstrated that CE exhibited anti-nociceptive and anti-inflammatory effects better than esculetin. The probable mechanism of anti-hyperalgesic effect of CE is related to inhibition of inflammatory cytokines IL-1 β and TNF- α .

1. Introduction

Pain, as defined by International Association for the Study of Pain, is “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, as described in terms of such damage”. Pain has been classified as acute or chronic based on duration only; bone, joint, cutaneous, superficial or deep, muscular or visceral based on location; and inflammatory, neuropathic or cancer pain based on cause or type [1].

Inflammation initiates upon tissue injury, and leads to a cascade of biochemical reactions that provoke the nervous system for pain sensation. In addition, long-term inflammation induces adaptive changes in the nervous system which can exaggerate or attenuate the sensation of pain [2]. Cytokines, the necessary mediators to conduct the inflammatory response from injured and infected sites, are also favoring factors for proper wound healing [3]. Exaggerated production of pro-inflammatory cytokines at the site of injury can be manifested systemically as hemodynamic and metabolic instability [3, 4].

Proinflammatory cytokines tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) are involved in the pathogenesis of pain. During the injury and inflammation, IL-1 β is released primarily by monocytes and macrophages as well as non-immune cells, such as fibroblasts and endothelial cells. It was found that IL-1 β is expressed in dorsal root ganglion (DRG) nociceptive neurons. Also, its expression is enhanced following trauma to peripheral nerves and brain injury in microglia and astrocytes. Moreover, IL-1 β has also been reported to produce hyperalgesia following either intraperitoneal, intracerebroventricular or

intraplantar injection. TNF- α , also known as cachectin, is another inflammatory cytokine that plays a well-established key role in some pain models. TNF- α reduces the activation threshold of type C peripheral nerve fibers for mechanical stimuli through extravasation of plasma and results in mechanical allodynia [3–6]. TNF- α acts on several different signaling pathways through two cell surface receptors, TNFR1 and TNFR2 to regulate apoptotic pathways, nuclear factor kappa B (NF- κ B) activation and activate stress-activated protein kinases (SAPKs). Its receptors are present in both neurons and glia and plays an important role in both inflammatory and neuropathic hyperalgesia [6].

The ability to mimic the clinical presentation of diseases such as pain, in animal models, is important to achieve reliable medical results[7]. Nowadays, bacterial lipopolysaccharide (LPS) is widely used worldwide for arrays of investigations such as modeling of diseases, inflammatory mechanism evaluation, psycho/neurologic and behavioral studies both in vivo and in vitro [7–9]. Moreover, It is also considered as a causative agent of different types of pain and currently is used to induce animal models of pain enhancement sensitivity, hyperalgesia and allodynia [7, 8]. Systemic LPS exposure leads to inflammatory responses in serum and spinal cord of rats. Also, intraperitoneal LPS administration inflammatory responses showed a tendency toward white matter injury. It was reported that systemic LPS administration induces CNS inflammation [8]. LPS activates the innate immune response and stimulates the secretion of inflammatory cytokines through NF- κ B signaling pathway. This triggers the synthesis of pro-inflammatory cytokines; TNF- α and IL-1 β , which stimulate the secretion of inflammatory mediators such as prostaglandins, leukotrienes, nitric oxide (NO) and substance P. Finally, these pro-nociceptive factors lead to inflammation, neuronal hypersensitivity and facilitate pain signaling in the peripheral nervous system and the spinal cord [6–8].

Classical drugs for pain management are non-steroidal anti-inflammatory drugs (NSAIDs) and narcotics. Some side effects, such as digestive disorders, kidney damage, respiratory failure and dependence, may occur with these medications[10, 11]. The use of new and effective analgesics with fewer side effects is a priority. Many studies focus on drugs used in traditional medicine due to the low cost, accepted effects and low risk of herbal medicines [12].

Cichorium intybus (CI) known as chicory belongs to the Asteraceae family and is widespread in Asia and Europe [13]. Roots are fleshy, tapering, white from inside and brownish-yellow from outside [14]. The root of chicory is a rich source of anti-oxidant, anti-inflammatory and analgesic compounds such as hexadecanoic acid; sesquiterpene lactones (SLs); 8-deoxylactucin and lactucopicrin, and flavonoids; quercetin and catechin which can be used to alleviate inflammatory disorders [15–18].

Esculetin (ESC)(6,7-dihydroxycoumarin), a coumarin derivative compound is one of the major bioactive compounds of *Cichorium intybus* L. and has shown many pharmacological activities like anti-inflammatory, antioxidant and antidiabetic effects [19, 20]. The aim of this study was to investigate the effect of chicory hydro-alcoholic extract and ESC on LPS-induced pain and inflammation in mice.

2. Materials And Methods

2.1. Preparation of chicory root extract (CE)

The roots of chicory were collected from high land rural areas around Guilan province, Iran. Roots were washed and dried in the natural condition at 24-26°C for 3-4 days. Then 100 grams of the chicory powder was soaked in 300 ml of methanolic hydro-alcoholic solution (800 ml methanol 96% with 200 ml water) and was extracted by soxhlet extractor. The resulting extract was dried in dry heat at 40-50°C for 3-4 days [21]. Dried extract was dissolved in saline and was applied to treatment groups.

2.2. GC-MS analysis of hydro-alcoholic extract of chicory

Analysis of extraction were carried out using GC-MS system (Agilent Technologies-Wilmington, DE, U.S.A) and gas chromatograph (Model 7890A, a model 5970B mass selective detector and electron ionization model 70 eV). An applied fused-silica capillary column (30 m × 250 m i.d., 0.25 m film thickness) prepared by Scitech Scientific was used. GC temperature program was set as follow: 120°C as initial temperature, 2 min held, increased to 140°C at a rate of 2°C/min, raised to 220°C by the rate of 3°C/min and finally increased more to 320°C by the rate of 7°C/min and held for 15 min. The split ratio was 1:12; sample injection temperature was 290°C, transfer line temperature set as 310°C, and ion source temperature considered 240°C. A quadruple mass spectrometer (MS 5975 C inrtXL EI/CI MSD with triple-axis detector) operated at 70 eV in the electron impact CTC PAL auto sampler [21].

2.3. Drugs

Esculetin (purity 98%) was purchased from Sigma Aldrich (St. Louis USA). LPS from Escherichia coli (Serotype 0111: B4) was produced from Sigma-Aldrich (St. Louis, USA). Measurement of plasma TNF- α and IL-1 β (Zellbio GmbH, Germany) was performed by enzyme-linked immunosorbent assay (ELISA) method. All other chemicals and reagents used for the study were analytical grade and were provided by approved organizations.

2.4. Animals

Sixty-four male NMRI mice (25-40 g) were used. Animals maintained at 22±2°C with a relative humidity of 55± 5%, regular 12h light-12h dark cycle, with food and water ad libitum. Experiments were carried on from 9:00 a.m. to 13:00 p.m. The ethical considerations of experiments were approved by Animal Care and Use Committee of Guilan University of Medical Sciences (IR.GUMS.REC.1396.336). Regarding the ethical principles for conducting experiments, it should be emphasized that all the steps of the experimental procedures were conducted according to the Guide for the Care and Use of Laboratory Animals.

2.5. Experimental groups

Mice were randomly divided into 8 groups (n=8): control: (Normal saline 10 ml/kg), CE300: (CE 300 mg/kg), CE500 (CE 500 mg/kg), ESC: (ESC 40 mg/kg), LPS (a single dose of 0.83 mg/kg), LPS+CE300, LPS+CE500, and LPS+ESC. Animals were administered intragastrically with CE and ESC once daily for 7

days. On day 7, LPS (0.83 mg/kg/i.p.) was injected 30 min after the last administration of these drugs. Blood samples were collected from eye socket 90 min after LPS injection and then centrifuged at 4500 rpm for 15 min. The serum was separated and stored at -80°C. Hot plate and formalin tests were performed 24 hours after LPS injection.

2.6. Behavioral evaluation tests

2.6.1. Hot plate test

Mice were individually placed in a transparent glass cylinder on the hot plate surface (55°C) and the reaction time of the animals was measured in seconds. The latency before the mice began licking their paws or jumping off were recorded [22].

2.6.2. Formalin test

Twenty microliters of formalin (1% v/v) were injected subcutaneously into the right hind paw of the animals. A 30-gauge needle was used to perform the injections. The animals were immediately placed in an acrylic observation chamber (20 cm high, 20 cm diameter) and the time spent licking and biting their injected paw was measured [22].

2.6.3. Determination of cytokines in serum

The TNF- α and IL-1 β serum levels were measured by ELISA according to the manufacturer's instructions. The results are reported as picograms per milliliter (pg/ml).

2.7. Statistical analysis

To investigate the normality of the data, the Shapiro-Wilk test was used. Analysis of the data was performed using one-way analysis of variance (ANOVA), followed by Tukey post-hoc test. The level of significance was set at $p < 0.05$.

3. Results

3.1. GC-MS (Gas Chromatography-Mass Spectrometry) analysis

GC-MS analysis of hydro-alcoholic extract of CE root are presented in Table1 and figure 1. The relative intensity peaks of different compounds revealed that the major ones were hexadecanoic acid (25.03%), sesquiterpene lactones; 8-deoxylactucin (17.15%) and lactucopicrin (12.08%), Anthocyanins; Cyanidin (7.32%), Delphinidin (6.54%) and flavonoids; Catechins (6.56%), Quercetin (5.81%).

Table 1
Compounds identified in the hydro-alcoholic extract of root chicory by GC-MS.

Name of the compound	Retention time (s)	Peak area %
Octadecatrienoic acid	10.123	5.62
Tetrahydro Benzene (Isoquinoline)	13.245	1.91
Catechins (flavonoid)	16.325	6.56
Quercetin (flavonoid)	20.369	5.81
Hexadecanoic acid	23.047	25.03
Diol acetate	26.82	4.52
Cyclolanost	30.874	1.11
8-deoxylactucin (sesquiterpene lacton)	33.61	17.15
lactucopicrin (sesquiterpene lacton)	37.321	12.08
Epoxy Naphtha	40.497	4.03
Hexadecene	44.125	1.1
Cyanidin (glycoside)	47.569	7.32
Delphinidin (glycoside)	50.048	6.54
Lupeol (Terpenoid)	54.71	1.22

3.2. The effect of chicory root extract and esculetin on the response time in hot plate test

As shown in Fig. 2, intraperitoneal injection of LPS (0.83 mg/kg) could significantly decrease the response time in the hot plate test compared to control mice ($p < 0.05$). By contrast, there was a significant response time elevation ($P < 0.0001$) when both doses of CE (300 and 500 mg/kg) were administered before LPS. Also, LPS+ESC (40 mg/kg) treatment increased the response time with a significance of $P < 0.001$ in comparison with LPS group.

3.3. The effect of chicory root extract and esculetin on the first phase of formalin test

Figure 3 shows that LPS significantly increased the licking and biting time in the first phase of formalin test compared to the control group ($P < 0.05$). By contrast, only pretreatment with the CE 500 mg/kg significantly decreased the licking and biting time in comparison with LPS group ($P < 0.01$).

3.4. The effect of chicory root extract and esculetin on the second phase of formalin test

LPS administration increased the licking and biting time in the second phase of formalin test ($p < 0.001$) compared to control group. Pretreatment with both CE doses (300,500 mg/kg) and esculetin (40 mg/kg) could significantly decrease licking and biting time in comparison with LPS group ($p < 0.0001$) (Fig. 4).

3.5. The effect of chicory root extract and esculetin on pro-inflammatory cytokines level in serum

ELISA analysis indicated that exposure to LPS, significantly increased serum TNF- α level ($p < 0.0001$), while CI extract could only reverse its level when applied at 500 mg/kg ($P < 0.001$) (Fig. 5). On the other hand, serum level of IL-1 β also increased after LPS administration ($P < 0.05$), but as has shown in Fig. 6, both CI (500 mg/kg) and ESC (40 mg/kg) could significantly reduce the IL-1 β level in serum ($P < 0.001$).

Discussion

The present study revealed that LPS significantly reduced the response time in hot plate test and increased the licking and biting time in both phases of formalin test. Both doses of CE (300, 500 mg/kg) and esculetin (40 mg/kg) increased the hot plate response time compared to LPS group. Both doses of the CE and ESC decreased the licking and biting time in the second phase of formalin test, but during the first phase of formalin test only 500 mg/kg of CE showed a significant reduction in comparison with LPS group. Moreover, LPS administration significantly increased serum TNF- α and IL-1 β levels. On the other hand, pre-treatment with the CE (only, 500 mg/kg) prevented LPS-induced serum production of TNF- α , but IL-1 β increase was inhibited by both doses of CE, and ESC.

Previous studies have shown that systemic administration of LPS can lead to a generalized hyperalgesia [8]. Various mechanisms including the release of pro-inflammatory cytokines and glial cell activation have been proposed to be responsible for the LPS-induced hyperalgesia [7, 8]. TNF- α and IL-1 β are two essential cytokines involved in the inflammatory response as well as neuropathic pain induction. IL-1 β release through p38 MAPK signaling pathway, enhances the expression of COX-2 and leads to production of PGE2 in the white and gray matter of spinal cord and DRG [6, 8]. TNF- α a cytokine which is secreted by immune and glial cells, induces MAPKs phosphorylation and NF- κ B activation. In addition to be an anti-inflammatory mediator, TNF- α can act as a potentiator to increase other inflammatory cytokines [23]. Also, in some in vitro studies on neuronal cells line and hippocampal slices, it has been reported that TNF- α can reduce the GABA inhibitory activity [24]. Based on this important evidence, one of our main conclusions is that an increase in TNF- α following LPS leads to neuropathic pain, and this may be due to its inhibitory effect on GABAergic neurotransmission.

Here, our findings demonstrated that CE inhibits LPS-induced hyperalgesia and this effect may be achieved by inhibiting the production of two pro-inflammatory cytokines, TNF- α and IL-1 β . Previous studies have demonstrated that hexadecanoic acid, sesquiterpene lactones and flavonoids - the three major CE compounds shown in GC-MS analysis- have anti-inflammatory and analgesic activities.

Medicated oils rich in n-hexadecenoic acid had rigorous use for the treatment of rheumatic symptoms in the traditional medical system of India, Ayurveda [15]. It is a phospholipase A2 (PLA2) competitive inhibitor that reduces the production of pro-inflammatory cytokines. Furthermore, it increases the activity

of aromatic amino acid decarboxylase and enhances DOPA production, which improves dopaminergic neurotransmission and relieves pain [15, 25].

Sesquiterpene lactones have potent anti-inflammatory activity (I). Anti-inflammatory effect of 8-deoxylactucin is mediated by two mechanisms: 1) inhibition of NF- κ B activity and 2) cyclooxygenase (COX-2) inhibition [26]. In an in vitro study, it has been shown that SLs extracted from chicory root, in a dose-dependent manner downregulated the genes expression of COX-2, inducible nitric oxide synthase (iNOS), TNF- α and IL-1 β in LPS-stimulated macrophages [27]. It was observed that lactucin and lactucopicrin, found in CI, exerted an analgesic effect in the tail-flick and hot plate tests in mice and their antinociceptive activity was similar to ibuprofen [17, 28].

The flavonoid quercetin is a well-established molecule with strong analgesic, anti-inflammatory and antioxidant activity. In vivo and in vitro studies revealed that these effects are associated with several mechanisms, including inhibition of NF- κ B, PLA₂, reduction of pro-hyperalgesic and inflammatory cytokines/mediators such as PGE₂, NO, TNF- α , IL-1 β and iNOS production and expression. It also exerts opioid like effects, stimulates GABA_A and serotonin receptors, decrease/downregulates TLR4 and COX2, acts as inhibitor of TRPV1/NMDA receptors and also decreases NMDA receptors functionality [16, 29, 30]. Studies indicated that flavonoids can cross the blood–brain barrier (BBB) [29]. The quercetin inhibits CNS and spinal cord glial cells activation, and consequently reduces cytokine production and oxidative stress resulting in reduced neuro-inflammation and hyperalgesia [30]. Catechin, belonging to the flavonoid family, can alleviate the inflammation and pain response in rats by decreasing the generation of TNF- α , PGE₂, and IL-1 β [31]. We now know that circulating matrix metalloproteinase 9 (MMP-9) plays an important role in neuro-inflammation and oxidative stress as two leading factors of the neuropathic pain. It has been proved that Catechin reduces MMP-9 in diabetic rats [1].

Some other CE compounds with lower percentages (shown in Table 1) may be involved in the anti-inflammatory and analgesic activities of chicory.

GC-MS identified two anthocyanin compounds in the CE, Cyanidin-3-glucoside and Delphinidin. Anthocyanins (ANCs) are phenolic compounds which belong to the subclass of flavonoids. glycosylation is essential to increase ANCs molecular stability [6]. The ANCs or their bioactive metabolites readily penetrate the BBB and act as centrally antinociceptives to reduce CNS inflammation and scavenge free radicals [6, 32]. It has been demonstrated that ANCs attenuate inflammation-induced thermal and mechanical hyperalgesia with similar efficacy to indomethacin (6). It has been reported that cyanidin-3-glucoside possesses antioxidant and anti-inflammatory properties (33). Delphinidin can reverse mechanical, thermal and chemical hyperalgesia, as well as local inflammation (32). Also, due to its antioxidant activity, delphinidin has been proposed as a potential clinical cure to treat inflammatory pain (34). Delphinidin downregulates NF- κ B signaling pathway and leads to decrease TNF- α level. Moreover, ANCs are strong COX inhibitors with inhibitory capacity even comparable to NSAIDs such as naproxen and ibuprofen, hence, abridging the inflammation and nociceptive sensitization. In addition, ANC was also demonstrated to suppress glutamate release from inflamed neurons (6).

Lupeol is a triterpenoid compound. A previous study showed that it reduces the inflammatory response through the modulation of p38 pathway. The researchers reported that lupeol inhibited LPS-induced κ B α /degradation and decreased the DNA binding activity of NF- κ B in the macrophages (4). It also down-regulates TNF- α gene expression (35).

The name Octadecatrienoic acid refers to many different structural and conformational isomers. Fatty acids can modulate immune responses by acting directly on T cells. Linoleic acid exerts anti-inflammatory effect by decreasing production of the inflammatory mediators such as PGE₂, IL-1 β , TNF- α and nitric oxide. Also, α -linolenic acid exhibits high anti-inflammatory effect (15). Furthermore, 9,12,15-octadecatrienoic acid- the other component which was found in the CE- has been introduced as an anti-inflammatory compound (36).

Although some studies have shown that the anti-nociceptive property of esculetin may be mediated by inhibition of lipooxygenase (5-LOX) and PGE₂ production, understanding the mechanisms underlying the analgesic activity of esculetin needs further studies (37). Recently, one study on the fibromyalgia, a syndrome of persistent widespread pain, showed that esculetin can reduce pain by decreasing TNF- α and IL-1 β levels in fibromyalgia (38). In consistence with these results, we demonstrated that esculetin inhibits LPS-induced hyperalgesia and this effect may be due to inhibition of IL-1 β , but not TNF- α production. This may be resulted from different ways to regulate IL-1 β and TNF- α mRNA expression. LPS induces IL-1 β mRNA expression by both protein kinase C (PKC) and calmodulin (CaM) kinase dependent pathways, while TNF- α expression depends on PKC pathway but not CaM kinase pathway, apparently [39]. So, it seems that esculetin interferes with PKC signaling pathway.

The present study showed that hydro-alcoholic extract of chicory (500 mg/kg) was more effective than esculetin 40 mg/kg in behavioral and biochemical tests. There are many compounds with anti-inflammatory and analgesic properties in the chicory root which obviously may improve the efficacy of chicory compared to esculetin.

Conclusion

The present study demonstrated that chicory root extract exhibited anti-nociceptive and anti-inflammatory effects more than esculetin. The probable mechanism of anti-hyperalgesic effect of CE is through the inhibitory effect on inflammatory cytokines IL-1 β and TNF- α production.

Declarations

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Financial interests

The authors declare they have no financial interests.

Conflicting interests

The authors have no relevant financial or non-financial interests to disclose

Author contribution

All authors contributed to the study conception and design. Material preparation and data collection and analysis were performed by [BehroozKhakpourTaleghani] and [Aria Haji Rasouli]. The first draft of the manuscript was written by [Aria Haji Rasouli,] and all authors read and approved the final manuscript.

Data Availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request

Ethics approval

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of University by Guilan University of Medical Sciences (Date: 2017-5-24 / No: IR. GUMS. REC. 1396.336)

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Figures

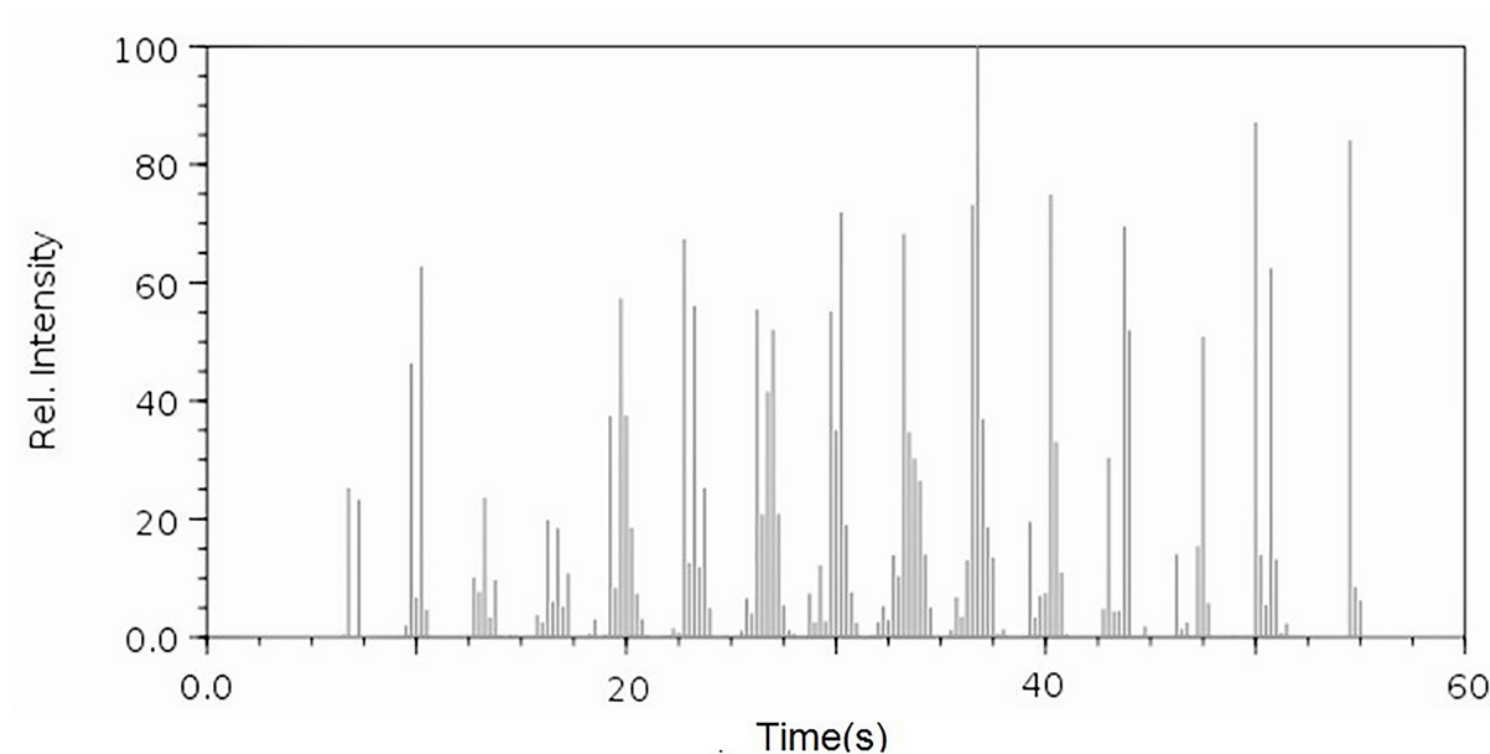


Figure 1

GC-MS chromatogram of CE hydro-alcoholic extract

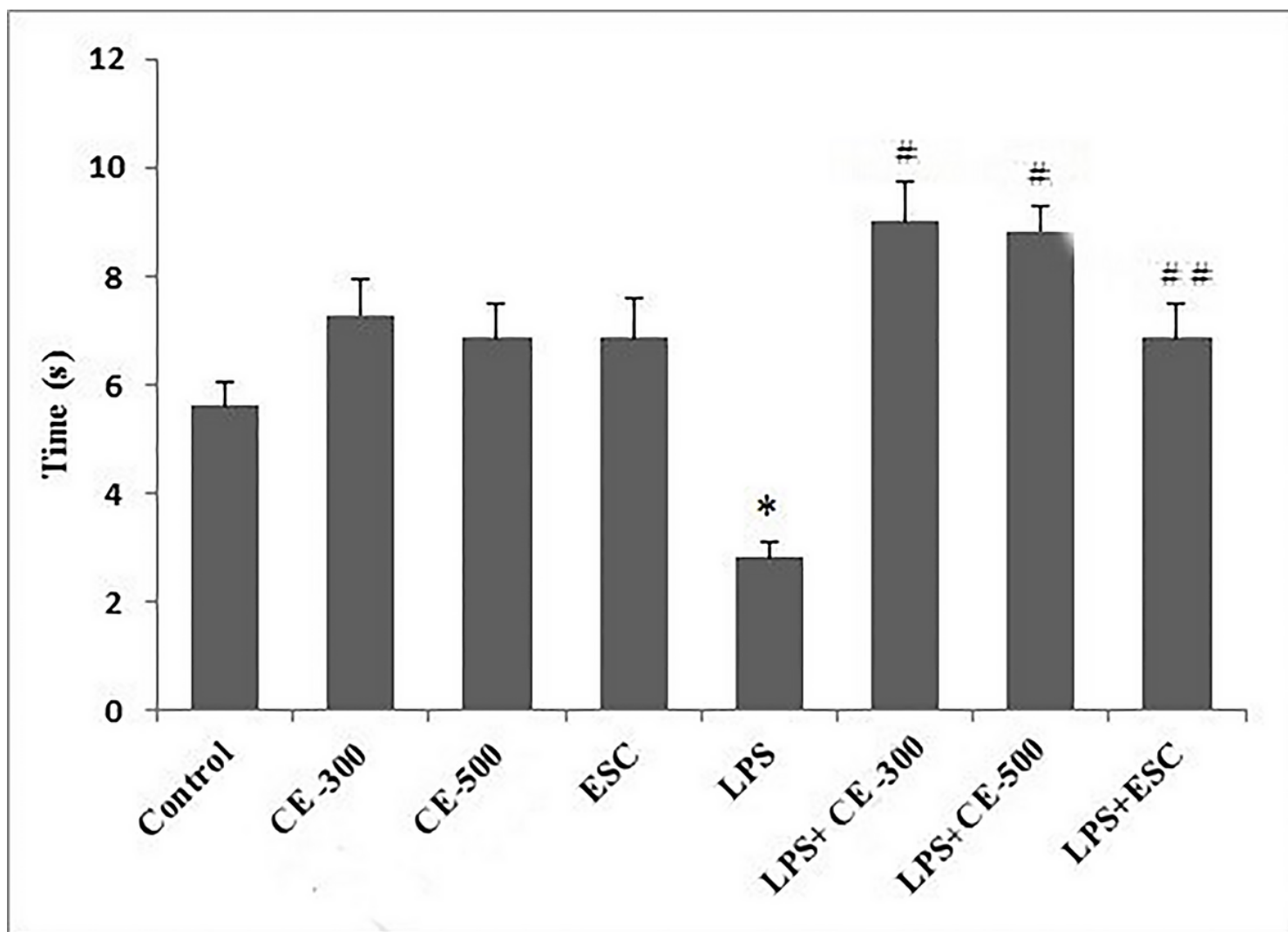


Figure 2

Effect of chicory root extract and esculetin on hot plate reaction time (n=8). Values are expressed as mean $\pm$ standard error of the mean. * $p < 0.05$ compared to control group, # $P < 0.0001$ and ## $P < 0.001$ compared to LPS group. LPS: Lipopolysaccharide. ESC: Esculetin. CE: Chicory root extract

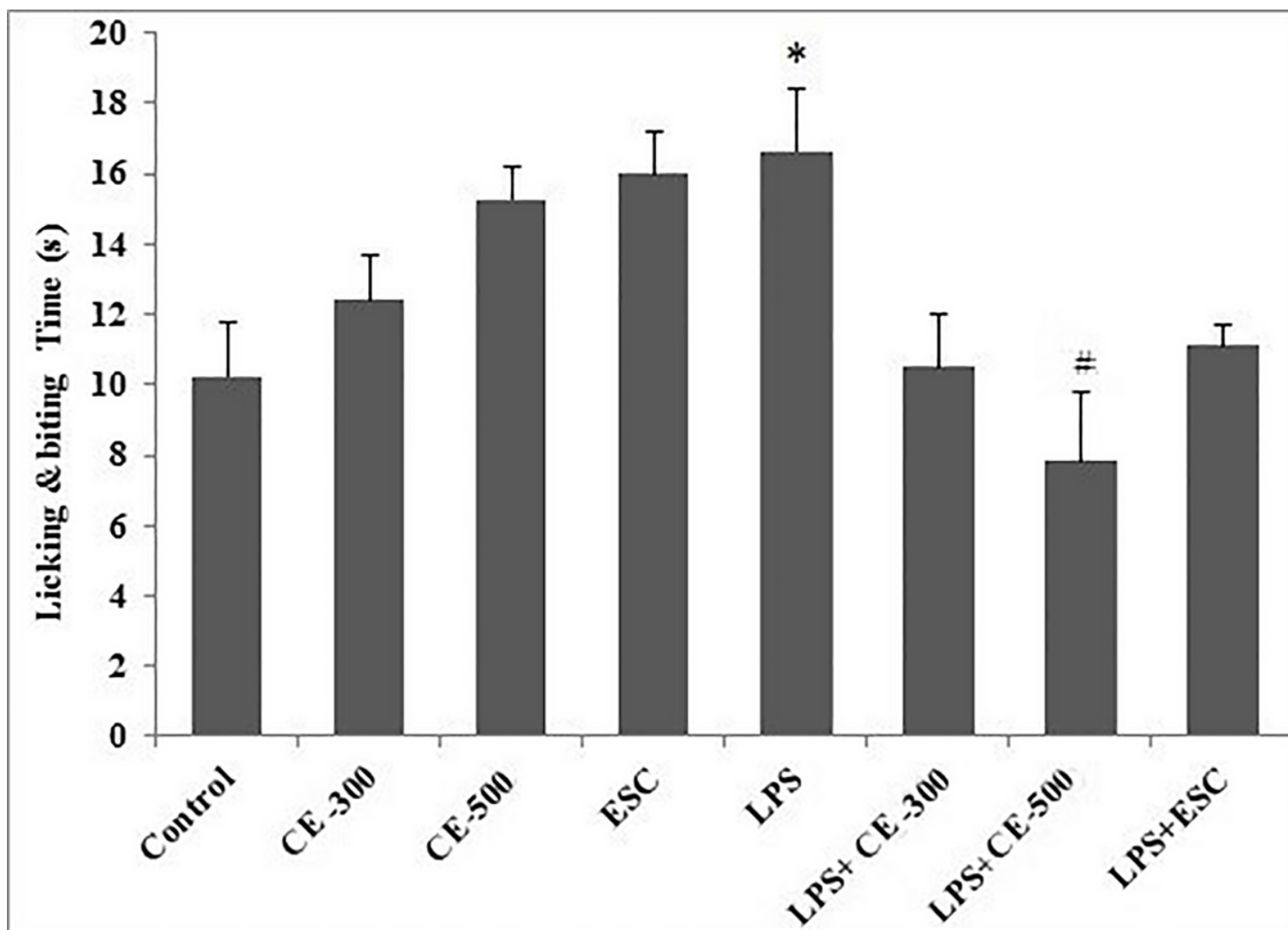


Figure 3

Effect of chicory root extract and esculetin on the first phase of formalin test (n=8). Values are expressed as mean $\pm$ standard error. * $p < 0.05$ compared to control group, # $P < 0.01$ compared to LPS group. LPS: Lipopolysaccharide. ESC: Esculetin. CE: Chicory root extract

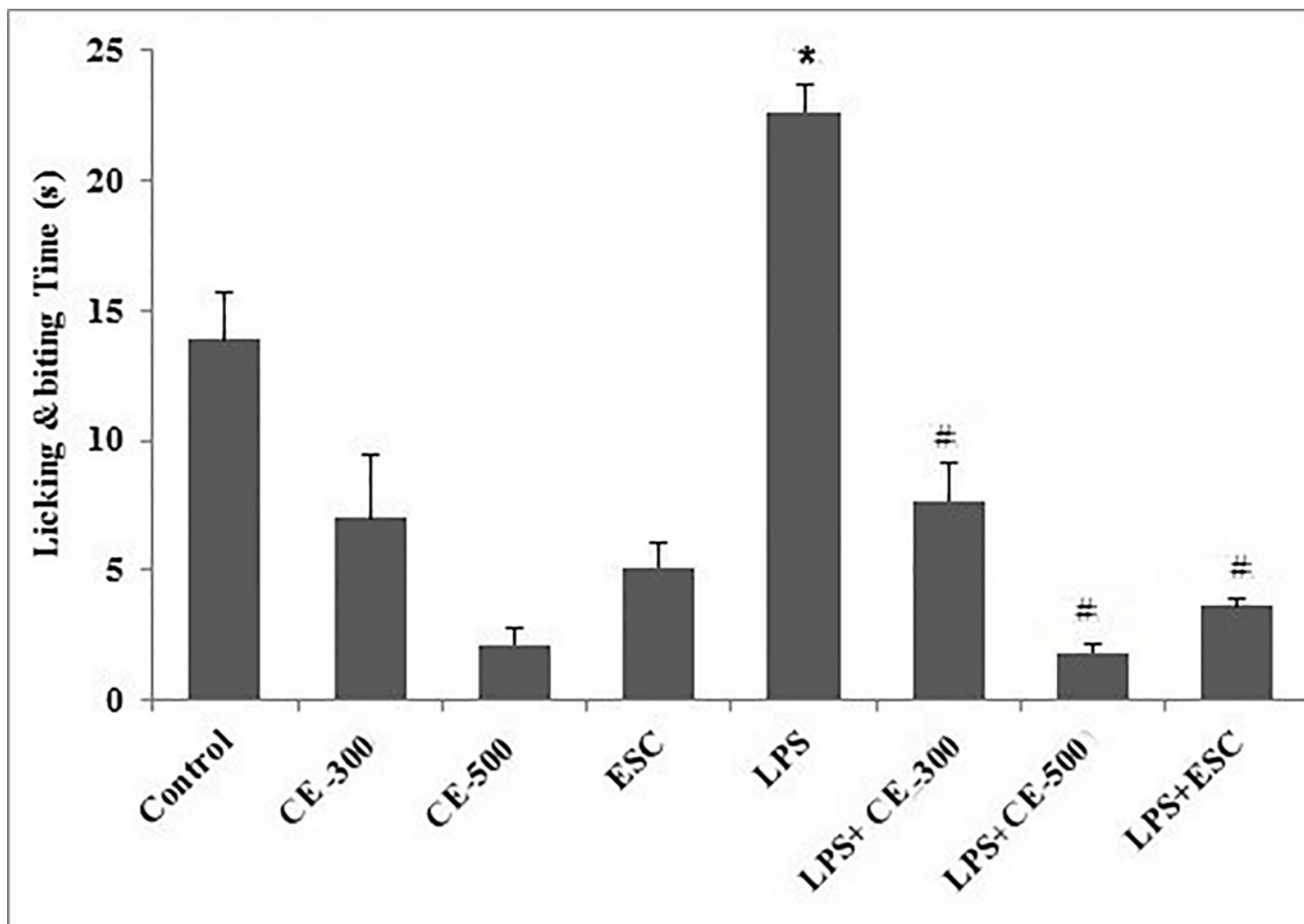


Figure 4

Effect of chicory extract and esculetin on the second phase of formalin test (n=8). Values are expressed as mean $\pm$ SE. * $p < 0.001$ compared to control group, # $p < 0.0001$ in comparison with LPS group. LPS: Lipopolysaccharide. ESC: Esculetin. CE: Chicory root extract

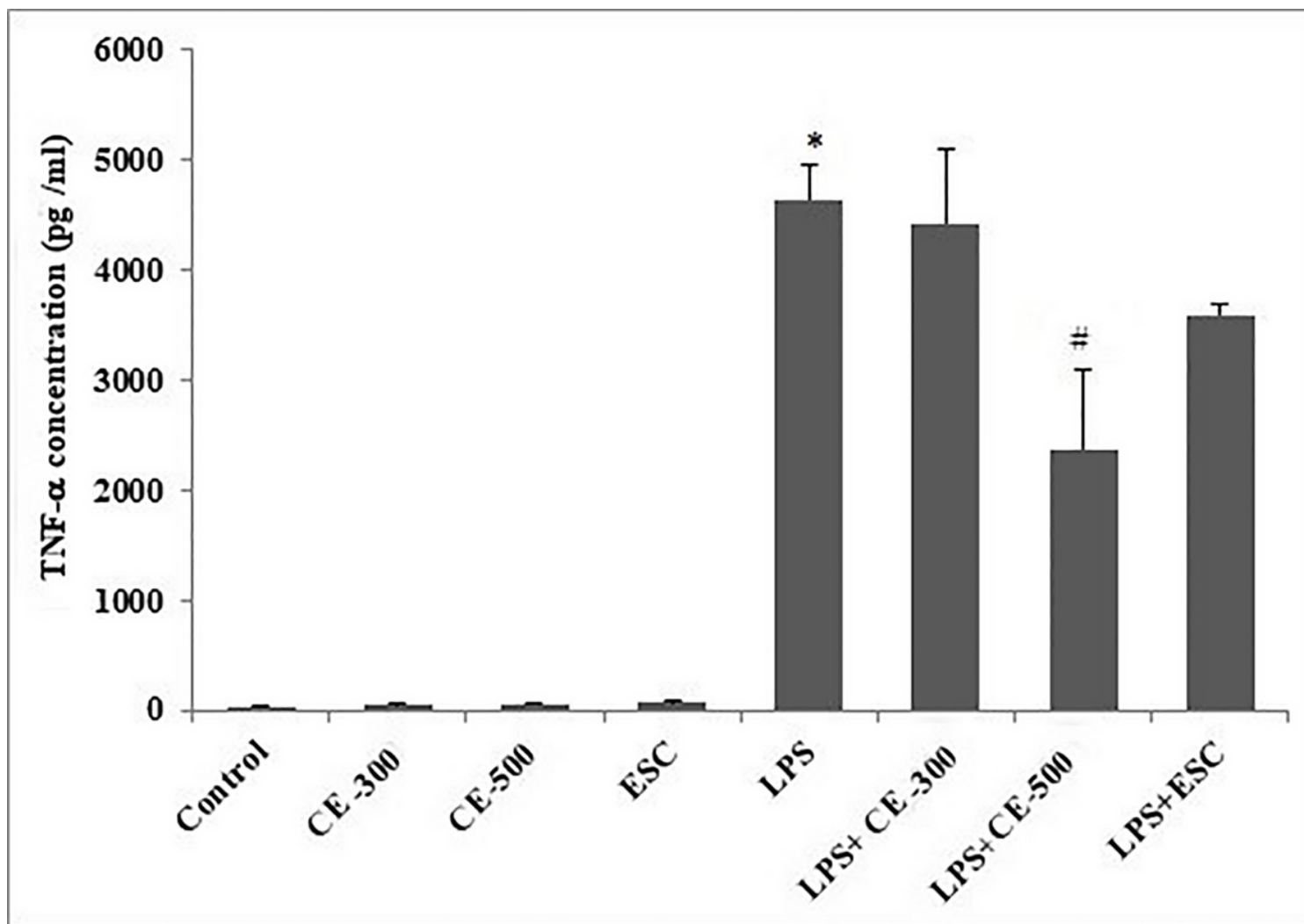


Figure 5

Effect of chicory root extract and esculletin on serum TNF- α concentration (n=8). Values are expressed as mean $\pm$ standard error. * $p < 0.0001$ compared to control group, # $p < 0.001$ in comparison with LPS group. LPS: Lipopolysaccharide. ESC: Esculetin. CE: Chicory root extract

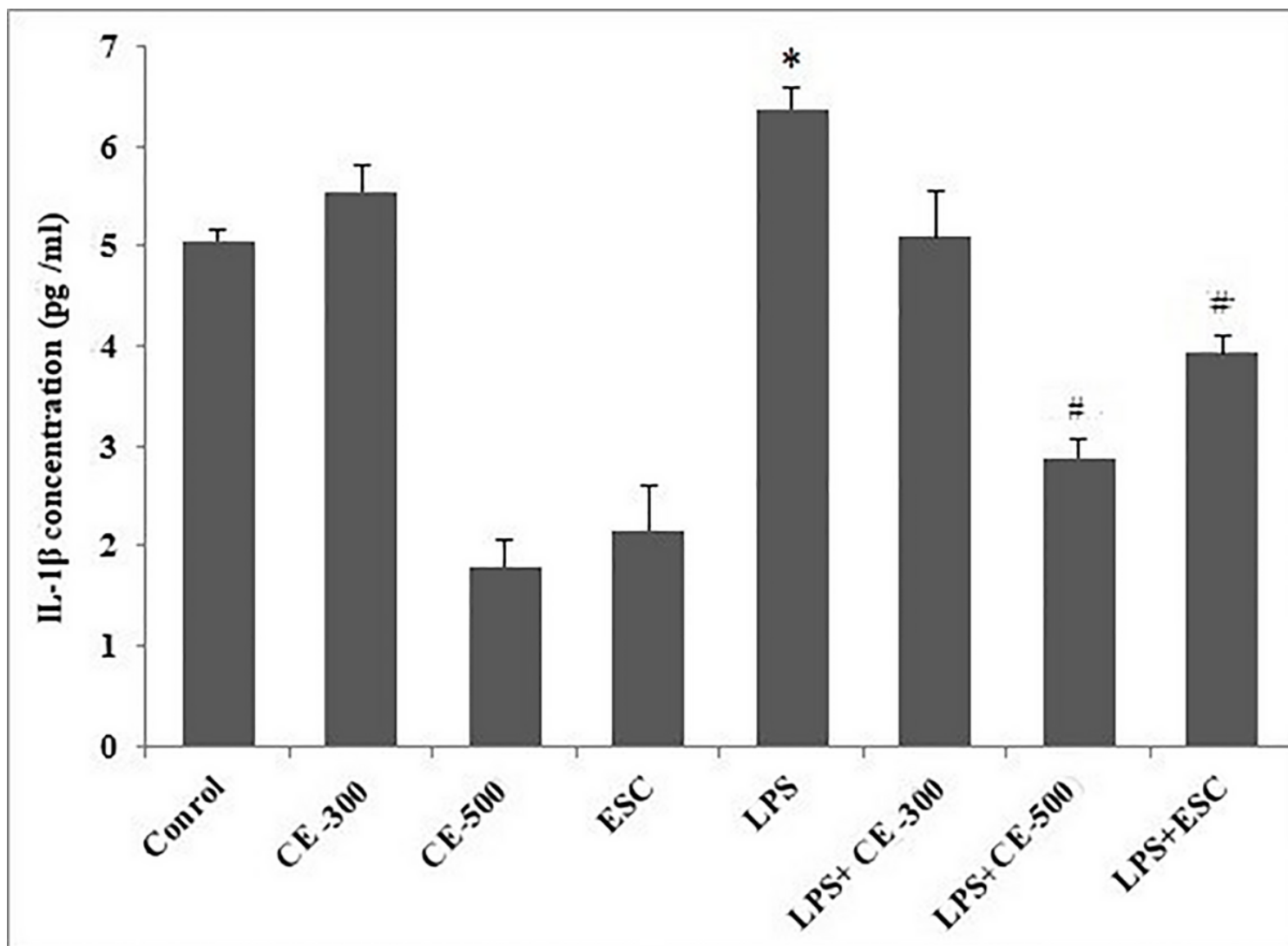


Figure 6

Effect of chicory root extract and esculetin on serum IL-1β concentration using ELISA test (n=8). Values are expressed as mean $\pm$ SE. * $p < 0.05$ compared to control group, # $p < 0.001$ in comparison with LPS group. LPS: Lipopolysaccharide. ESC: Esculetin. CE: Chicory root extract