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Extract of *Oenothera biennis* L. stem inhibits LPS-induced inflammation by regulating MAPK and NF-κB signaling pathways

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Abstract: *Oenothera biennis* L. is a perennial herb distributed across America, Asia, and Europe. The pharmacological effect of *Oenothera biennis* L. stem is poorly understood. We demonstrated that lipopolysaccharide (LPS)-induced the high production of inflammatory mediators nitric oxide (NO) and prostaglandin E_2 (PGE_2), and the pro-inflammatory cytokines tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IL-1β in peritoneal macrophages (PMs) were significantly inhibited by the crude extract The inflammation related signaling extra cellular signal-regulated ERK, P38 of MAPK and NF-kappaB (NF-κB) activated by LPS dramatically inhibited. In conclusion, our results suggested that the stems of *Oenothera biennis* L.possess a high anti-inflammatory property, thus, can be used in the industrial production of medicinal products as the raw material in the future.

Keywords: *Oenothera biennis* L. stems, anti-inflammation, MAPK, NF-κB, resource utilization.

INTORDUCTION

Oenothera biennis L. (*O. biennis*) is a perennial herb distributed across America, Asia, and Europe (Bayles *et al.*, 2009), which contains kinds of constituents like fatty acids, steroids, terpenoids, flavonoids, and tannins (Srivastava *et al.*, 1998). At present, different parts of the *O. biennis* plant are used for edible and medicinal purposes, for instance, the leaves have been converted to tea or directly eaten (Zhang, 2006), roots applied to make a poultice for treating diseases (Church, 2006), and seeds used for the extraction of abundant quantities of bioactive compounds (Wettasinghe *et al.*, 2002). To date, the oil extracted from *O. biennis* seeds has been paid the most attention and used as dietary supplement for more than 40 years because of its high concentration of γ-linolenic acid (Hudson, 1984).

Meanwhile, the oil exerts medicinal effects, including high anti-inflammatory activity, and prevents and treats various diseases, such as rheumatoid arthritis (Brzeski *et al.*, 1991), atopic dermatitis (Schäfer *et al.*, 1991), and premenstrual syndrome (Cerin *et al.*, 1993). However, the leaves and stems of *O. biennis* after seeds harvesting are considered as the non-medicinal parts, and are used as a raw material in the textile and paper industries (Zhong, 2004).

During product production or cultivation of medicinal plants, the waste is unavoidably generated and often abandoned. Studies have reported that wastes formed during processing of plant products can be utilized as reusable resources due to rich biofibers, multivitamins, minerals and other chemical elements in wastes. Thus, the pomaces of olives (Cioffi *et al.*, 2010), apples (Juśkiewicz *et al.*, 2012), and grapes (Jara-Palacios *et al.*, 2015) have been used as raw materials in the production of pharmaceutical products and industrial materials (Liu *et al.*, 2006). On the other hand, the non-medicinal parts of the plants have been found to possessing potential medicinal values. For example, the roots of *Rheum officinale* (*Polygonaceae*) and *Scutellaria baicalensis* (*Lamiaceae*) are the main medicinal parts, but the stem of these species was found to containing bioactive compounds such as anthraquinones in *R. officinale* (Tan *et al.*, 2012) and flavonoids in *S. baicalensis* (Miao *et al.*, 2014) For *Dendrobium officinale*, the stems are generally used and roots are discarded. However, the root extract of *D. officinale* was found to possessing a hypoglycemic effect on mouse models of type II diabetes (Mi *et al.*, 2015). These findings indicate that plant organs except main application parts also have the potential medicinal value in various plant species, including *O. biennis* stem, thereby suggesting that the comprehensive utilization of *O. biennis* stems has a critical significance for saving resources and protecting the environment.

To date, studies on medicinal effects of *O. biennis* have focused on seed extracts, but for the stems are limited (Granica *et al.*, 2013). In general, the stems of oil plants accounts for more than 50% of the biological yield, these abundant parts are always discarded resulting in the environmental destruction and resource waste. Hence, to use the resource sufficiently, we attempted to verify the pharmacological property of the *O. biennis* stem. In this study, the anti-inflammatory property of *O. biennis* stem extract on lipopolysaccharides (LPS)-induced inflammation were investigated by using *in vitro* methods to provide a reference for the efficient use of *O. biennis* resources.

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MATERIALS AND METHODS

Chemicals and reagents

The fetal bovine serum (FBS) and DMEM were procured from Gibco Co. (NY, USA). NO and PGE_2 enzyme-linked immunosorbent assay (ELISA) kits were procured from Elabscience Biotechnology Co., Ltd. (Wuhan, China). The TNF-α, IL-6 and IL-1β ELISA kits were procured from BD Bioscience (SD, USA). SyBr and PrimeScript™ PT reagent Kit was obtained from TaKaRa (Shiga, Japan). The phospho-p38 (p-p38), p-ERK, p-JNK and β-actin antibodies were procured from Cell Signaling Technology (MA, USA). NF-κB p65 was procured from Santa cruz biotechnology (CA, USA). Alexa Fluor 594-conjugated AffiniPure Goat Anti-mouse IgG was procured from Jackson immune Research (PA, USA). Hoechst was procured from InvivoGen Co. (CA, USA). The LPS (Escherichia coli 0111: B4), adenosine triphosphate (ATP), Triton X-100 and other chemicals were purchased from Sigma Aldrich (MO, USA). The rutin, gallic acid and tannic acid were purchased from Maya reagent (Zhejiang, China).

Preparation of O. biennis extracts

The dry entire plants of *O. biennis* were purchased from Haozhou Dadetang Pharmaceutical Co. (Bozhou, China), which were authenticated by Dr. Xueli Quan, a taxonomist from Yanbian University and a voucher specimen of the plant was deposited at the key laboratory of Natural Resources of Changbai Mountain & Functional Molecules, Ministry of Education (YBU-0010). The stems and seeds of *O. biennis* were separately collected and extracted. For extraction, the sample of stem or seeds (50g) was ground with a mortar and pestle and extracted thrice with 70% ethanol under reflux for 24h. The extracted solution was filtered and evaporated at 40°C; the recovery rate reached 4.9%. The extracts were lyophilized and stored at -20°C for further use.

Animals

C57BL/6 (21-24g, 6 weeks old) were obtained from the Changchun Yisi Experimental Animal Co. (Changchun, China) [SPF, SCXK (J) 2016-0003]. The mice were housed in an experimental animal room under 23°C ± 3°C, humidity of 50 %-65 %, and a 12h light/dark cycle. All experiments were conducted in accordance with the guidelines of the Yanbian University Experimental Animal Center.

Preparation of obtain peritoneal macrophages (PMs)

The method of used C57BL/6 to obtain peritoneal macrophages (PMs) has been described in previous (Han *et al.,* 2018). Briefly, mice intraperitoneally injected with 4% thioglycolate medium 4 d before sacrifice, then used PBS to collect PMs and cultured with DMEM.

Cell viability on PMs

Cell viability was determined as previously described (Han *et al.,* 2018). The optical density value was measured at 540 nm by using a spectrophotometer. The cell viability of the treatment groups was compared with the control group.

Determination of pro-inflammatory mediators

The methods of determined have been described in previous (Han *et al.,* 2018). Briefly, used various concentrations (12.5, 25, and 50μg/mL) of stem and seeds extracts to incubated PMs for 1h and LPS (0.1μg/mL) stimulation for various hours. And for IL-1β, PMs were treated with LPS for 3h, extracts for 1h and 5mM ATP 1h. Levels of NO, PGE_2, IL-6, TNF-α and IL-1β were measured following the manufacturer's instructions.

The method of determined the mRNA expression of TNF-α, IL-6 and IL-1β by quantitative PCR (qPCR) assay has been described in previous (Han *et al.,* 2018). Briefly, used various concentrations (12.5, 25, and 50μg/mL) of stem and seeds extracts to incubated PMs for 1h and LPS (0.1μg/mL) stimulation for 3 h. RNA was extracted using TRIzol and reverse-transcribed into cDNA, and subjected to qPCR. The primers were purchased from Sangon Biotech (Shanghai, China) (table 1).

Determination of iNOS, COX-2, and MAPK-molecules expression by Western Bloting assay

The methods of determined the iNOS, COX-2, and MAPK-molecules expression by Western Blotting have been described in previous (Han *et al.,* 2018). Briefly, PMs were treated with various concentrations (12.5, 25, and 50μg/mL) of stem and seeds extract for 1h, followed by LPS (0.1μg/mL) stimulation for different time. The cell lysis were obtained for further determination.

Immunohistochemistry

PMs were seeded in 48-well plates (1.0×10^5 cells/well) and 50μg/mL of stem extract were added and incubated for 1h, followed by LPS (0.1μg/mL) stimulation for 30 min for determining the nuclear translocation of NF-κB.

Table 1: Primer sequences for the real-time PCR

Target	Forward primer (5'-3')	Reverse primer (5'-3')
IL-6	AGACAAAGCCAGAGTCCTTCAGAGA	GCCACTCCTTCTGTGACTCCAGC
TNF-α	TGGGCCTCTCATGCACCACC	GAGGCAACCTGACCACTCTCCCT
IL-1β	ATGGGCAACCACTTACCTATTT	GTTCTAGAGGCTGCCTAATG
Cyclophilin	CATCCGTAAAGACCTCTATGCCAA	ATGGAGCCACCGATCCAC

Table 2: Comparison of bioactive compound contents in stems and seeds of *Oenothera biennis*

Compounds	Stems	Seeds
	Content (mg/g DW)	
Total Flavonoids	43.29±2.59	71.42±2.59
Quercetin	4.87 ±0.81	1.13±0.12
Myricetin	4.35 ±0.64	1.84±0.17
Epicatechin gallate	6.79±0.13	4.32±0.32
Quercetin-3-O-glucoside	2.45±0.11	2.72±0.11
Kaempferol-3-O-rutinoside	5.03±0.14	6.90±0.53
Total polyphenols	35.66±1.86	49.23±0.83
Total tannins	0.99±0.04	1.82±0.02

Data are the mean ± standard error (n=3)

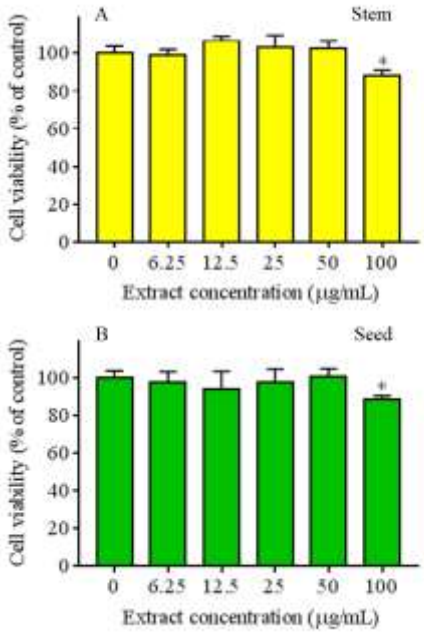

Fig. 1: Effect of *Oenothera biennis* stem (A) and seed (B) extracts cell viability of PMs. Data represents the mean ± standard error of three replicates. Differences between mean values were assessed by Student's *t*-test.*p<0.05 versus the control group untreated with extract (0μg/mL).

PMs were fixed and processed for immunostaining as described previously (Liberatore, Jackson-Lewis *et al*., 1999).

Determination of bioactive compound contents

The contents of total flavonoids, polyphenols and tannins of *O. biennis* stem and seed samples were determined by the spectrophotometry. The contents of flavonoid monomers (quercetin, myricetin, epicatechin gallate, quercetin-3-O-glucoside and kaempferol-3-O-rutinoside) were determined by the high performance liquid chromatography (HPLC). The method of total flavonoid (Piao *et al*., 2017), total polyphenol (Piao *et al*., 2017), total tannin (Sze-Tao *et al*., 2001) contents and five flavonoids (Piao *et al*., 2017) determination was described in previously.

STATISTICAL ANALYSIS

Data of the *in vitro* experiment were expressed as mean ± standard error of three independent experiments in replicates. Statistical significance of differences was assayed by Student's *t*-test (GraphPad Prism 5, Graphpad Software, Inc., CA, USA). Data with p value <0.05 were considered as significant.

RESULTS

Effects on in vitro anti-inflammatory property

The non-toxic concentrations of extracts were selected by determining the cell viability. Fig. 1 shows that the concentrations of extracts from 0μg/mL to 50μg/mL have no effect on cell viability, but significantly decreased after

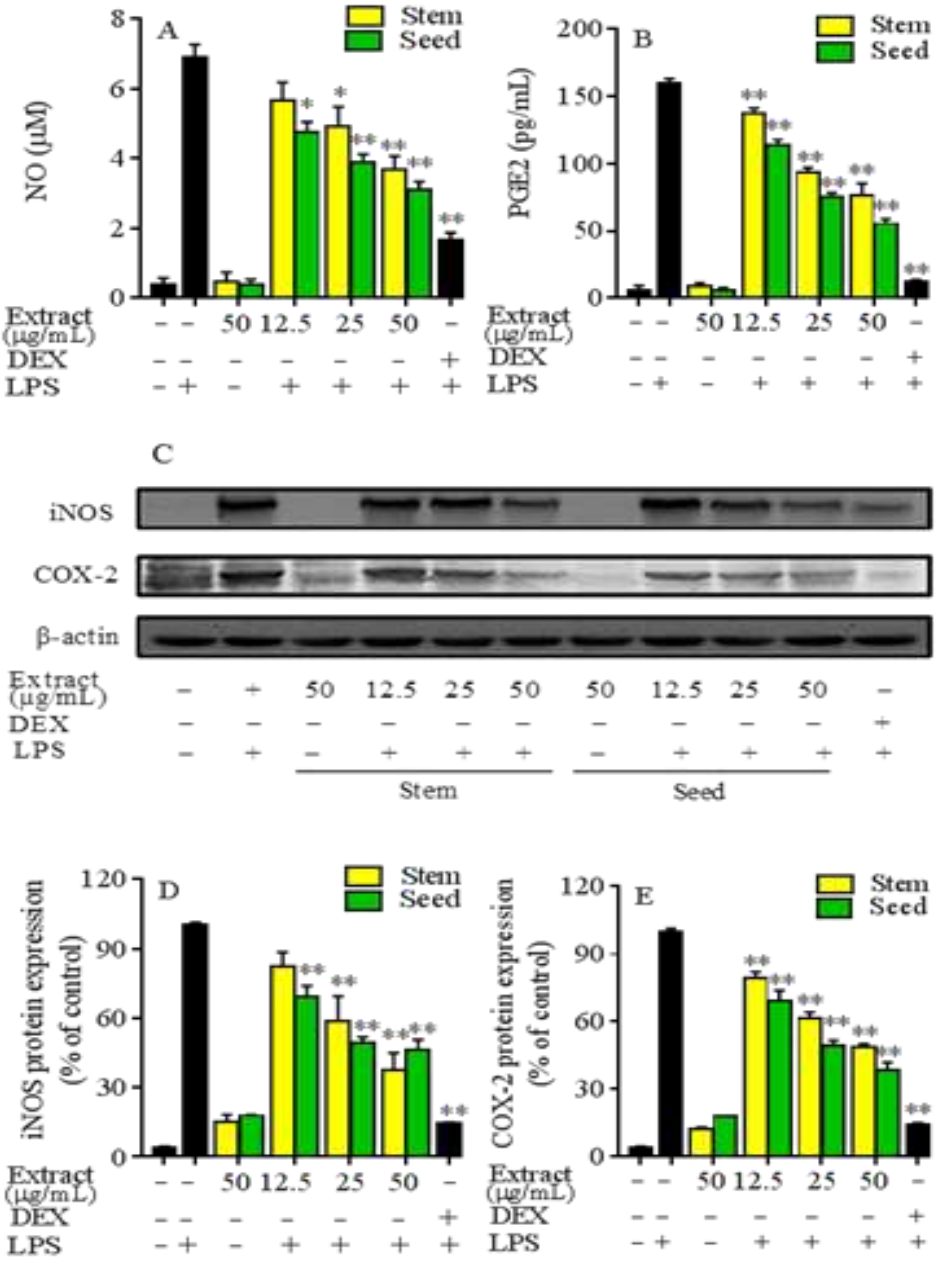

Fig. 2: Effects of different concentrations of *Oenothera biennis* stem and seed extracts on the induction of NO, PGE_2 and their synthetases (iNOS and COX-2) in LPS-induced PMs. (A) NO production. (B) PGE_2 production. (C) Western blot analysis. (D) Relative expression of iNOS. (E) Relative expression of COX-2. Data represents the mean ± standard error of three replicates. Differences between mean values were assessed by Student's *t*-test.**p*<0.05 and ***p*<0.01versus the LPS treatment alone.

treatment with 100μg/mL. This result suggests that both extract concentrations lower than 50μg/mL are not cytotoxic. Therefore, both extracts of 12.5, 25 and 50μg/mL were used for the present experiment.

The inhibitory effect of stem extract on information of pro-inflammatory mediators was compared with the seed extract. Fig. 2 shows both extracts of stems and seeds all affected the induction of NO, PGE_2 and their synthases (iNOS and COX-2). Although the inhibitory effect of the stem extract on NO (fig. 2A) and PGE_2 (fig. 2B) formation was lower than that of the seed extract, notably decreased trend was determined after the treatment of stem extract at 25 & 50μg/mL for NO and that at all con-

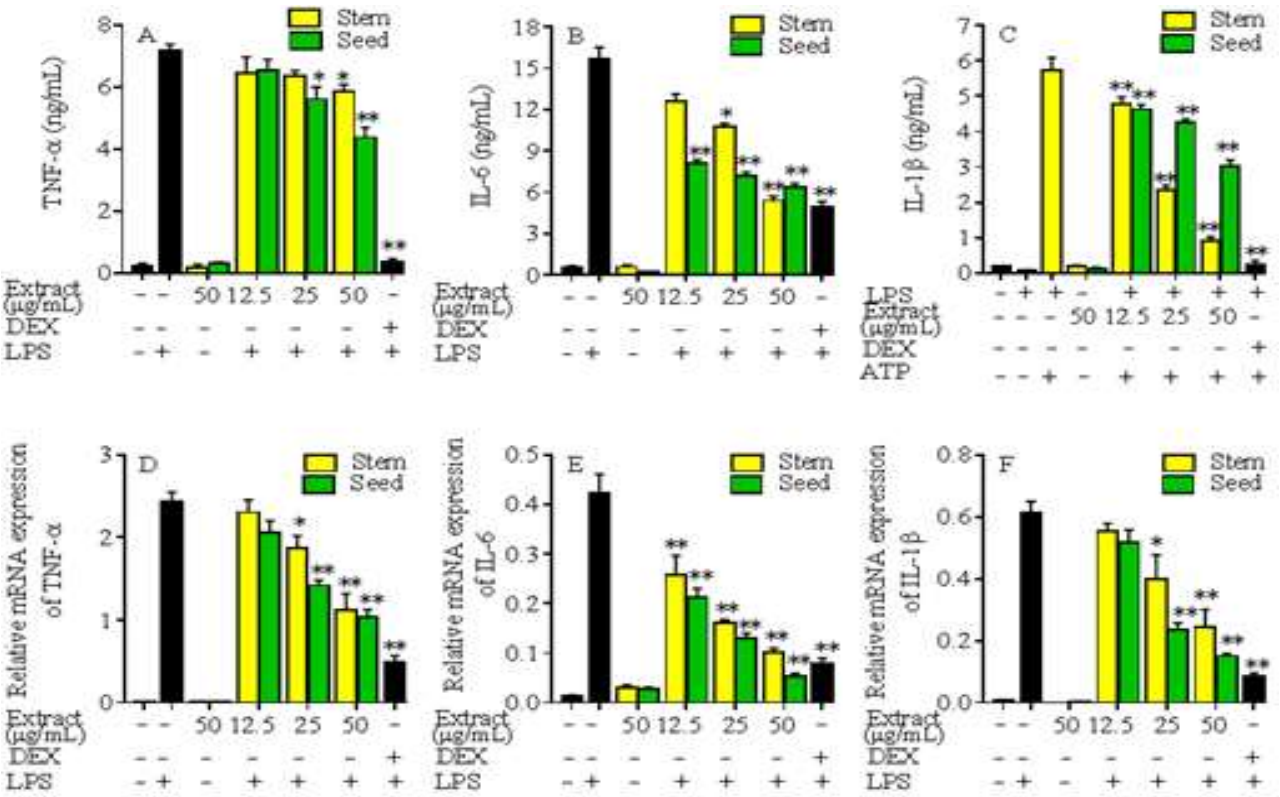

Fig. 3: Effects of different concentrations of *Oenothera biennis* stem and seed extracts on pro-inflammatory cytokines in LPS-induced PMs. A, B and C indicate the section of TNF-α, IL-6 and IL-1β determined by ELISA assay, respectively. D, E and F indicate the mRNA expression of TNF-α, IL-6 and IL-1β determined by Real-time PCR assay, respectively. Data represents the mean ± standard error of three replicates. Differences between mean values were assessed by Student's *t*-test.**p*<0.05 and ***p*<0.01versusthe LPS treatment alone.

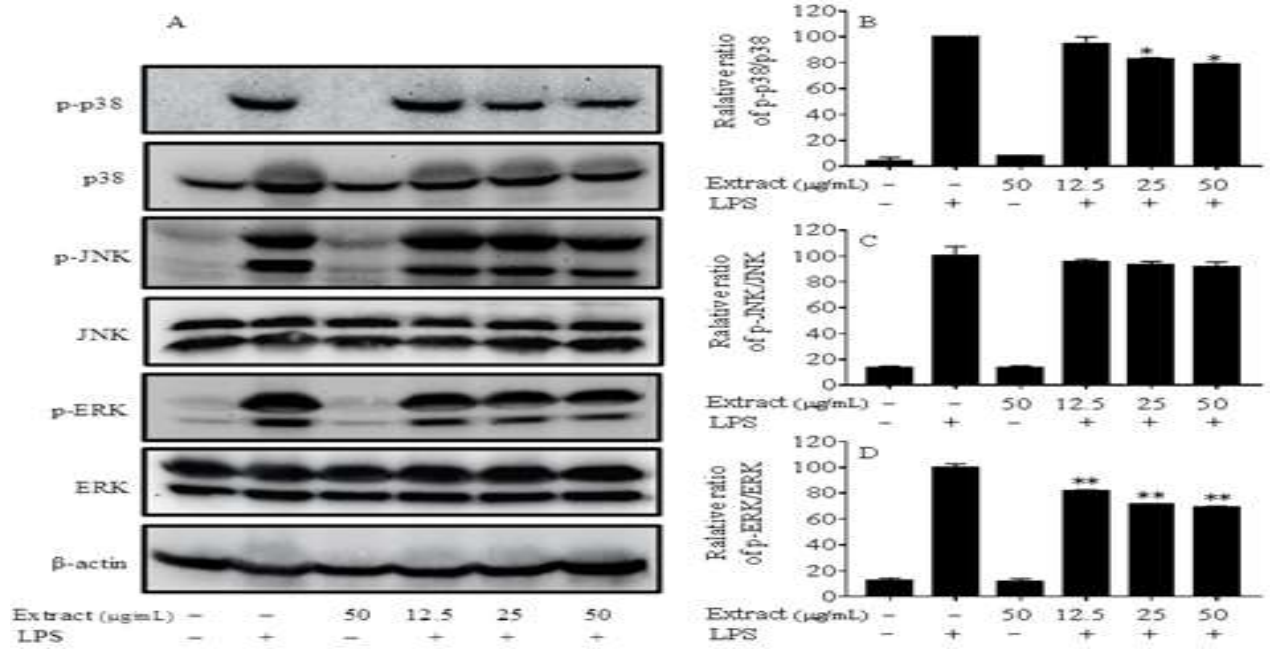

Fig. 4: Effects of different concentrations of the stem extract on the expression of phosphorylated MAPKs-moleculesin LPS-induced PMs. (A) Western blot analysis. (B) Relative ratio of p-p38/p38. (C) Relative ratio of p-JNK/JNK. (D) Relative ratio of p-ERK/ERK.

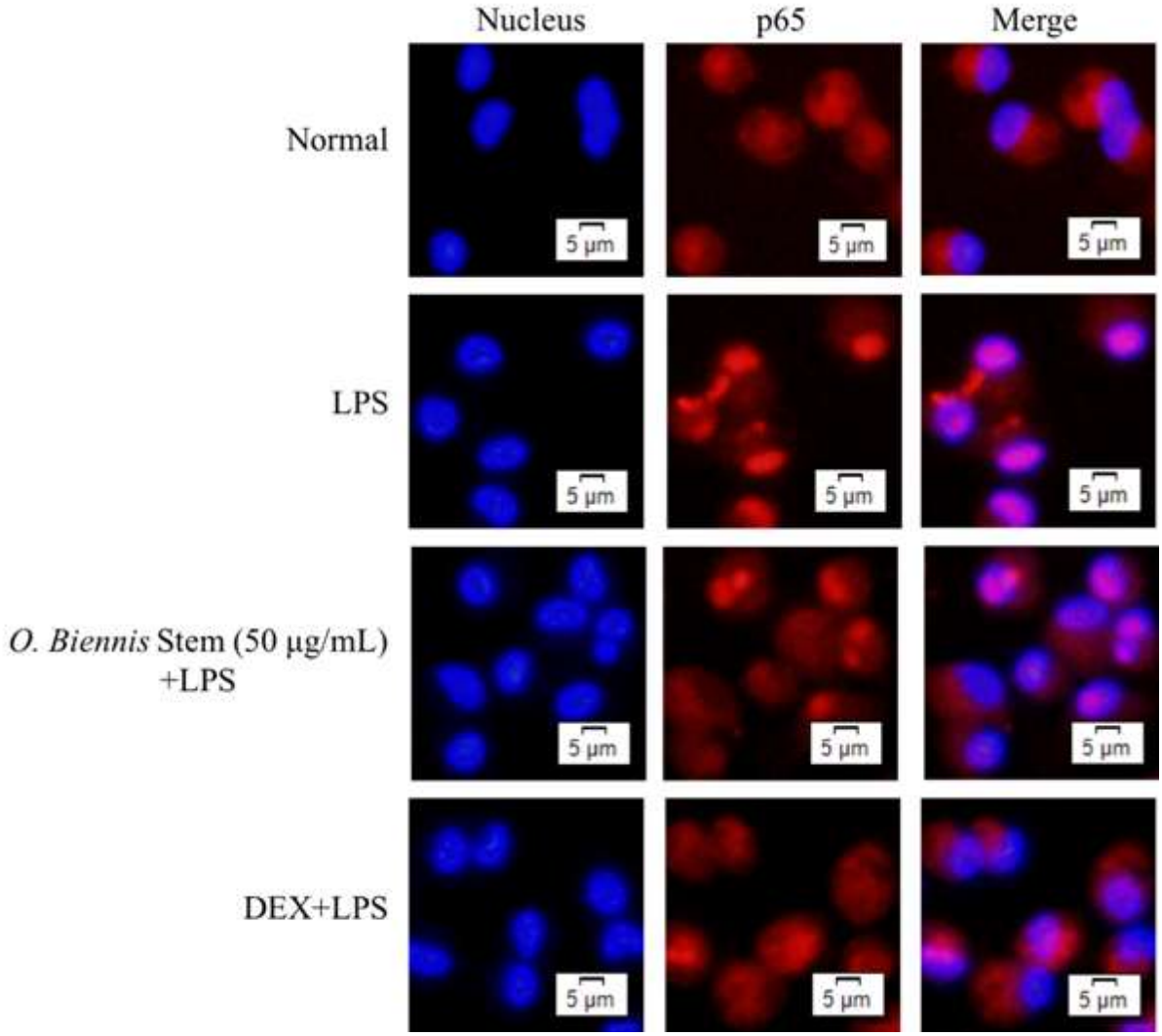

Fig. 5: Effects of stem extract on nuclear translocation of NF-κB.

centration for PGE_2 and in a dose-dependent manner. The extracts also inhibited the induction of the iNOS and COX-2 (fig. 2C). The relative expression of the iNOS significantly decreased at all concentrations of the stem extracts (fig. 2D), whereas the obviously decreased expression was found at both concentrations of 25 and 50 g/mL for the COX-2 (fig. 2E).

A high inhibitory effect on the TNF-α, IL-6 and IL-1β was determined in seeds and also in stems (fig. 3). Stem extracts at varying concentrations differently affected the formation of the pre-inflammatory cytokines, namely, the level of the TNF-α (fig. 3A) decreased only at 50μg/mL and IL-6 (fig. 3B) at 25 and 50μg/mL, but IL-1β (fig. 3C) at all the concentrations. After the stem extract treatment, the mRNA expression of the TNF-α (fig. 3D), IL-6 (fig. 3E) and IL-1β (fig. 3F) was down expressed and in dose-dependent manner. The finding demonstrates that the stem of *O. biennis* presents a high anti-inflammatory activity and is a potential resource to be developed.

To understand the effect of the stem on the expression of several proteins related to the inflammatory pathway, we investigated changes of MAPK molecules (p38, JNK and ERK) in LPS-stimulated PMs using the Western blotting assay (fig. 4). The phosphorylation degree of p38 (fig. 4A), JNK (fig. 4B) and ERK (fig. 4C) in PMs weakened after treating with the stem extract of 12.5, 25, and 50 μg/mL, which resulted in the decrease of the protein expression of p-p38, p-JNK and p-ERK. This result is well consistent with the anti-inflammatory activity determined in the present study.

It is well known that LPS-induced inflammation can regulate NF-kB signaling pathway. In this study, the effect of *O. biennis* stem extract on the nuclear translocation of NF-kB in LPS-stimulated PMs was detected to investigated whether the *O. biennis* stem extract inhibited of NF-kB pathway activation, which is closely related to the transcriptional regulation of inflammatory mediators. Fig.5 shown that *O. biennis* stem

extract can attenuate the LPS-induced translocation of NF-kB p65 subunit into nucleus which suggested *O. biennis* stem extract has strong anti-inflammation effect might also be linked through the blocking of NF-κB signaling pathway in activated PMs.

Comparison of bioactive compound content between stems and seeds

In our study, we have found that *O. biennis* stem has a good anti-inflammation effect. Many studies have demonstrated that lots of bioactive compounds in plants have significant anti-inflammatory effect, such as flavonoids (Adebayo *et al*., 2013), polyphenols (Nichols *et al*., 2010) and tannins (Mota *et al*., 1985). Thus, the present study determined these bioactive compound contents in stems and compared with seeds. Table 2 shows that a high amount of total flavonoids (43.29mg/g DW) was found in stems although that was relatively lower than in seeds. In stems, flavonoid monomer contents of quercetin, myricetin, and epicatechin gallate were higher, quercetin-3-O-glucoside was similar, and kaempferol-3-O-rutinoside was slight lower when they compared with seeds. In addition, 35.66mg/g DW of polyphenols and 0.99mg/g DW of tannins were also determined in stems, which were no difference compared with seeds (table 2). In general, the medical value of seed-removed stems of *O. biennis* is often been ignored, but the present study found that rich bioactive compounds (flavonoids and polyphenols) of the anti-inflammation presented in stems, which has important meaning for utilizing *O. biennis* resources efficiently without wasting.

SUPPLEMENT


中药材合格证
Chinese Herbal Medicine Certificate

符合 2010 年版《中华人民共和国药典》标准
Conforming to standards of Pharmacopoeia of People's Republic of China （2010 edition）

品名：月见草
Materials Name：*Oenothera biennis L.*

产地：吉林省长白山
Origin: Changbai mountain, Jilin

收获日期：2014 年 9 月
Harvest date: September, 2014

批号：14100
Lot number:14100

运销商：亳州市大德堂药业有限公司
Marketing agency: Bozhou Dadetang Pharmaceutical Co. (Bozhou, Anhui, China)

供货日期：2014 年 10 月 14 日
Delivery date：October 14, 2014

Sup.1:Chinese Herbal Medicine Certificate

Sup. 2: *Picture of Oenothera biennis* L.
The aerial part, stem and seed of *Oenothera biennis* L. (Left to right).

DISCUSSION

During the inflammation reaction, macrophages is the key role in host immune defense during infection and disease development (Schluger *et al*., 1998). The LPS is widely

used as an irritant in inflammation experiments *in vitro* and *in vivo*, which is the main pathogenic component of gram-negative bacteria. The pro-inflammatory mediators (NO, PGE_2, TNF-α, IL-6, and IL-1β) secreted by macrophages are challenged with the LPS; consequently, the inflammatory reaction and tissue injury, such as pulmonary fibrosis (Coker *et al*., 1998), atherosclerosis (Libby *et al*., 2002) and asthma (Tak *et al*., 2001) are intensified. In this study, we compared the anti-inflammatory activity of stems and seeds *in vitro* (fig. 3). After treatment of both extracts, the formation of NO, PGE_2, TNF-α, IL-6 and IL-1β were all significantly inhibited (fig. 2 and 3). The different information of pro-inflammatory mediators in both extract is match with the result of the flavonoid and polyphenol content analysis in the present study, where higher amount of total flavonoids determined in both stems and seeds. Thus the finding of our study demonstrates the anti-inflammatory activity of stems, we further investigated changes of MAPKs-related molecules (p38, JNK and ERK) and NF-κB in LPS-stimulated PMs. At present, LPS-induced MyD88-dependent (MAPKs) or -independent pathway has well confirmed (Latz *et al*., 2013). In the regulation of macrophages activation, the MAPK family plays key roles and the phosphorylation of MAPKs results in the expression of pro-inflammatory mediators. We found that the low expression of the phosphorylated-p38, JNK and ERK exerted in LPS-stimulated PMs after the treatment of the stem extract of *O. biennis* (fig. 4), which was well associate with the pro-inflammatory cytokines decrease. NF-kB pathway, which is implicated in the transcriptional regulation of inflammatory mediators. *O. biennis* stem extract can attenuate the LPS-induced translocation of NF-kB p65 subunit into nucleus which suggested *O. biennis* stem extract has strong anti-inflammation effect might also be linked through the blocking of NF-κB signaling pathway in activated PMs (fig. 5). The *in vitro* experiments sufficiently proved that *O. biennis* stems possesses a high anti-inflammatory property, and can be used in the production of related medicinal products as a raw material resource.

Flavonoids and polyphenols in plants are closely related to the inflammation and tannins, a kind of polyphenols, also possess the anti-inflammation ability. Chemical studies of *Oenothera* species have shown that flavonoids, polyphenols and tannins (oenothein A and oenothein B) contain in the whole plants (Singh *et al*., 2012). In this study, compared with tannins, large quantities of flavonoids and polyphenols were found in both stems and seeds of *O. biennis* (table 2), thereby resulting in their high anti-inflammatory activity. In addition, although the flavonoid and polyphenol contents in stems were relatively lower than that in seeds, flavonoid monomers of quercetin, myricetin and epicatechin gallate in stems were significantly higher, which was intriguing enough to warrant further studies of the anti-inflammatory property.

CONCLUSION

O. biennis is an important medicinal crop, but the stem after seeds harvesting has considered as the non-medicinal part. The experiment of anti-inflammatory property indicated that pro-inflammatory mediators (NO, TNF-α, IL-6, and IL-1β) observably reduced by the stem extract treatment and the phosphorylated p38, JNK and ERK lowly expressed, and blocking the nuclear translocation of NF-κB. Although we have demonstrated the anti-inflammatory effect of *O. biennis* stem *in vitro*, there are still more *in vivo* experiments on inflammation-related diseases should be considered for future applications. And the present study provides a reference for the stems using in the industrial production of medicinal products as the raw material in the future.

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