Anti-inflammatory activity of β -patchoulene isolated from patchouli oil in mice

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Abstract:

β -Patchoulene (β -PAE) is a tricyclic sesquiterpene isolated from the oil of *Pogostemon cablin* (patchouli oil), which has been widely used in traditional Chinese medicine for the treatment of inflammatory diseases. However, as one of the major principle of patchouli oil, the biological activity of β -PAE has not been explored so far. In the present study, the anti-inflammatory activity *in vivo*, and the underlying mechanism, of β -PAE was investigated on experimental mice models of acute inflammation, i.e. xylene-induced ear edema, acetic acid-induced vascular permeability and carrageenan-induced paw edema. The results showed that β -PAE evoked a significant dose-dependent inhibition of ear edema induced by xylene, paw edema induced by carrageenan and suppressed the increase of vascular permeability elicited by acetic acid. Histopathological analysis indicated that β -PAE could markedly decrease the cellular infiltration in paw tissue. β -PAE was also shown to significantly decrease the malondialdehyde (MDA) level and myeloperoxidase (MPO) activity in edema paw. In addition, carrageenan-induced production of some pro-inflammatory cytokines: tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), interleukin-6 (IL-6), prostaglandin E₂ (PGE₂) and nitric oxide (NO), were suppressed in a dose-dependent manner in mice subjected to β -PAE pretreatment, and it also significantly down-regulated the protein expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). Further analysis revealed that β -PAE also inhibited the translocation of nuclear factor- κ B (NF- κ B) from the cytoplasm to the nucleus and stabilize the conversion of nuclear factor- κ B α (I κ B α) level. These results provided additional chemical and pharmacological basis for the traditional application of *P. cablin* in inflammatory disorders.

Key words: β -Patchoulene; Patchouli oil; Anti-inflammation; Carrageenan; NF- κ B

1. Introduction

Inflammation is the body's common physiologic response to damage to by pathogens, toxins, and local injuries such as biological, chemical or physical stimuli (Medzhitov, 2008; Weiss, 2008), and it is characterized by the classic signs such as redness, swelling, heat and hyperalgesia (Grivennikov et al., 2010). Persistent or over inflammatory response can produce different inflammatory mediators that have insidious effects and can result in damage of tissue and organs (Medzhitov, 2008; Shin et al., 2008). Nowadays, non-steroidal anti-inflammatory drugs (NSAIDs) are among the most commonly prescribed therapeutics for the treatment of pain and inflammation (Rainsford, 2007). However, owing to the side effects such as gastrointestinal lesion, hemorrhage and peptic ulcers induced by long-term clinical usage, NSAIDs are becoming highly controversial (Egbe et al., 2016; Rainsford, 2007). Therefore, finding novel and safer anti-inflammatory drugs become extremely urgent and important. In particular, the ethnological medicinal plants have been drawing more and more attention for their less toxicities and disadvantageous effects (Sucher, 2013).

Pogostemon cablin (Blanco) Benth. (Labiatae) is an important traditional medicine in both Chinese and southeastern Asian countries such as Malaysia and India, used to relieve inflammatory disorders for thousands of years (Chen et al., 2013). The essential oil produced by *P. cablin* is thought as the most important bioactive material which contains various sesquiterpenes and hydrocarbons, such as patchouli alcohol, pogostone and β -patchoulene to produce diverse properties. Of which anti-inflammatory effect is one of the most striking biological properties of patchouli oil. In our previous investigation, patchouli alcohol (Li et al., 2011; Xian et al., 2011; Zheng et al., 2014) and pogostone (Chen et al., 2015a; Chen et al., 2015b) was demonstrated to

exert attractive anti-inflammatory activity in various animal and cell models.

To date, aiming at finding natural products with anti-inflammatory property and providing insight into the mode of action, our research group have conducted a systematic study on extracts and active component from *P. cablin* (Chen et al., 2015a; Chen et al., 2015b; Li et al., 2013; Li et al., 2014; Lin et al., 2014; Xian et al., 2011; Zheng et al., 2014). In the present work, we managed to isolate β -patchoulene (β -PAE, $C_{15}H_{24}$, the chemical structure shown in Fig. 1), one of the representative hydrocarbon sesquiterpenoids from patchouli oil, and investigated whether oral application of β -PAE could exhibit *in vivo* anti-inflammatory effect by using three common inflammatory animal models, i.e. xylene-induced ear edema in mice, acetic acid-induced vascular permeability in mice and carrageenan-induced paw edema in mice.

To further elucidate the possible anti-inflammatory action and the underlying mechanisms, the effect of β -PAE on histological alternation, oxidative indicators such as malondialdehyde (MDA) level and myeloperoxidase (MPO), inflammatory mediators such as tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), interleukin-6 (IL-6), prostaglandin E2 (PGE₂) and nitric oxide (NO), expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), and nuclear factor- κ B (NF- κ B) signaling pathway were probed in the carrageenan-induced mice paw.

2. Materials and methods

2.1. Materials and reagents

Patchouli oil (Lot.150305) was purchased from Guangzhou Baihua Flavours and Fragrances Company Ltd. (Guangzhou, China). Carrageenan (Carr) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Evans blue, xylene and Tween-80 were purchased from Sinopharm (Shanghai,

China). Indomethacin (Indo) was obtained from Huanan Pharmacy Company (Guangzhou, China).

All other reagents and chemicals used in the study were of analytical grade.

2.2. Isolation and purification of β -PAE

β -PAE was isolated from patchouli oil according to the published literature with some modification (Su et al., 2014). Briefly, the patchouli oil (700g) was placed in the round-bottom flask of fractionating tower and the pressure of this system was kept at 6 kPa. It was heated at 135.0 °C until refluxing, after that maintained at 135.0 °C for 3 h. Subsequently, the concentration of β -PAE in distillates was analyzed by gas chromatography-mass spectrometer (GC-MS) every 30 min while the temperature was kept at 135.0 °C. According to the respective distilled-off temperature, the distillates were designated into four fractions and fraction A (35.18g, containing β -PAE $\geq 80\%$) was cooled at 15 °C for 20 min. Subsequently, fraction A was subjected to silica gelcolumn chromatography eluted with petroleum ether and evaporated to dryness, the yellowish oily liquid of β -PAE was finally obtained. As shown in Fig. 2, the purity of β -PAE was found to be $>98\%$ based on the GC-MS analysis, and the chemical structure of β -PAE was identified by comparing its spectral data (GC-MS fingerprint) with those literature data published previously (Hu et al., 2006).

2.3. Animals

Male and female Kun Ming (KM) mice (18–22 g) were provided by the Medical Experiment Animal Center of Guangzhou University of Chinese Medicine, Guangzhou, China. The animals were maintained on a 12-h light/dark cycle under regulated temperature (22 ± 2 °C) and humidity ($50\pm 10\%$) and fed with forage and clean water *ad libitum*. All experimental procedures were

performed in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. The mice were allowed for 7 days in the pre-experimental period to adapt to the laboratory conditions. In all but acute toxicity experiments, animals of either sex were randomly assigned to 6 groups (n = 10) in the study. The intact and vehicle groups received intragastrically (i.g.) 0.1% Tween 80 throughout the process of the experiments. The prevention groups received (i.g.) different doses of β -PAE (10, 20 and 40 mg/kg, dissolved in 0.1% Tween 80) and indomethacin (Indo, 10 mg/kg, reference drug, dissolved in 0.1% Tween 80) respectively for a period of 7 days. All drugs were administered once daily.

2.4. Acute toxicity test

The median lethal dose (LD₅₀) of β -PAE was determined in mice according to the Chemical-Test method of acute oral toxicity of the PRC National Standard (GB/T 21603-2008) and the food security of the PRC National Standard (GB 15193.3-2014). Briefly, KM mice were randomly assigned to six groups (10 mice per group of either sex) to evaluate the acute toxicity of β -PAE after a single oral dose. The mice were administered orally with β -PAE (0, 100, 316, 1000, 3160, 10000 mg/kg) in each of the test groups. After administration, the mice were then allowed free access to forage and water *ad libitum*, and observed under open-field conditions for 2 weeks for any mortality or behavioral changes, and the number of deaths and indications of clinical toxicity within this period of time were recorded.

2.5. Xylene-induced ear edema in mice

The xylene-induced ear edema test with modifications was performed according to a previously described method (Li et al., 2016). Briefly, on the last day, all mice were administrated

152 intragastrically 60 min before induction of ear edema by topical application of 20 μ L xylene on the
153 anterior and posterior surface of the right ear. The left ear was used as a control. After another 60
154 min of xylene application, all mice were killed by cervical dislocation. Circular sections of both
155 ears were collected with an 8 mm diameter punch and were immediately weighed. The extent of
156 ear edema was evaluated by the weight difference between the right and the left ear biopsies of the
157 same animal.

158 *2.6. Acetic acid-induced vascular permeability in mice*

159 The acetic acid-induced vascular permeability test was performed according to a previously
160 described method with modifications (Yesilada et al., 1988). Briefly, 60 min after the
161 administration on the last day, each animal was intravenously injected 1% Evans blue in
162 physiological saline solution at dose of 10 ml/kg followed by an intraperitoneal injection of 0.6%
163 acetic acid at 0.1 ml/10 g body weight. 20 min later mice were killed by cervical dislocation and
164 the peritoneal exudates were collected after being washed with 5ml of physiological saline and
165 centrifuged for 15min at 550 $\times$ g. The absorbance values of supernatants were measured at 590 nm
166 wavelength using an ultraviolet-visible spectrophotometry. The vascular permeability effects were
167 expressed according to the absorbency of dyestuff that had leaked into the peritoneal cavity.

168 *2.7. Carrageenan-induced mouse paw edema model*

169 *2.7.1. Paw edema assay and the preparation of supernatants*

170 The carrageenan-induced mouse hind paw edema test was used for the assessment of
171 anti-inflammatory activity (Albano et al., 2013). Briefly, on the last day, 60 min after final
172 administration, each mouse was given a subcutaneous injection of 25 μ L of 1% freshly prepared

carrageenan suspension in physiological saline into the plantar side of right hind paw. The paw volume was measured before the injection as the basal volume (V_o) and carried on measuring at the 1st, 2nd, 3rd, 4th, 5th and 6th h after the injection as the pathological volume (V_i) using a MK101CMP plethysmometer (Muromachi Kikai Co. Ltd., Japan). The degree of swelling was calculated using the following formula: % edema degree = $(V_i - V_o)/V_o \times 100$. Percentage inhibition of swelling was presented using the following formula: % inhibition = $(\text{Mean edema degree (Carr)} - \text{Mean edema degree (test)})/\text{Mean edema degree (Carr)} \times 100$.

In the secondary experiment, another set of mice was used and the grouping and administration were the same as the above experiment. Four h after carrageenan treatment, mice were killed and the hind paws were immediately harvested. Then the hind paws were placed in cold PBS (1:9, v/w) and homogenized by using a Tissue Lyser II high-throughput tissue homogenization system (Qiagen Co. Ltd., Hilden, Germany). The homogenate was incubated on ice for 15min and centrifuged for 15min at $10000 \times g$ at 4°C . The supernatants were collected and stored at -80°C for further inflammatory factor assay.

2.7.2. Histological analysis

Histological evaluation of paw edemas was collected on mice which were not subject to supernatants collection. Tissue sections were removed and fixed with 4% paraformaldehyde for 1 week at room temperature, dehydrated and embedded in paraffin. Tissue sections were cut into 5 μm sections, then stained with hematoxylin and eosin (H&E stain) subsequently and examined with a BX60 microscope (Olympus Corporation, Kyoto, Japan). Every 3 to 5 tissue slices were randomly chosen from each group. Paw swellings and enlarged cavities of every tissue slice were evaluated ($40\times$ and $200\times$) and the images were captured with a Nikon DS-U3 (Nikon Corporation,

195 Shanghai, China).

196 2.7.3. *MDA assay*

197 MDA activity was evaluated by the method of thiobarbituric acid reacting substance (TBARS).
198 The whole experiment procedure was conducted according to the manufacturer's instructions
199 (Jiancheng Institute of Biotechnology, Nanjing, China). Briefly, MDA reacted with thiobarbituric
200 acid in the acidic high temperature and formed a red complex TBARS, which could be measured
201 by the absorbance at 532 nm.

202 2.7.4. *MPO assay*

203 The MPO assay kit (Jiancheng Institute of Biotechnology, Nanjing, China) was used to measure
204 MPO activity according to the manufacturer's recommendations. One unit of MPO activity was
205 defined as that required to degrade 1 μmol hydrogen peroxide at 37°C. MPO activity was
206 expressed as units per milligram (U/g). The enzyme activity of each reaction mixture was
207 measured at 460 nm.

208 2.7.5. *Nitric oxide assay*

209 The concentration of nitrite was measured to reflect the production of NO using a Griess
210 reagent system kit (Jiancheng Institute of Biotechnology, Nanjing, China) according to the
211 manufacturer's instructions. Briefly, the supernatant was mixed with the Griess reagent for 10 min
212 at room temperature. Nitrite products in the supernatants were measured by the absorbance at 550
213 nm with NaNO_2 being used for a standard curve. The results were expressed as $\mu\text{mol/g}$ protein.

214 2.7.6. *Measurement of TNF- α , IL-1 β , IL-6 and PGE2 by ELISA*

The levels of TNF- α , IL-1 β , IL-6 and PGE₂ in the supernatants were determined using commercially available enzyme linked immunosorbent assays (ELISA) kit (R&D Systems, Abingdon, UK) according to the manufacturer's instructions. TNF- α , IL-1 β , IL-6 and PGE₂ were determined from a standard curve. The optical densities were measured by the absorbance at 450 nm and results were expressed as pg/mg tissue.

2.7.7. Western blot analysis

Western blot analysis was performed to evaluate the effect of tested compounds on iNOS, COX-2, phosphor-I κ B α (p-I κ B α) and I κ B α protein expressions and the expressions of NF- κ B (p65) in cytosol and nucleus. Briefly, the total protein, cytosol protein and nuclear protein were extracted by using Nuclear-Cytosol Extraction Kit (Cell Signaling Technology, USA). Nuclear and cytosolic extracts were prepared and subjected to western blot analysis. Lamin B (nuclear protein) and β -actin (cytosolic protein) were used as controls, and protein concentration was determined using the BCA protein assay kit. Equal protein extracts were separated on 10% SDS-PAGE gel and transferred to polyvinyl difluoride (PVDF) membranes. The membrane was blocked with 5% skim milk, sequentially incubated with primary antibody (Cayman Co., USA) and horseradish peroxidase-conjugated secondary antibody followed by enhanced chemiluminescence detection (Amersham International plc., Buckinghamshire, U.K.).

2.8. Statistical analysis

All the data were expressed as mean $\pm$ S.E.M. with a value of $P < 0.05$ considered statistically significant, and statistical analysis was carried out using one-way analysis of variance (ANOVA), followed by Dunnett's test. All analyses were made with Statistical Product and Service Solutions

(SPSS) software (version 20.0).

3. Result

3.1. Acute toxicity

Intragastric administration of stepwise, graded doses of β -PAE in mice gave an LD₅₀ value greater than 10000 mg/kg. During the time of observation the animals did not cause any behavioral changes, and there was no convulsions and death, which indicated that the β -PAE had a relatively wide margin of safety.

3.2. Effect of β -PAE on xylene-induced ear edema in mice

As illustrated in Fig. 3A, compared with the vehicle control, pretreatment with β -PAE (10, 20 and 40 mg/kg) exerted dose-dependent inhibition on xylene-induced ear edema, with inhibition rate of 26.24% ($P < 0.05$), 40.94% ($P < 0.05$), and 45.17% ($P < 0.05$), respectively, while the positive control (10 mg/kg) significantly inhibited the ear edema by 43.3% ($P < 0.05$).

3.3. Effect of β -PAE on acetic acid-induced vascular permeability in mice

As shown in Fig. 3B, pretreatment with β -PAE (10, 20 and 40 mg/kg) significantly suppressed the increase of vascular permeability in a dose-dependent manner and the inhibitory effect of β -PAE (10, 20 and 40 mg/kg) was 36.48% ($P < 0.05$), 45.14% ($P < 0.05$), and 59.39% ($P < 0.01$), respectively. The inhibitory effect of β -PAE at the dosage of 40 mg/kg was as potent as the reference drug indomethacin.

3.4. Effect of β -PAE on carrageenan-induced paw edema in mice

As illustrated in Fig. 4, after the carrageenan treatment, the volume of paw edema was

obviously increased in a time-dependent manner and peaked at 3 h (Fig. 4A). Pretreatment with β -PAE (10, 20 and 40 mg/kg) significantly inhibited the increase of paw edema in a dose-dependent manner from the 1st to the 6th h when compared with the vehicle control (Fig. 4B). The edema of mouse paw was more significantly reduced when pretreated with β -PAE at the dosage of 40 mg/kg compared to the positive control. And the inhibition ratios of the 40mg/kg pretreatment with mice at 4, 5 and 6 h were 56.86% ($P < 0.001$), 62.63% ($P < 0.001$) and 68.00% ($P < 0.001$), respectively.

3.5. Histopathological analysis

Paw biopsies of the vehicle control showed marked tissue destruction, swelling and obvious cellular infiltration in the connective tissue compared with the intact control, and the infiltrates accumulated between collagen fibers and into the intercellular spaces (Fig. 5A and B). As for paw biopsies of mice pretreated with β -PAE and Indo, edematous condition was obviously abated and none of cellular infiltrations was shown in intercellular spaces. When compared to the vehicle control, the collagen fibers of the β -PAE and Indo group were regular in shape and showed a reduction of intercellular spaces. The inflamed cells and cellular infiltration were also decreased (Fig. 5C, D, E and F).

3.6. Effects of β -PAE on MDA and MPO levels

In Fig. 6, after the carrageenan stimulation, MDA (Fig. 6A) and MPO (Fig. 6B) levels in the paw edema increased sharply compared to the intact control ($P < 0.001$). Pretreatment with β -PAE (10, 20 and 40 mg/kg, all $P < 0.001$) and Indo (10 mg/kg, all $P < 0.001$) significantly decreased the accumulations of MDA and MPO in comparison with the vehicle control. Moreover, the

inhibitory effect of β -PAE at the dosage of 40 mg/kg was similar to Indo (10 mg/kg) at the 4th h after induction.

3.7. Effects of β -PAE on TNF- α , IL-1 β , and IL-6 levels

In this study, the levels of TNF- α , IL-1 β and IL-6 were examined to further explore the anti-inflammatory effect of β -PAE on mice treated with carrageenan. As shown in Fig. 7, the concentrations of TNF- α (Fig. 7A), IL-1 β (Fig. 7B) and IL-6 (Fig. 7C) levels increased significantly after the carrageenan treatment when compared with that of the intact control ($P < 0.001$). However, pretreatment with β -PAE (10, 20 and 40 mg/kg, all $P < 0.001$) and Indo (10 mg/kg, $P < 0.001$) significantly decreased the productions of TNF- α , IL-1 β and IL-6 in a dose-dependent manner.

3.8. Effects of β -PAE on NO, PGE₂ levels and iNOS, COX-2 protein expressions

The effect of β -PAE on the NO level and iNOS protein expression were shown in Fig. 8A and Fig. 8B, carrageenan treatment increased the level of NO and the protein expression of iNOS of the vehicle control. However, pretreatment with β -PAE (10, 20 and 40 mg/kg) strongly decreased the level of NO and down-regulated the iNOS protein expression dose-dependently. Moreover, as shown in Fig. 8C and Fig. 8D, the PGE₂ level (all $P < 0.001$) and COX-2 protein expression were observed to increase significantly after the carrageenan treatment. Pretreatment with β -PAE (10, 20 and 40 mg/kg) also significantly suppressed the PGE₂ level (all $P < 0.001$) and down-regulated the COX-2 protein expression in a dose-dependent manner, as compared to the vehicle control.

3.9. Effects of β -PAE on NF- κ B and I κ B protein expressions in mice paw edema

NF- κ B is a central molecule in the inflammatory cascade. To study whether the

anti-inflammation effects of β -PAE involved the inhibition of NF- κ B activation, we also examined the effects of β -PAE on the protein level of phosphor-I κ B α (p-I κ B α), I κ B α and NF- κ B (p65) from the cytosol to the cell nucleus translocation after activation. As shown in Fig. 9A, Fig. 9B and Fig. 9C, mice treated with Carr resulted in a significant decrease in I κ B α levels and a marked increase markedly in p-I κ B α levels. However, pretreatment with β -PAE (10, 20 and 40 mg/kg), as well as the positive control, could attenuate these effects. As shown in Fig. 9D, Fig. 9E and Fig. 9F, NF- κ B (p65) just distributed in the cytosol in intact control. When stimulated by Carr, NF- κ B (p65) level increased in cell nucleus. However, administration of β -PAE (10, 20 and 40 mg/kg) inhibited the translocation of NF- κ B (p65) from the cytosol to the nucleus by the stimulation of Carr in a dose-dependent manner compared with the vehicle control. These results suggest that β -PAE administration could significantly inhibit the activation of NF- κ B.

4. Discussion

Modern pharmacological researches on patchouli oil and *P. cablin* were predominantly focused on the major component patchouli alcohol and pogostone, seldom endeavor has been made to explore the biological effects of other sesquiterpenes. Therefore, whether another major sesquiterpenes β -PAE harbored anti-inflammatory potential remained poorly understood. Despite its relative abundance in patchouli oil, difficulties in access and supply has hampered studies on its biological properties, largely due to the complexity of sesquiterpenes contained therein and the lack of efficient and practical methods in separating this sesquiterpenes. In our present study, the feasible and suitable method for the isolation of β -PAE from patchouli oil has been achieved, therefore making it possible to explore the possible pharmacological property of β -PAE. Therefore, further attempt was made to investigate whether the isolated sesquiterpenoids β -PAE might

contribute to the anti-inflammatory effect of patchouli oil and *P. cablin* via three common animal model of acute inflammation.

Xylene-induced ear edema in mice is a simple animal model to evaluate the level of vasodilatation and plasma extravasations of neurogenic inflammation (Li et al., 2011). In this assay, pretreatment with β -PAE (10, 20 and 40 mg/kg) led to a dose-dependent inhibition of the xylene-induced mice ear edema, suggesting that β -PAE might b attenuate vasodilatations and plasma extravasations of neurogenic inflammation. Therefore, β -PAE might produce the anti-inflammatory effect by controlling the early stage of acute inflammation in this model.

To further validate the effect of β -PAE on acute inflammation, the acetic acid-induced vascular permeability mouse model was used. Under the stimulus of acetic acid, the level of inflammatory mediators such as histamine, serotonin, bradykinin and prostaglandins (PGs) will increase, which in turn lead to vasodilatation and an increase in vascular permeability (Nardi et al., 2007). In this study, administration of β -PAE (10, 20 and 40 mg/kg) dose-dependently decreased the acetic acid-induced Evans blue dye leakage in peritoneal cavity of mice. The result indicated that the anti-inflammatory effect of β -PAE might be associated with prevention of vasodilation and inhibition of the above inflammatory mediators.

The carrageenan-induced paw edema has been commonly used as an experimental model of acute inflammation to screen the acute anti-inflammatory agents (Shukla et al., 2010). Thus, this model was used in the study and the paw edema degree was tested. Experimental data showed that pretreatment with β -PAE (10, 20 and 40 mg/kg) significantly and effectively inhibited the increase of paw edema in a dose-dependent manner after carrageenan treatment. Consistent with the above results, histopathological analysis demonstrated that pretreatment with β -PAE significantly abated

the carrageenan-induced inflammatory response in mice. Therefore, according to the anti-inflammatory effects of β -PAE on the aforementioned animal inflammation models, it is worth testing its anti-inflammatory mechanism.

Free radical generation at the site of inflammation is proposed to be the major cause of the tissue damage accompanied by many inflammatory diseases (Huang et al., 2014). MDA production is due to free radical attack on the plasma membrane and it forms covalent protein adducts with the subsequent development of oxidative stress. Thus, the inflammatory effect would result in the accumulation of MDA. MPO activity is another important index of neutrophil infiltration and the marker of oxidative stress in inflammation (Xian et al., 2012). The results demonstrated that β -PAE and Indo markedly suppressed MDA and MPO activity in inflamed tissues. In addition, it was previously reported that sesquiterpenes compounds of patchouli oil can block the signaling pathways involved in oxidative stress protect cell components from the harmful actions of free radicals (Ghantous et al., 2010; Manoj et al., 2012; Murakami, 2009). Hence, β -PAE might be one of the sesquiterpenes responsible for anti-oxidative activity of patchouli oil.

The development of carrageenan-induced paw edema commonly involved two phases through sequential release of several mediators. The first phase of edema (0–2 h) involves the release of histamine and serotonin followed by kinin and finally through bradykinin, PGs, and lysosome. The second phase (2–6 h) is closely related to the enhanced production of PGs, NO, IL-1 β , IL-6 and TNF- α , which could make the inflammatory response become worsen (Huang et al., 2011; Oka et al., 2007). These pro-inflammatory cytokines are prone to be produced in response to the inflammatory stimuli at the preliminary stage and then in turn induce the production of various

inflammatory mediators such as NO and PGs, which result in amplification of inflammation (Dinarello, 2000). In the current study, β -PAE significantly inhibited carrageenan-induced inflammatory response by acting more effectively in the second phase than in the initial phase. Furthermore, administration of β -PAE (10, 20 and 40 mg/kg) and Indo significantly decreased the concentrations of IL-1 β , IL-6 and TNF- α . Thus, another possible mechanism behind the anti-inflammatory effect of β -PAE might be closely related to the inhibition on pro-inflammatory cytokines including IL-1 β , IL-6 and TNF- α .

During inflammatory processes, the formation of PGE₂ generated by COX-2 and NO generated by iNOS is commonly recognized as inflammatory sign (Lu et al., 2012). COX-2 is the key enzyme that synthesizes PGE₂ from arachidonic acid. Serving as the chemotactic and activating factors for inflammatory cells, PGE₂ is considered to be one of the vital lipidic inflammatory mediators in the pathogenesis of inflammation. NO is also a crucial mediator contributing to the generation of inflammatory response in carrageenan-induced inflammation and it was generated via the oxidation of the terminal guanidine nitrogen atom of L-arginine by iNOS (Ye et al., 2014). Moreover, NO could stimulate COX activity and result in the exaggerated production of PGs in carrageenan-induced paw edema (Hua et al., 2008). Therefore, it can be an effective strategy to suppress these cytokines in the treatment of various inflammatory diseases. In the present study, the possible effect of β -PAE on NO and PGE₂ was investigated. The results showed that administration of β -PAE (10, 20 and 40 mg/kg) provoked remarkable inhibition on the release of NO and PGE₂ in a dose-dependent manner. To determine whether β -PAE mediated inhibition of NO and PGE₂ release was regulated at the translational level, we performed the western blot analysis for NO and PGE₂ expression. Results indicated that treatment with carrageenan

significantly up-regulated the protein levels of iNOS and COX-2, whereas this effect was suppressed by β -PAE pretreatment, indicating that β -PAE possibly exhibited the inhibitions of NO and PGE₂ production by suppressing iNOS and COX-2 expression. Therefore, the ability of β -PAE to inhibit NO and PGE₂ production and to down-regulate the expression of iNOS and COX-2 was likely to mediate, at least in part, the anti-inflammatory activities of β -PAE *in vivo*.

The NF- κ B is required for transcription of many pro-inflammatory mediators with a heterotetramer consisting of two proteins (p65 and p50), which play a significant role in the generation of acute inflammation. The ability of NF- κ B to regulate pro-inflammatory gene expression is controlled by interactions with other proteins, notably members of the I κ B family (Salminen et al., 2008). When encounters various stimuli such as IL-1 β and TNF- α , I κ B becomes phosphorylated and the I κ B form then becomes ubiquitinated and degraded by the proteasome. The phosphorylation of I κ B leads to its degradation and the subsequent translocation of NF- κ B to the nucleus where it activates a variety of genes (Lawrence, 2009). To investigate the potential role of NF- κ B in the anti-inflammatory effect of β -PAE, the phosphorylation patterns of relevant signaling molecules were examined to determine the effect of β -PAE on signaling events upstream from NF- κ B translocation, a major transcription factor controlling inflammatory gene expression. The carrageenan-mediated increase in the level of phosphorylated I κ B α was suppressed by β -PAE. The result showed that β -PAE could significant increase I κ B α levels, but inhibited markedly the carrageenan-mediated increase in p-I κ B α levels, and suppressed the translocation of NF- κ B (p65) from the cytosol to the nucleus. Therefore, these results suggest that β -PAE might protect carrageenan-induced inflammation by inhibiting the transactivation of NF- κ B via stabilization of I κ B α .

To the best of our knowledge, this is the first report investigating the anti-inflammatory property of β -PAE, and the results demonstrated that oral administration of β -PAE has potent anti-inflammatory activity in animal models of inflammation, and the observed anti-inflammatory action of β -PAE might be mediated through pro-inflammatory cytokines, down-regulate the protein expressions of iNOS and COX-2, as well as suppress the transactivation of NF- κ B. In addition, it might be associated with its anti-oxidation effect. This study also provided further experimental evidence for the folklore medicinal uses of *P. cablin* and patchouli oil to treat various ailments related to inflammatory processes. And it is worthwhile to further explore β -PAE's anti-inflammatory mechanism as well as the other pharmacological activities.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Figure legends

Fig.1. Chemical structure of β -patchoulene.

Fig.2. GC–MS total ion chromatograms for β -patchoulene. ^a: m/z, relative intensity shown in parenthesis, and the ion of relative intensity 100 was used for the quantification.

Fig.3. Effect of β -PAE on xylene-induced ear edema in mice (A) and acetic acid-induced vascular permeability in mice (B). (A) The ear edema was represented as the ear edema which was assessed as the weight difference between the right and the left ear biopsies of the same animal. (B) The capillary permeability was represented by the amount of Evans blue extruded into peritoneal cavity, which was measured by the OD of the supernatant. Data are expressed as means $\pm$ S.E.M. (n = 10), * $P < 0.05$, ** $P < 0.01$ vs the vehicle control.

Fig.4. Effect of β -PAE on the carrageenan-induced paw edema in mice. (A) Paw edema degree was represented as the mean $\pm$ S.E.M. (n = 10). (B) Suppression of paw edema (%) was represented as the ratio of the mean paw size increase of β -PAE treatment groups (%) on that of the vehicle control (%). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs the vehicle control.

Fig.5. Histopathological examinations on carrageenan-induced paw tissue swelling, edema, haemorrhage and leukocyte infiltration. (A) Intact control. (B) Vehicle control. (C) β -PAE (10mg/kg). (D) β -PAE (20mg/kg). (E) β -PAE (40mg/kg). (F) Indo (10mg/kg). The tissue sections were observed under a microscope at 400 $\times$.

561

562 **Fig.6.** Effect of β -PAE on carrageenan-induced the activities of MDA (A) and MPO (B) in mouse
563 paw edema. Data are expressed as means $\pm$ S.E.M. (n = 10), ### $P < 0.001$ vs the intact control;
564 *** $P < 0.001$ vs the vehicle control.

565

566 **Fig.7.** Effect of β -PAE on carrageenan-induced the levels of TNF- α (A), IL-1 β (B) and IL-6 (C) in
567 mouse paw edema. Data are expressed as means $\pm$ S.E.M. (n = 10), ### $P < 0.001$ vs the intact
568 control; *** $P < 0.001$ vs the vehicle control.

569

570 **Fig.8.** Effect of β -PAE on carrageenan-induced the levels of NO (A), PGE₂ (C) and iNOS, COX-2
571 expression. Protein expression of iNOS (B) and COX-2 (D) were measured by western blot assay.
572 β -Actin was used as controls. Data are expressed as means $\pm$ S.E.M. (n = 10), ### $P < 0.001$ vs the
573 intact control; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs the vehicle control.

574

575 **Fig.9.** Effect of β -PAE on carrageenan-induced the protein expression of phosphor-I κ B α (A), I κ B α
576 (B), phosphor-I κ B α /I κ B α (C) and NF- κ B (p65) in the cell nucleus (D), the cytosol(E), as well as
577 the cell nucleus/the cytosol (F) was measured by western blot assay. β -Actin and Lamin B were
578 used as controls. Data are expressed as means $\pm$ S.E.M. (n = 10), ### $P < 0.001$ vs the intact control;
579 * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs the vehicle control.

Response to Reviewers' Comments

Dear Editors and Reviewers,

On behalf of my co-authors, we thank you very much for giving us the opportunity to revise our manuscript. We appreciate editor and reviewers very much for their careful considerations and valuable comments. Those comments are all valuable and very helpful for revising and improving our manuscript, as well as the important guiding significance to our researches. We have revised the manuscript in accordance with the reviewers' comments and the checklist for style revision. The revised portion has been highlighted with red color for indication. The main corrections in the paper and the responses to the reviewer's comments are as following:

Reviewer #1:

1. Which is the name of the compounds, since appears: *P. cablin*; β -Patchoulene (β -PAE); *Pogostemon cablin* (Blanco) Benth. (Labiatae).

Response: Thanks for your comments. As indicated in the manuscript, β -Patchoulene is the test article investigated in the work, and β -PAE is the abbreviation of β -Patchoulene. It is one of the representative hydrocarbon sesquiterpenoids from *Pogostemon cablin* (Blanco) Benth., an important Chinese medicinal plant of the Labiatae family. While *P. cablin* is the abbreviation of *Pogostemon cablin* (Blanco) Benth.

2. Which means with i.g to the mice?

Response: Thank you for your questions. The term "i.g." is the common and standard abbreviation for intragastric administration, and it has now been widely used (Kim et al., 2016; Liu et al., 2016; Sartori et al., 2015). In our revised manuscript, the word "intragastrically" has been added in Page 7, line 135 for more clear expression.

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3. The discussion could be related only the present results.

Response: Thanks for your constructive suggestion. According to your recommendation, the expressions unrelated to the present results in discussion have been deleted in the revised manuscript. The deletions read as this: "Some researches demonstrate that carrageenan-induced inflammation is associated with free radicals, and the hydrogen peroxide, superoxide, prostaglandin and NO will be released after carrageenan treatment." in Page 17, lines 346-348, "IL-1 β , IL-6 and TNF- α are known to be involved in the neutrophil migration in carrageenan-induced inflammation(Lu et al., 2011)." in Page 18, lines 364-365 and "It has been proved that the generation of cytokines such as IL-1 β and TNF- α has a mutual promotion with PGs synthesis in the inflammatory diseases due to the induction of the COX-2 expression (Liao et al., 2014)." in Page 18, lines 375-377.

4. The references must be up-to-date.

Response: Thank you for your suggestion. Some old references in the manuscript have been updated in the revised manuscript. For more details, please refer to the revised manuscript shown in red.

References (up-to-date):

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Reviewer #2:

1. In Fig.3 and 4, why did authors not show the data of intact control group?

Response: We sincerely thank the reviewer for the professional comments. As shown in the manuscript, Fig.3 depicted the effect of β -PAE on xylene-induced ear edema in mice and acetic acid-induced vascular permeability in mice, while Fig.4 displayed the effect of β -PAE on the carrageenan-induced paw edema in mice. In the research of anti-inflammatory effect of candidate compounds or botanical extracts, these three inflammatory animal models are commonly used, in which the comparison of the test article and vehicle group was usually performed to investigate its possible anti-inflammatory effect, without the involvement of intact group. Since the intact animal would not give reference meaning for the potential candidate. Hence, our grouping regime was widely deemed as standard and reliable for anti-inflammatory activity screening of potential candidates, and predominantly adopted by researchers of this field. Indeed, numerous studies have evaluated the anti-inflammatory activities and mechanisms of the tested samples by comparing the treatment group (tested articles plus challenge) with the vehicle group (only challenge), seldomly employing an intact group without challenge. (Albano et al., 2013; Freitas et al., 2013; Jiang et al., 2015; Latief et al., 2015; Liu et al., 2015; Niu et al., 2015; Peng et al., 2014; Yrbas

et al., 2015) However, we sincerely appreciated reviewer's valuable comments on our experimental design, and we will pay special attention about the experimental study in our future study.

References:

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2. Authors should add vehicle group into the analysis of Fig. 4B.

Response: We sincerely thank the reviewer for this suggestion. Fig. 4B depicted the inhibition of paw edema, calculated as the ratio of treatment group to the vehicle group. That is to say, Fig. 4B represented the ratio of the mean paw size increase of β -PAE treatment groups or Indo treatment group (%) to that of the vehicle control (%). (Li et al., 2013; Mulisa et al., 2015; Wang et al., 2015) Hence, in our present work, the vehicle group was indeed embodied and not directly re-shown in Fig. 4B.

References:

Li, C.W., Wu, X.L., Zhao, X.N., Su, Z.Q., Chen, H.M., Wang, X.F., Zhang, X.J., Zeng, H.F., Chen, J.N., Li, Y.C., Su, Z.R., 2013. Anti-inflammatory property of the ethanol extract of the root and rhizome of *Pogostemon cablin* (Blanco) Benth. *TheScientificWorldJournal* 2013, 434151.

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3. Scale bar is missing in Fig. 5.

Response: Thanks for being observant and pointing this out. The scale bar has been added accordingly in Fig. 5.

4. Authors had better add the ratio of p-IκB / IκB and p65-nuclear/p65-cytosolic.

Response: We sincerely thank the reviewer for the constructive suggestion. We have been added the ratio of p-IκB / IκB and p65-nuclear/p65-cytosolic (Fig. 9C and Fig. 9F). And the Fig. 9C and Fig. 9F are as follow.

Fig.9C. Effect of β -PAE on carrageenan-induced protein expression of phosphor-I κ B α /I κ B α measured by western blot assay. Data are expressed as means $\pm$ S.E.M. (n = 10), ### P<0.001vs the intact control; *** P<0.001vs the vehicle control.

Fig.9F. Effect of β -PAE on carrageenan-induced protein expression of NF- κ B (p65) in the cell nucleus/the cytosol measured by western blot assay. Data are expressed as means $\pm$ S.E.M. (n = 10), ###P<0.001vs the intact control; ***P<0.001vs the vehicle control.

We appreciate for Editors/Reviewers' warm work earnestly, and hope that the correction will meet with approval. Should you have any questions, please don't hesitate to contact us at the address below. Once again, thank you very much for your comments and suggestions.

Best regards.

Yours sincerely,

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Guangzhou University of Chinese Medicine

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Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Intact control

Vehicle control

β-PAE 10mg/kg

β-PAE 20mg/kg

β-PAE 40mg/kg

Indo 10mg/kg

Figure 6

Figure 7

A

B

C

Figure 8

Figure 9