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Chemical Constituents of Supercritical Extracts from *Prunus yedoensis*, *Saururus chinensis*, *Zanthoxylum piperitum* and their Anti-inflammatory Activities

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Abstract: Essential oils, volatile compounds from plants, are attractive natural ingredients possessing wide range of applications in cosmetics, foods, household products and alternative medicines. In this study, the three different oils from *Prunus yedoensis* (PYE), *Saururus chinensis* (SCE), *Zanthoxylum piperitum* (ZPE) were prepared by supercritical carbon dioxide extraction and their chemical components were analyzed. In addition, their anti-inflammatory activities were investigated by measuring the inhibition of pro-inflammatory cytokines. Monoterpenes α-Pinene (22.4%), 2-β-pinene (20.1%) and camphor (13.1%) were found as the major components in PYE. In the case of SCE, sesquiterpenes δ-cadinol (22.5%) and δ-cadinene (19.7%) as well as a diterpene *trans*-phytol (13.7%) were mainly identified. The major chemical constituents of ZYE were in the order of octanoic acid (13.4%), n-heptanol (9.8%) and 1-octanol (8.1%). As a bioactivity study, the effects of PYE, SCE and ZPE on nitric oxide (NO), tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6 production in lipopolysaccharide-activated RAW 264.7 macrophages were examined. These essential oils (PYE, SCE, ZPE) were appeared to considerably suppress the production of pro-inflammatory cytokine and mediators in dose-dependent manner. The results indicate that PYE, SCE and ZPE can be useful natural agents to manage inflammatory symptoms and diseases.

Key words: Supercritical carbon dioxide, essential oils, chemical composition, *Prunus yedoensis*, *Saururus chinensis*, *Zanthoxylum piperitum*, anti-inflammation

INTRODUCTION

Supercritical Fluid Extraction (SFE) is a separation process where supercritical fluid, mostly liquid state carbon dioxide (CO_2), is used as an extracting solvent. SFE has beneficial feature over conventional techniques such as solvent extraction or steam distillation because it could eliminate a step for removal of the used organic solvent (Reverchon and De Marco, 2006). Therefore, SFE with CO_2 fluid is in increasing demand to produce high-quality extracts from plant source with medicinal properties (Ocana-Fuentes *et al*., 2010) including *Sideritis scardica* (Tadic *et al*., 2010) and *Thymus vulgaris* (Grosso *et al*., 2010).

Essential oils produced by aromatic plants are mixture of volatile compounds with characteristics of strong odour. The major chemical components in the oils are terpenoids, diverse class of natural compounds with basic skeleton of isoprene. Identification of the individual chemical constituents in the essential oil could be accomplished by the use of several techniques. One of the most popular methods is the use of gas chromatography-mass spectrometry (GC-MS), where identification of specific compounds in an essential oil is achieved through comparison of their relative retention times/indices as well as their fragmentation peaks in mass spectra.

Although, essential oils which are commonly used as fragrance, are already in rampant use, the renewed interest in natural products has made it imperative to study the different modes of biological action in order to identify new applications of these products in human health care. Studies in both industrial and academic fields are increasingly focusing on medicinal and aromatic plants and their biological properties which could be used to treat skin disorders (Kim *et al*., 2008). Therefore, in our search for commercially useful essential oils, we selected *Prunus yedoensis*, *Saururus chinensis* and *Zanthoxylum piperitum* as candidate plants in this study. *Prunus yedoensis* (Rosaceae) is a medium-sized

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deciduous tree known as Yoshino Cherry. It blooms in April and its flower displays a beautiful white or light-pink color. *Saururus chinensis* is a perennial herb distributed in China and Korea. It belongs to a family Saururaceae and grows up to about 1 m. *Zanthoxylum piperitum* is a deciduous aromatic shrub belonging to a Family Rutaceae and its fruit is pulverized and used as Japanese Pepper in different type of cuisines. All of these plants are commonly growing in Jeju, an island located at the southernmost part of Korea. The objectives of the present study were to identify the main constituents of the *Prunus yedoensis*, *Saururus chinensis* and *Zanthoxylum piperitum* essential oils prepared by SFE method and to evaluate their biological activities such as their anti-inflammatory activities for application to human skin.

MATERIALS AND METHODS

Plant materials: An ethnobotanical survey was conducted in the Halla Mountain of Jeju Island from April to December 2012. Voucher specimens were identified by Dr. C.G. Hyun and deposited in Department of Chemistry. The plant was identified immediately after collection and air-dried at room temperature for later analysis. In the case of *Prunus yedoensis*, only the flower part was used as the material in this experiment. The leaves and branches were used together for the plants *Saururus chinensis* and *Zanthoxylum piperitum*.

Supercritical fluid extraction: Each plant material (2,000 g) was milled and sieved. The materials with an average particle diameter of 0.4 mm (collected between sieves of 0.2 and 0.6 mm) were used for the experiments. Extractions with supercritical carbon dioxide (SC CO_2) were performed on laboratory scale equipment. The apparatus was manufactured by Supercritical Laboratory (Model No. SFE0350R1) located in South Korea. The pressure and temperature conditions for the extraction of the first fraction were 10 Mpa and 40°C, respectively while the SC CO_2 flow rate was 0.67 kg h^{-1} (sample name: EO-CO_2). After the plant material was exhausted, the pressure was raised to 35 Mpa and the extraction of the second fraction followed. The SC CO_2 flow rate was 0.32 kg h^{-1} (sample name: AO-CO_2). Commercial carbon dioxide (99% purity) supplied by Tehnogas (Messer-Tehnogas, Serbia) was used for SC CO_2. Dichloromethane and methanol (GC purity, Sigma-Aldrich, Germany) was used for dissolution of supercritical extracts prior to GC-FID-MS analyses.

Gas chromatography-mass spectrometry: Gas chromatographic analyses were performed on a Hewlett-Packard 5890 gas chromatograph equipped with a polar Supelcowax column (30 m×0.25 mm×0.25 μm), an apolar DB-1HT column (30 m×0.25 mm) and a split-splitless injection port (split mode). The temperature was set at 40°C for 5 min, ramped to 210°C at rate of 10°C min^{-1} and held at 250°C for 28 min. Compounds were identified by their retention indices on both columns and by GC-MS using a Hewlett-Packard MSD 5972 mass spectrometer at 70 eV coupled to an HP 5890GC equipped with a DB-1HT column (30 m×0.32 mm×0.1 μm). The retention indices and mass spectra of each compound were compared with those in the literature.

Cell culture and viability: Murine RAW 264.7 macrophages were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin, all from GIBCO (Grand Island, NY, USA), in an incubator at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Cell viability was determined by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. RAW 264.7 cells were cultured in 96-well plates for 18 h, followed by treatment with various concentrations of the PYE, SCE and ZPE. After 24 h incubation, MTT was added to the medium for 4 h. Finally, the supernatant was removed and formazan crystals were dissolved in DMSO. Absorbance was measured at 540 nm. The percentage of cells showing cytotoxicity was determined relative to the control group.

Determination of nitric oxide (NO) products: After pre-incubation of RAW 264.7 cells (2.0×10^5 cells mL^{-1}) with LPS (1 μg mL^{-1}) for 24 h, the quantity of nitrite in the culture medium was measured as an indicator of NO production. Briefly, 100 μL of cell culture medium was mixed with 100 μL of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid), the mixture with different concentration (12.5, 25.0 and 50.0 μg mL^{-1}) of oils was incubated at room temperature for 10 min and the absorbance at 540 nm was measured in a microplate reader. Fresh culture medium was used as a blank in every experiment. The quantity of nitrite was determined from a sodium nitrite standard curve.

Measurement of pro-inflammatory cytokine (TNF-α, IL-1β and IL-6) production: The inhibitory effects of PYE, SCE and ZPE on pro-inflammatory cytokine (IL-1β,

IL-6 and TNF-α) production in LPs-treated RAW 264.7 cells were determined by ELISA as described in the manufacturer's instructions (R & D Systems, Minneapolis, MN).

Statistical analysis: The data are expressed as the Mean±Standard Error (SE). A statistical comparison was performed via., a one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (Duncan 1955). p-values of less than 0.05 were considered to be significant.

RESULTS AND DISCUSSION

As part of our ongoing alternative medicine program, we have directed our attention to explore essential oils possessing biological effects including anti-inflammatory, anti-aging and anti-cancer activities (Kim *et al*., 2009a, 2011; Yang *et al*., 2010; Yoon *et al*., 2009, 2010). Herein, we report the chemical compositions and anti-inflammatory activities of the plants, PYE, SCE and ZPE. The essential oils were obtained by supercritical extraction from the blossoms of *P. yedoensis*, the leaves and branches of *S. chinensis* and *Z. piperitum*. The identified compounds and quantitative analytical results from Gas Chromatography equipped with Mass detector (GC/MS) are shown in Table 1-3 according to their elution orders on a DB1-HT column. The GC/MS retention indices were calculated using a homologous series of n-alkanes, C_6-C_{31}.

Analysis of GC/MS for the PYE led to the identification of 10 different components, most of which are volatile monoterpenoids, representing 100% of the total oil (Table 1). The major components detected in the oil were α-pinene (22.38%), 2-β-pinene (20.09%), camphor (13.07%), sylvestrene (7.45%), caryophyllene oxide (9.39%), *trans*-caryophyllene (8.76%), junipene (7.73%), germacrene-d (4.18%), δ-cadinene (3.60%) and camphene (3.35%).

Table 1: Chemical composition (%) of *Prunus yedoeusis* essential oils

RT	RI	Name	Area (%)
10.43	933.4	α-pinene	22.4
10.97	947.8	Camphene	3.4
12.01	976.0	2-β-pinene	20.1
13.89	1029.4	Sylvestrene	7.5
17.78	1141.1	Camphor	13.1
32.32	1402.0	Junipene	7.7
32.99	1419.4	*Trans*-Caryophyllene	8.7
35.41	1482.3	Germacrene D	4.2
36.88	1541.4	δ-Cadinene	3.5
38.67	1633.9	(-)-Caryophyllene oxide	9.4
Total			100.0

The general chemical profiles of the SCE including the content percentage and the retention indices are summarized in Table 2. Selecting only components with MS data matches over 80%, a total of 13 components were characterized through a typical library search which represented about 97% of SCE oil. The major components detected in the oil were bicyclic sequiterpenes, δ-cadinol (22.51%) and δ-cadinene (19.74%). On the other hand, C_6-C_9 saturated linear-chain alcohols and their acids such as octanoic acid (13.37%) and n-heptanol (9.82%) were identified as the major components in ZYE (Table 3).

The Nitric Oxide (NO) is produced by activated human macrophages and play critical roles in inflammatory diseases like sepsis and arthritis (Murakami and Ohigashi, 2007). Hence, the inhibition of NO by down-regulation of its synthetic enzyme iNOS in inflammatory cells offers a new therapeutic strategy for

Table 2: Chemical composition (%) of *Saururus chinensis* essential oils

RT	RI	Name	Area (%)
16.32	1101.3	Linalool	5.5
32.98	1419.3	*Trans*-Caryophyllene	4.7
34.62	1461.9	Farnesol	3.3
35.28	1478.9	γ-Muurolene	4.2
35.41	1482.3	Germacrene D	3.6
35.90	1495.1	sesquisabinene hydrate	3.9
36.12	1501.0	α-Muurolene-(-)	2.4
36.60	1513.3	δ-Cadinol	22.5
36.87	1520.5	δ-Cadinene	19.7
37.89	1546.9	Germacrene B	3.6
38.67	1567.1	(-)-Caryophyllene oxide	5.4
44.97	1951.9	6,10,14-Trimethyl-2-pentadecanone	4.3
50.23	2223.0	*Trans*-Phytol	13.7
Total			96.8

Table 3: Chemical composition (%) of *Zanthoxylum piperitum* essential oils

RT	RI	Name	Area (%)
5.46	802.6	Hexanal	2.4
8.18	873.8	1-Hexanol	2.1
9.33	903.8	n-heptanal	4.2
11.94	974.3	1-heptanol	9.8
12.43	987.4	Hexanolic acid	3.8
13.06	1005.0	Octanal	6.5
13.89	1029.5	β-phellandrene	3.2
13.99	1032.4	1,8-Cineole	2.1
15.40	1074.4	1-Octanol	8.1
15.66	1081.9	Heptanoic acid	5.4
16.47	1105.5	Nonanal	4.6
18.10	1150.0	Citronella	7.6
18.97	1173.7	Octanoic acid	13.4
21.44	1225.0	*b*-Citronellol	1.7
24.07	1268.5	Nonanoic acid	5.4
32.98	1419.3	*Trans*-Caryophyllene	5.6
35.40	1482.3	Germacrene D	1.8
36.01	1498.1	2-Tridecanone	2.5
36.88	1541.3	γ-Cadinene	3.0
38.10	1608.3	Nerolidol	2.1
44.93	2248.8	Farnesyl acetate 3	2.8
Total			98.1

the treatment of inflammation. In order to validate the potentials of the above essential oils as anti-inflammatory agents in traditional Korean medicine, we investigated the effects of PYE, SCE and ZPE on the production of NO in LPS-activated RAW 264.7 macrophages. RAW 264.7 cells were treated with various concentrations of samples for 24 h and cell viability was assessed using an MTT assay. The nitrite concentration was determined in the supernatant after treatment with LPS (1 μg mL^{-1}) alone or co-treated with PYE, SCE and ZPE (3.13-200 μg mL^{-1}) for 24 h using Griess reagent. As shown in Fig. 1a, the oils PYE, SCE and ZPE suppressed LPs-induced NO formation significantly in dose-dependent fashion. Among these, ZPE gave the most reliable result, where 62% NO inhibition was observed with 25 μg mL^{-1} oil sample.

As shown in Fig. 1b, the numbers of viable activated macrophages were never affected by either of PYE, SCE or ZPE as determined by MTT assays, thus indicating that NO inhibition by these essential oils is not caused by cytotoxicity or cell death but is involved by other biological pathways.

It has been reported that cytokines such as TNF-α, IL-6 and IL-1β have proinflammatory effects both *in vitro* and *in vivo*. Moreover, TNF-α production is crucial to the synergistic induction of NO synthesis in IFN-γ and/or LPS-stimulated macrophages. TNF-α elicits a number of physiological effects such as septic shock, inflammation, cachexia and cytotoxicity while IL-6 is believed to be an endogenous mediator of LPS-induced fever (Feldmann, 2008; Ding *et al*., 2009; Kim *et al*., 2009b; Sugita, 2009; Yoon *et al*., 2010). Thus, the inhibition of

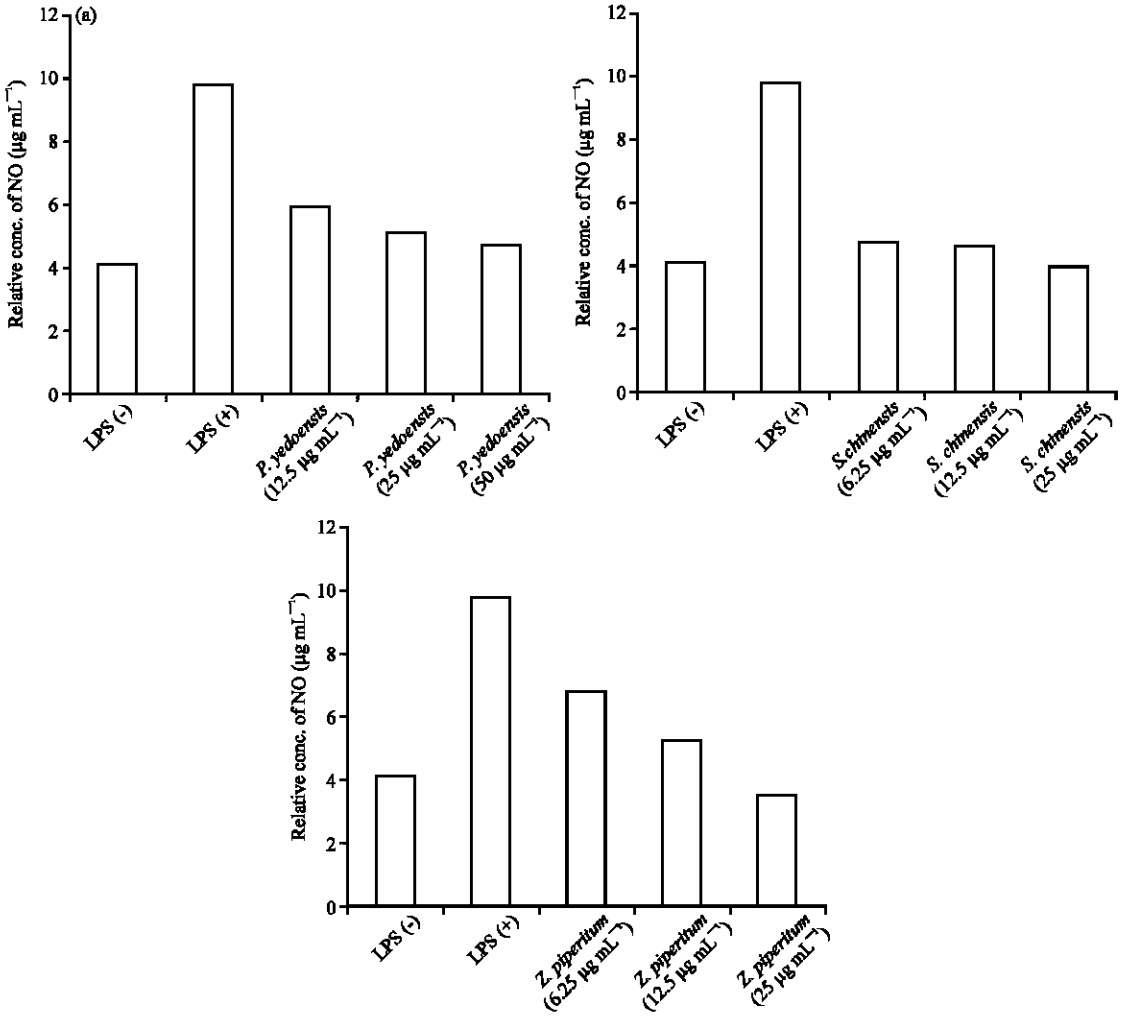

Fig. 1(a-b): Continue

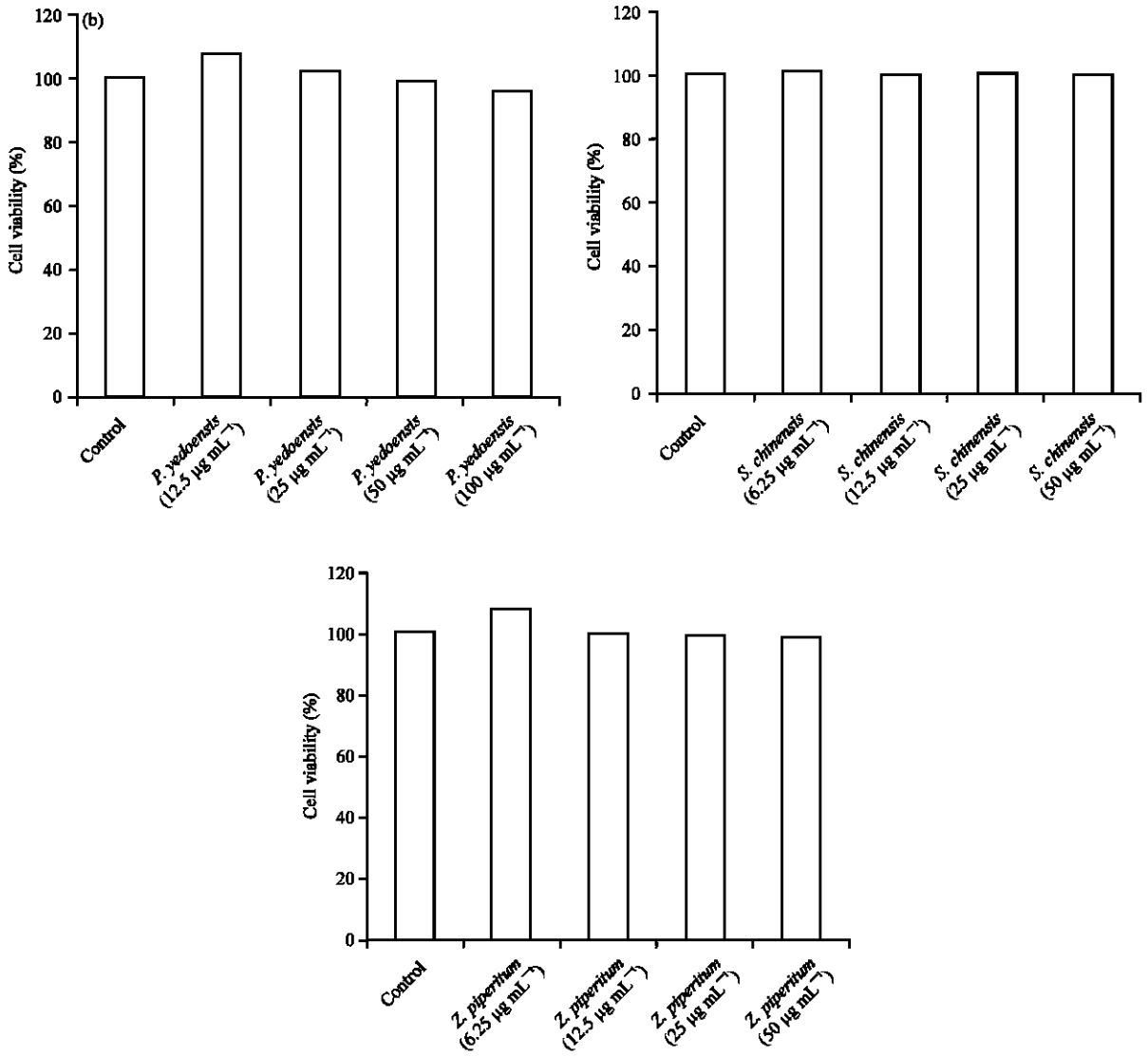

Fig. 1(a-b): Effects of PYE, SCE and ZPE on the production of nitric oxide (A) and cyotoxicity (B) in RAW 264.7 cells. The production of nitric oxide was assayed from culture medium of cells stimulated with LPS (1 μg mL^{-1}) in the presence of PYE, SCE and ZPE. NO production was determined by ELISA method. Parameters on x-axis denote as follows, LPS (-): without LPS-treatment; LPS (+): LPS-treated; (A): *P. yedoensis* (12.5 μg mL^{-1}): *P. yedoensis* (12.5 μg mL^{-1}) added to LPS-treated cell; (B): Control: without additives; *P. yedoensis* (12.5 μg mL^{-1}): *P. yedoensis* (12.5 μg mL^{-1}) added to control cell. Cytotoxicity was determined using MTT method. The data represent the Mean±SD of triplicate experiments

cytokine production or function is a key mechanism in the control of inflammation. In this experiment, levels of IL-1β, TNF-α and IL-6 in the culture supernatants were measured using ELISA kits. Lipopolysaccharide (1 μg mL^{-1}) stimulation for 24 h led to marked increases of TNF-α, IL-6 and IL-1β levels in the cell supernatants (Fig. 2). As shown in Fig. 2a, treatment of the oils PYE, SCE or ZPE inhibited the production of TNF-α in dose-dependent manner in RAW 264.7 cells. Among the tested samples, SCE exhibited the more prominent inhibition profile. In the examination of IL-6 production, PYE and SCE exhibited considerable inhibition activities but the effect of PYE was rarely appeared (Fig. 2b). Compared to TNF-α and IL-6, the decrease of IL-1β contents by the oils was observed only in a slight degree. The inhibitory effect of PYE was rarely observed in the same way (Fig. 2c).

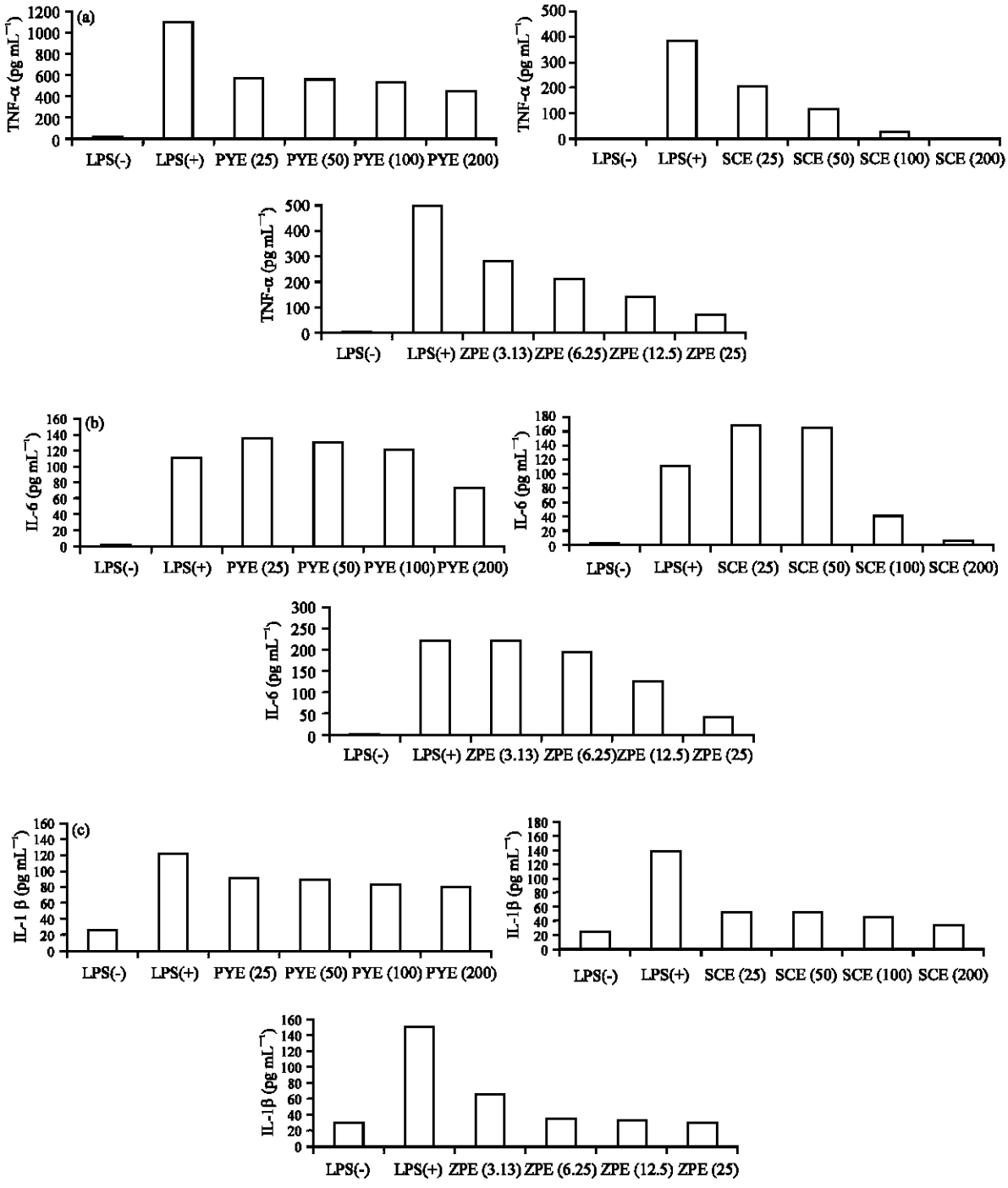

Fig. 2(a-c): Effects of PYE; SCE and ZPE on TNF-α; IL-6 and IL-1β production in LPS-stimulated RAW 264.7 cells. The cells were stimulated with 1 μg mL^{-1} of LPS alone or with a combination of LPS and various concentrations of PYE; SCE and ZPE for 24 h. (a) TNF-α (b) IL-6 and (c) IL-1β produced and released into the culture medium was assayed by the ELISA method. Parameters on x-axis denote as follows; LPS (-): without LPS-treatment; LPS (+): LPS-treated; (a) PYE (25): *P. yedoensis* oil (25 μg mL^{-1}) added to LPS-treated cell; SCE (25): *S. chinensis* oil (25 μg mL^{-1}) added to LPS-treated cell; ZPE (3.13): *Z. piperitum* oil (3.13 μg mL^{-1}) added to LPS-treated cell. The data represent the Mean±SD of triplicate experiments

CONCLUSION

The chemical composition and anti-inflammatory activities of supercritical extracted essential oils from *P. yedoensis* (PYE), *S. chinensis* (SCE), *Z. piperitum* (ZPE) were investigated for the first time. We have demonstrated that treatment of RAW 264.7 cells with PYE, SCE and ZPE can decrease levels of pro-inflammatory mediators and cytokines in LPS stimulated cells. In fact, the oils PYE, SCE and ZPE considerably inhibited the release of NO, TNF-α, IL-6 and IL-1β in a concentration-dependent manner. Based on these findings, essential oils of *P. yedoensis*, *S. chinensis* and *Z. piperitum* appear to possess suitable properties as effective anti-inflammatory agents for the treatment of a variety of inflammatory diseases.

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