academicJournals

Vol. 7(33), pp. 2451-2458, 3 September, 2013
DOI: 10.5897/JMPR12.360
ISSN 1996-0875 ©2013 Academic Journals
http://www.academicjournals.org/JMPR

Journal of Medicinal Plants Research

Full Length Research Paper

Inhibition of lipopolysaccharide-induced expression of cyclooxygenase-2 by *Zingiber cassumunar* Roxb. constituents in human dental pulp cells

Visakha Aupaphong[1], Thaweephol Dechatiwongse Na Ayudhya[1,2] and Sittichai Koontongkaew[1,2*]

[1]Faculty of Dentistry, Thammasat University, Thailand.
[2]Medicinal Herb Research Unit for Asthma, Thammasat University, Rangsit campus, Pathum-Thani, Thailand.

Accepted 4 May, 2012

Dental pulp inflammation expresses many pro-inflammatory cytokines; interleukins, cyclooxygenase (COX)-2 and prostaglandin E_2 (PGE_2). Inflammatory reduction and promoting regeneration of dental pulp cells are advantage for dental pulp healing. *Zingiber cassumunar* Roxb. (Phlai) has anti-inflammatory activity which may be applied to decrease inflammation in dental pulp tissues. The present study had aimed to evaluate inhibitory effect of Phlai constituents on COX-1, COX-2 and PGE_2 expression inhuman dental pulp cells stimulated with lipopolysaccharide (LPS). After cells were inhibited with dexamethasone (cox-inhibitor) or Phlai constituents for 2 h, LPS were added and concentration adjusted to 100 µg/ml. COX-1 and COX-2 gene expressions were obtained by reverse-transcription polymerase chain reaction (RT-PCR) and realtime RT-PCR after incubating for 24 h. COX-1 and COX-2 protein expressions were identified by Western blot and PGE_2 level was detected by enzyme linked immunosorbent assay (ELISA) after incubating for 72 h. These Phlai constituents as *cis*-3-(3',4'-Dimethoxyphenyl)-4-[(*E*)-3''',4'''-dimethoxystyryl]cyclohex-1-ene (compound B), *cis*-3-(2',4',5'-Trimethoxyphenyl)-4-[(*E*)-2''',4''',5'''-trimethoxystyryl]cyclohex-1-ene (compound C) and (*E*)-1-(3',4'-Dimethoxyphenyl)but-1,3-diene (DMPBD) could reduce PGE2 level and COX-2 expression. *cis*-3-(3',4'-Dimethoxyphenyl)-4-[(*E*)-2''',4''',5'''-trimethoxystyryl]cyclohex-1-ene (compound C′) reduced PGE_2 levels but had less effect on COX-2. All constituents showed no significant change on COX-1. Our results suggest that some constituents of Phlai can reduce inflammation in human dental pulp cells by reducing COX-2 and PGE_2 production.

Key words: *Zingiber cassumunar*, Phlai, cyclooxygenase-1, cyclooxygenase-2, lipopolysaccharide, anti-inflammation, dental pulp cells, prostaglandin E_2.

INTRODUCTION

Dental pulp inflammation can be provoked by mechanical, chemical and microbial stimuli. Cyclooxygenase -2 and prostaglandins E_2 are increased in dental pulp inflammation (Chang et al., 2003, 2006; Hirao et al., 2009; Nakanishi et al., 2001; Tokuda et al., 2001). Dental caries is an infectious disease that usually leads to inflammation of the tooth pulp. Infected pulpal tissue consists of a mixture of microorganism infection (Lana et

*Corresponding author. E-mail: koontongkaew@yahoo.com. Tel. 66-2-9869213 ext 7126, 7127, Fax 66-2-5165385.

al., 2001; Sundqvist, 1992). Gram-negative bacteria which have lipopolysaccharide (LPS) on cell wall are particularly detected in infected root canals and necrotic pulp. LPS is a virulence factor of gram-negative bacteria. Previous studies demonstrated that LPS and gram negative bacteria enhanced the production of pro-inflammatory cytokines and mediators, such as COX-2, and PGE_2 in cultured pulp cells (Chang et al., 2003, 2005; Coil et al., 2004).

Basically, the objective of pulp treatments is to promote regeneration of dental pulp tissue function. Bioactive molecules and natural materials have been introduced to promote pulp regeneration. *Zingiber cassumunar* Roxb. (Family Zingiberraceae), known as "Phlai" in Thai, has been widely used as medicinal herb in Thailand. The rhizome contains many compounds which contain diverse biological activities such as smooth muscle relaxant (Anantasan, 1982; Anantasan and Nopadonratanakhun, 1980), anti-asthmatic (Kiatyingungsulee et al., 1979), anti-inflammatory (Han et al., 2005; Jeenapongsa et al., 2003; Krisanaprakornkit et al., 2005; Pongprayoon et al., 1997a, b), anti-allergic (Tewtrakul and Subhadhirasakul, 2007), local anesthetic (Anantasan, 1977), analgesic (Panthong et al., 1990), and anti-oxidant activity (Masuda et al., 1995; Nagano et al., 1997). There are many constituents in Phlai which has anti-inflammatory activity (Han et al., 2005; Ozaki et al., 1991; Panthong et al., 1990; Pongprayoon et al., 1997a, b). Most of pulpal diseases are caused by inflammation; therefore, the constituents of Phlai might play an advantage in reducing pulpal inflammation and promotion of tissue regeneration. However, little is known about active constituents and their mechanism in reducing inflammation in dental pulp cells. Thus, we need to investigate on active constituents of Phlai on COX-1, COX-2 and PGE_2.

MATERIALS AND METHODS

Preparation of plant materials

Dried, sliced rhizomes of *Z. cassumunar* Roxb. were purchased from a local herbal store and their identity was confirmed at the Medicinal Plant Research Institute, Department of Medical Sciences, Ministry of Public Health, Thailand. A specimen sample has been preserved in laboratory for future reference. Dry pulverized rhizome (60 g) was extracted with ethanol in a Soxhlet extractor for 6 to 8 h. The liquid extract was evaporated by a rotary evaporator at 50°C to provide ethanolic extract (5.5 g). The extract was chromatographed on a silica gel column using a hexane-ethyl acetate (AcOEt) gradient system as the eluting solvent to give many fractions which were combined to give fractions 1 to 9.

Fraction 2 was separated by repeated preparative layer chromatography (PTLC) (hexane: AcOEt, 8:2) which extracted compound 1 as a pale yellow oil (324.4 mg). Fractions 5 to 7 were purified by PTLC (hexane: AcOEt, 7:3), giving out three crystalline compounds which were recrystallized from ethanol to give compound 2 (56.7 mg, melting point (m.p.) 97.9°C), compound 3 (15.2 mg, m.p. 113.3°C) and compound 4 (13.9 mg, m.p. 133.6°C) (Figure 1). The isolated compounds were identified as (*E*)-1-(3',4'-Dimethoxyphenyl)but-1,3-diene (DMPBD), *cis*-3-(3',4'-Dimethoxyphenyl)-4-[(E)-3''',4'''-dimethoxystyryl]cyclohex-1-ene (compound B) *cis*-3-(3',4'-Dimethoxyphenyl)-4-[(*E*)-2''',4''',5'''-trimethoxystyryl]cyclohex-1-ene (compound C') and *cis*-3-(2',4',5'-Trimethoxyphenyl)-4-[(*E*)-2''',4''',5'''-trimethoxystyryl]cyclohex-1-ene (compound C), respectively. Their structures are shown in Figure 2. All compounds exhibited thin layer chromatographic characteristics, electron spray mass spectra (ESMS; LCT Micromass) and proton nuclear magnetic resonance spectra (Bruker Avance 500) in agreement with previously published data (Amatayakul et al., 1979; Dechatiwongse, 1976; Kuroyanagi et al., 1980; Pongprayoon et al., 1997b; Thai Pharmacopoeia Committee, 1995). Isolated compounds were separately combined in polyvinyl polyvinylpyrrolidone (PVP; Sigma, St.Louis, MO) with 1:10 ratio (compound: PVP). Each residue was then dissolved in water and kept at -20°C for use as the stock solution.

Isolation and culture of human dental pulp cells

Dental pulp cells (DPCs) were isolated from teeth extracted for orthodontic or tooth impact reasons in the dental clinic, Faculty of Dentistry, Thammasat University. This protocol was approved with Thammasat University ethic committee. The teeth were split immediately by carbide bur and elevator. Pulp tissue was removed and kept in Dulbecco's Modified Egle Medium (DMEM; Gibco, Grand Island, NY) to be carried to laboratory room. Pulp tissue was cut into small pieces and then cultured by an explant technique in working media, [DMEM containing 10% fetal bovine serum (FBS; Invitrogen™, Carlsbad, CA), 100 IU/ml to 100 µg/ml penicillin/streptomycin (Invitrogen™) and 10 µg/ml amphoterin-B (PAA, Austria)], humidified atmosphere of 95% air and 5% CO_2 at 37°C. DPCs in passage numbers of 3 to 6 were used in the study.

Treatments

Confluent cells were trypsinized, counted and plated in 6 well plates and allowed to achieve confluence. The cells were incubated with compound B, compound C′, or compound C 50 µg/ml, DMPBD 5 µg/ml, or dexamethasone (Dexa; Sigma) 1 µM for 2 h. Lipopolysaccharide (*Escherichia coli* 0111:B4; Sigma) was added into wells and adjusted to the concentration at 100 µg/ml except the control group. Cells were collected after further incubation of 24 h. Gene expression was determined using semi-quantitative reverse-transcription polymerase chain reaction (RT-PCR) and real-time PCR. For protein analysis, cells were harvested after incubation for 72 h. Western blot and enzyme-linked immunosorbent assay (ELISA) were used for protein analyses. Non-treated cells were used as the control. All tested compounds, including Phlai constituents, LPS and dexamethasone were tested for cell cytotoxicity before treatments.

Semi-quantitative RT-PCR

Total RNA was extracted with Trizol reagents (Invitrogen™) according to manufacturer's instruction. RNA samples (500 ng) were reverse-transcribed and amplified in a typical PCR using Super Script™ III Platinum® one-step Quantitative RT-PCR Kit

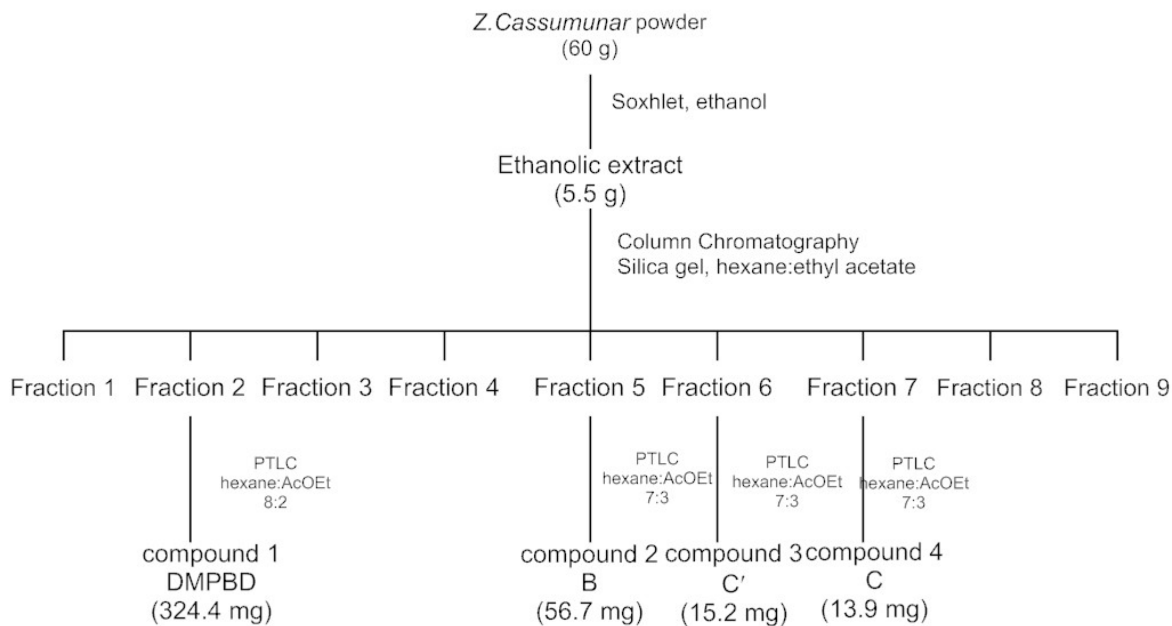

Figure 1. Extraction and isolation steps.

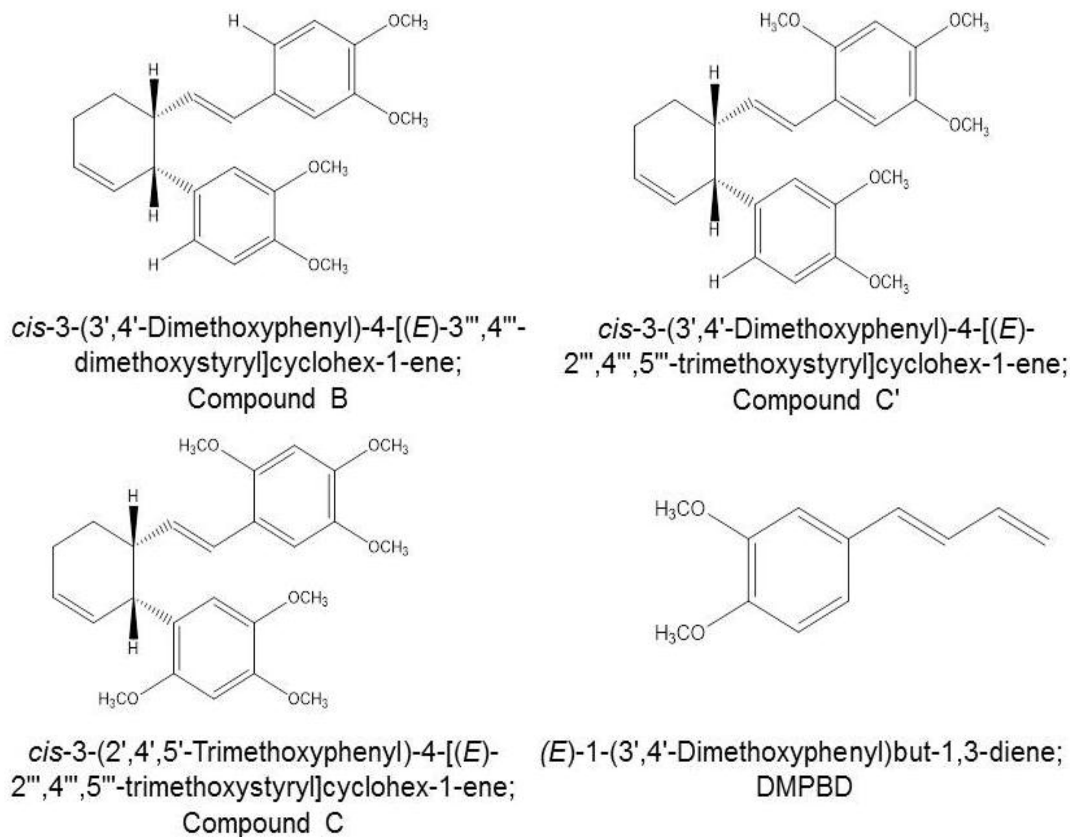

Figure 2. Chemical structure of Phlai constituents.

(Invitrogen^{TM}) 30 cycles. Primer sequences and annealing temperatures were detailed as follows; COX-2 (NM_000963.1) forward primer: 5′-GCAGTTGTTCCAGACAAGCA-3′, reverse primer: 5′-CAGGATACAGCTCCACAGCA-3′, size: 539 bp; COX-1 (NM_080591.1) forward primer: 5′-GAGTCTTTCTCCAACGTGAGC-3′, reverse primer: 5′-ACCTGGTACTTGAGTTTCCCA-3′ size: 350 bp and D-glyceral-dehyde-3-phosphate-dehydrogenase (GAPDH, NM_082046.3) forward primer: 5′-ACGCATTTGGTCGTATTGGG-3′, reverse primer: 5′-TGATTTTGGAGGGATCTCGC-3′, size: 239 bp (BSU, NSTDA, Thailand). Each cycle consisted 15 s at 95°C for denaturation, 30 s at 60°C for annealing, and 1 min at 72°C for extension. The PCR products were analyzed by 1.5% agarose gel electrophoresis. After normalizing with glyceraldehyde 3-phosphate dehydrogenase (GAPDH), densitometric values were expressed as the mean ± standard error (SE) of three independent experiments.

Real-time RT-PCR

Three cDNA samples were diluted five-fold in sterile distilled water, and 5 µl were subjected to real-time PCR using SYBR Green dye I. The reactions were performed in 15 µl of an iQ^{TM} SYBR$^{®}$ Green Supermix (Bio-Rad, Hercules, CA). The primers were designed using Beacon Designer 7.2 software as; COX-2 (NM_000963) forward primer: 5′-TTGGTCTGGTGCCTGGTC-3′, reverse primer: 5′-AGTATTAGCCTGCTTGTCTGG-3′; COX-1 (NM_000962) forward primer: 5′-GCCACCTTCATCCGAGAG-3′, reverse primer: 5′-GCAGAATACGAGTGTAATAGC-3′ and GAPDH (NM_002046) forward primer: 5′-ACAGCCTCAAGATCATCAG-3′, reverse primer: 5′-GAGTCCTTCCACGATACC-3′. The assay was carried out on an iCycler multicolor real-time PCR detective system (Bio-Rad) and analyzed using iQ^{TM}5 Optical System Software (version 2.0, Bio-Rad). PCR consisted of 40 cycles of 95°C for 10 s and 60°C for 30 s. Real-time PCR was performed in three independent experiments.

Western blotting

DPCs were lysed in RIPA buffer for 10 min. Then, cell lysates were centrifuged at 10,000 g at 4°C. The protein concentrations were determined using Bradford reagents with bovine serum albumin as a standard (Pierce, Rockford, IL). Sample proteins of 40 µg/ml were loaded on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. The membranes were blocked with 5% skim milk for 1 h, washed with Tris Buffered Saline Tween-20 (TBST), incubated in primary COX-2 goat polyclonal antibody (1:1000, Santa Cruz biotechnology, Santa Cruz, CA), COX-1 rabbit polyclonal antibody (1:1000, Santa Cruz biotechnology), or actin rabbit polyclonal antibody (1:5000, Sigma) with 3% bovine serum albumin for 2 h at room temperature. Then the membrane were washed with TBST and incubated with rabbit anti-goat or goat anti-rabbit antibody (1:40,000, Pierce) for 1 h at room temperature. After final washes, enhanced chemiluninescence was performed for 5 min with supersignal substrate (Pierce). Semi-quantitative determination of protein expression was performed using a densitometer. After normalized with actin, densitometric values were expressed as the mean ± SE of three independent experiments.

PGE_2 Assay

The amounts of PGE_2 in the conditioned medium were determined using a commercial ELISA kit (Parameter^{TM}**,** R&D Systems, UK) according to the manufacture's instruction. Three independent experiments were performed and the data were converted to pg/ml.

Statistical analysis

All experiments were performed in three independent samples. Significant differences were determined using One-way analysis of variance followed with Dunnett's multiple comparison. Differences with *p* values ≤ 0.05 were considered significant.

RESULTS

COX-1 and COX-2 gene expression

After exposure to Phlai constituents and then stimulated with LPS for 24 h, COX-2 gene was decreased significantly compared with that of the only LPS-treated group ($p < 0.05$). However, compounds B, C and DMPBD could not reduce mRNA of COX-2 levels to the baseline. In contrast, LPS could not enhance COX-1 expression in DPCs. COX-1 gene expression did not obviously change in cells treated with compounds B, C' and C (Figures 3 and 4).

COX-1 and COX-2 protein expression

After exposure to dexamethasone or Phlai constituents and stimulated with LPS for 72 h, COX-2 protein increased significantly after incubating only with LPS ($p < 0.05$). Compounds B, C, and DMPBD had the tendency to reduce COX-2 protein expression compared with LPS while dexamethasone inhibited COX-2 protein production significantly ($p < 0.05$). Little changes occurred on COX-1 protein compared with controlled samples (Figure 5).

PGE_2 production

LPS significantly enhanced PGE_2 synthesis. After exposure to compounds B, C′, C, and DMPBD or dexamethasone and stimulated with LPS for 72 h, PGE_2 decreased significantly compared to that of the only LPS-treated group ($p < 0.05$) (Figure 6).

DISCUSSION

Cultured dental pulp cells increased the expression of pro-inflammatory cytokine as COX-2 and prostaglandin E_2 after inducing with LPS (Hirao et al., 2009; Nakanishi et al., 2001). The constituents of Phlai could reduce inflammation of dental pulp cells via r eduction of COX-2

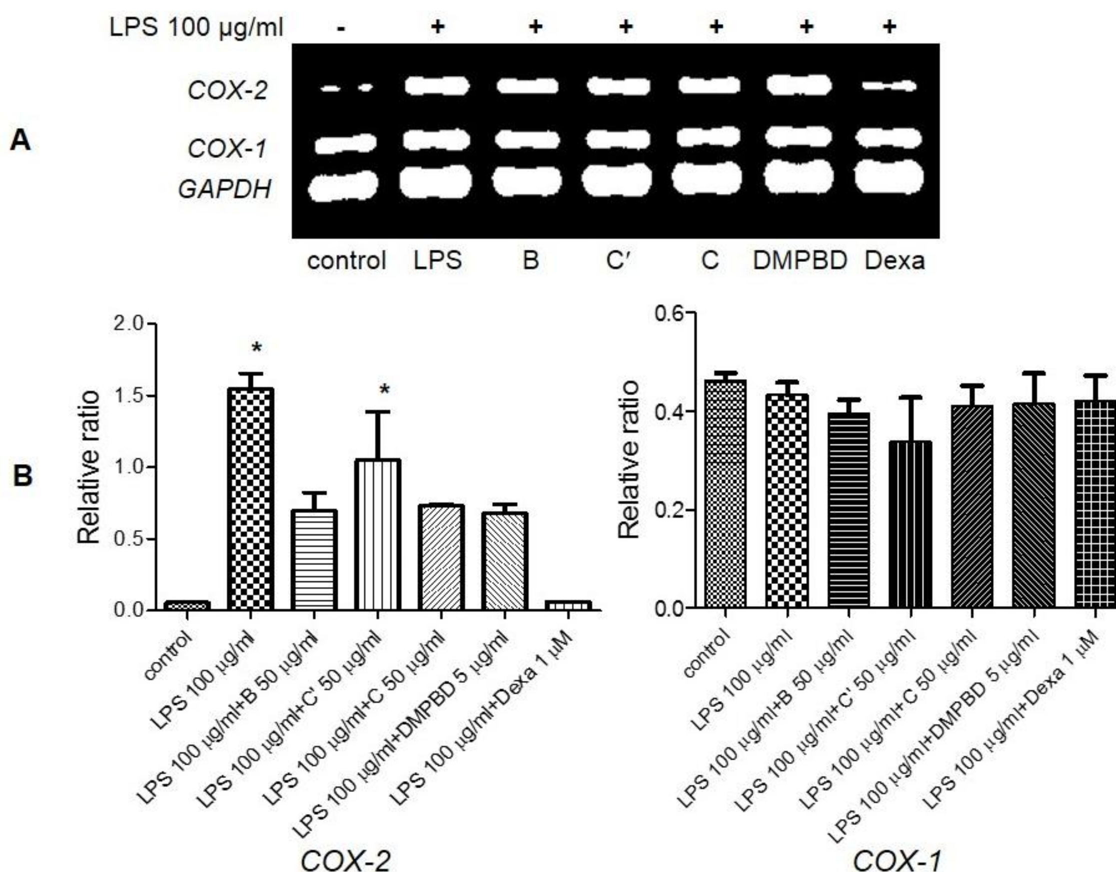

Figure 3. (A) RT-PCR band of COX-2, COX-1 and GAPDH gene. (B) Relative ratio of COX-2 and COX-1 mRNA after normalizing by GAPDH. LPS significantly increased COX-2 mRNA, compared with the control. Compound B, compound C, DMPBD and dexamethasone (Dexa) significantly decreased COX-2 mRNA. There is no change of COX-1 gene expression in all treated cells compared to the control. Data represent mean ± SE of 3 independent samples (**p* < 0.05 versus LPS).

and PGE_2 production but has less effect on COX-1. This might involve in the homeostatic function of prostaglandins. Previous studies showed that *cis*-3-(3',4'-Dimethoxyphenyl)-4-[(*E*)-3''',4'''-dimethoxystyryl]cyclohex-1-ene, *cis*-3-(2',4',5'-Trimethoxyphenyl)-4-[(*E*)-2''',4''',5'''-trimethoxystyryl]cyclohex-1-ene exerted potent topical anti-inflammatory in 12-*O*_tetradecanoylphorbol-13 acetate-induced rat ears (Pongprayoon et al., 1997a and b) and (*E*)-1-(3',4'-Dimethoxyphenyl)but-1,3-diene (DMPBD) had anti-inflammatory activity twice as potent as diclofenac (Pongprayoon et al., 1997a). DMPBD has been found to inhibit the rat ear edema induced by ethyl phenylpropiolate (EPP), arachidonic acid (AA) and 12-*O*-tetradecanoylphorbol 13-acetate. It posseses a potent anti-inflammatory activity through the inhibition of cyclooxygenase and lipooxygenase pathways (Jeenapongsa et al., 2003). Thus, (*E*)-1-(3',4'-Dimethoxyphenyl)but-1,3-diene (DMPBD); *cis*-3-(3',4'-Dimethoxyphenyl)-4-[(*E*)-3''',4'''-dimethoxystyryl]cyclohex-1-ene (compound B) and *cis*-3-(2',4',5'-Trimethoxyphenyl)-4-[(*E*)-2''',4''',5'''-trimethoxystyryl]cyclohex-1-ene (compound C) might be the active constituents of Phlai which could reduce dental pulp inflammation. Prostaglandin E_2 is produced by the action of COX-2 enzymes. NF-κB is one of the transcription factors of COX-2 expression. For this reason, inhibition of NF-κB activation should result in a decrease of COX-2 and PGE_2 expression. Previous study of *Centella asiatica* showed that asiatic acid exhibited anti-inflammatory properties through inhibiting of COX-2 production. The inhibition occurs through suppression of IKK and MAPkinase (p38, ERK1/2 and JNK) phosphorylation and resulted in down-regulation of NF-κB (p65 and p50) (Yun et al., 2008). Therefore, it is possible that Phlai constituents might decrease the production of inflammatory mediators including prostaglandin E_2 of dental pulp cells through interfering with the NF-κB activation.

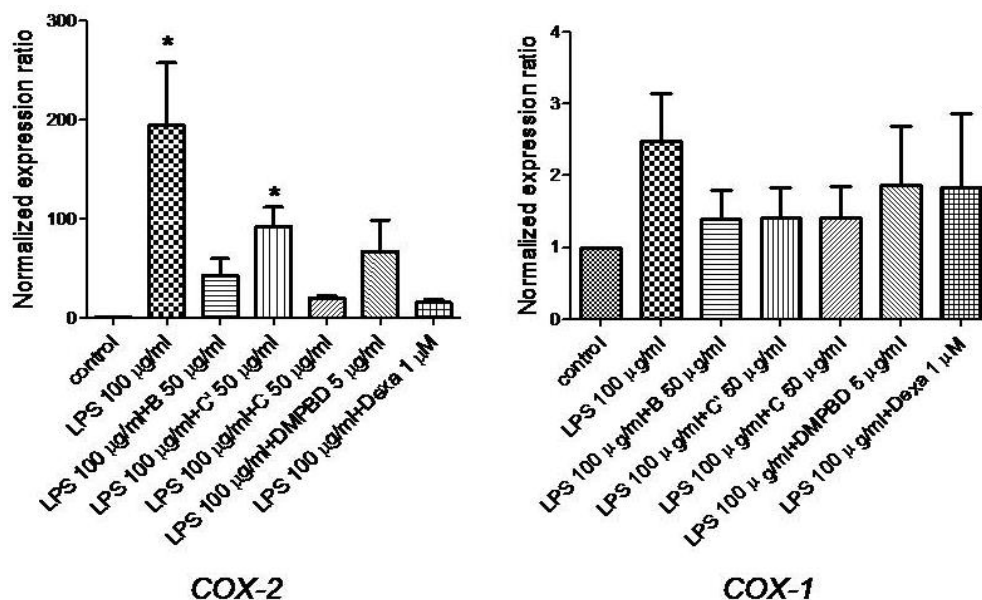

Figure 4. Real time-PCR of COX-1 and COX-2 mRNA. The result showed that compound B, compound C and DMPBD significantly decreased COX-2 mRNA (*$p < 0.05$ versus LPS). A small decrease in COX-1 mRNA was observed in cells treated with LPS and tested compounds. Data represent mean ± SE of 3 independent samples.

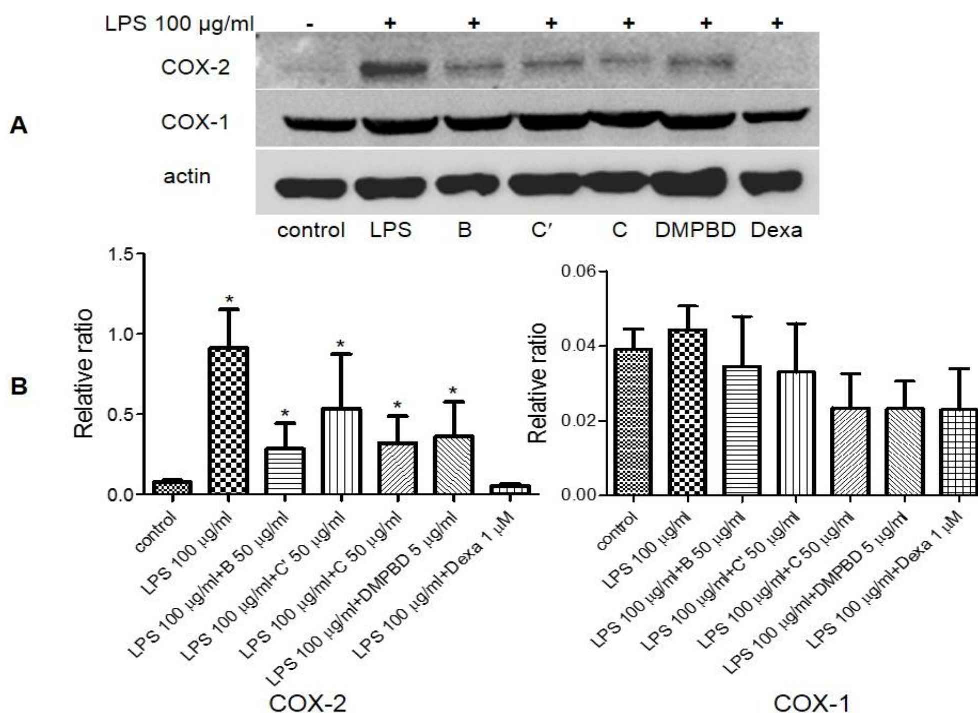

Figure 5. (A) Protein bands of COX-2, COX-1 and actin using Western blotting. (B) Relative ratio of COX-2 and COX-1 protein after normalizing by actin protein. Dexamethasone (Dexa) significantly decreased COX-2 mRNA (*$p < 0.05$ versus LPS). Compound B, compound C and DMPBD had trended to reduce COX-2 gene and no significant changes on COX-1 protein. Data represent mean ± SE of 3 independent samples.

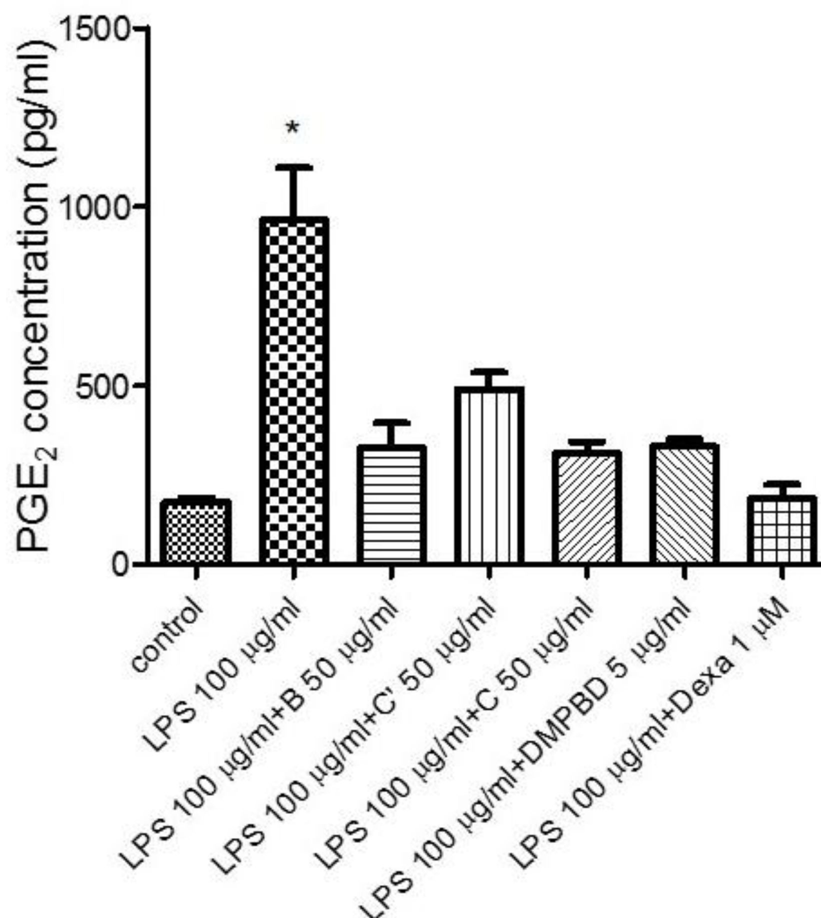

Figure 6. Concentration of PGE2 in conditioned media of dental pulp cells after exposure to isolated constituents. Compound B, compound C′, compound C, DMPBD and dexamethasone (Dexa) significantly decreased PGE2 production (**p*<0.05 vs. LPS). Data represent mean±SE of 3 independent samples.

Conclusion

Our results suggest that *cis*-3-(3',4'-Dimethoxyphenyl)-4-[(*E*)-3''',4'''-dimethoxystyryl]cyclohex-1-ene (compound B), *cis*-3-(2',4',5'-Trimethoxyphenyl)-4-[(*E*)-2''',4''',5'''-trimethoxystyryl]cyclohex-1-ene (compound C), (*E*)-1-(3',4'-Dimethoxyphenyl)but-1,3-diene (DMPBD) and *cis*-3-(3',4'-Dimethoxyphenyl)-4-[(*E*)-2''',4''',5'''-trimethoxystyryl]cyclohex-1-ene (compound C′) could suppress PGE_2 synthesis in LPS-stimulated dental pulp cells. Therefore, Phlai is a potential medicinal herb for use in the treatment of dental pulp inflammation.

ACKNOWLEDGEMENTS

Special thanks to Mr. Chakapong Intaraudom, BIOTEC, NSTDA, Thailand, Dr. Kan Chanpromma, Vice Rector for Research, Walailak University and Dr. Bordin Chitkul, Department of Chemistry, Faculty of Science, Ramkhamhaeng University for their advising technique of physical and chemical identification in plant extract. This work was supported in part by Thammasat University and by the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission.

REFERENCES

Amatayakul T, Cannon JR, Dampawan P, Dechatiwongse T, Giles RGF, Huntrakul C, Kusamran K, Mokkhasamit M, Raston CL, Reutrakul V, White A (1979). Chemistry and crystal structures of some constituents of *Zingiber cassumunar*. Aust. J. Chem. 32:71-88.

Anantasan V (1977). Medical plants "Plai or Puu Loei" and researches in phamacology. J. Pharm. Assoc. Thailand 31:381-388.

Anantasan V (1982). A Phamacological study of plai (*Zingiber cassumunar* Roxb.) water extract on smooth muscle (section II) J.

Natl. Res. Counc .Thailand 14:1-24.
Anantasan V, Nopadonratanakhun L (1980). A Phamacological study of Plai (*Zingiber cassumunar* Roxb.) water extraxt on smooth muscles (Section I) J. Natl. Res. Counc. Thailand 12:51-68.
Chang MC, Chen YJ, Tai TF, Tai MR, Li MY, Tsai YL, Lan WH, Wang YL, Jeng JH (2006). Cytokine-induced prostaglandin E2 production and cyclooxygenase-2 expression in dental pulp cells: downstream calcium signalling via activation of prostaglandin EP receptor. Int. Endod. J. 39:819-826.
Chang J, Zhang C, Tani-Ishii N, Shi S, Wang CY (2005). NF-kappaB activation in human dental pulp stem cells by TNF and LPS. J. Dent. Res. 84:994-998.
Chang YC, Huang FM, Yang SF, Liu CM, Lai CC, Chan Y, Hsieh YS (2003). Induction of cyclooxygenase-2 mRNA and protein expression in human pulp cells stimulated with black-pigmented bacteroides. J. Endod. 29:240-243.
Coil J, Tam E, Waterfield JD (2004). Proinflammatory cytokine profiles in pulp fibroblasts stimulated with lipopolysaccharide and methyl mercaptan. J. Endod. 30:88-91.
Dechatiwongse T (1976). Isolation of constituents from the rhizome of Plai (*Zingiber Cassumunar* Roxb.). Bull. Dept. Med. Sci. 3:75-79.
Han AR, Kim MS, Jeong YH, Lee SK, Seo EK (2005). Cyclooxygenase-2 inhibitory phenylbutenoids from the rhizomes of *Zingiber cassumunar.* Chem. Pharm. Bull. 53:1466-1468.
Hirao K, Yumoto H, Takahashi K, Mukai K, Nakanishi T, Matsuo T (2009). Roles of TLR2, TLR4, NOD2, and NOD1 in pulp fibroblasts. J. Dent. Res. 88:762-767.
Jeenapongsa R, Yoovathaworn K, Sriwatanakul KM, Pongprayoon U, Sriwatanakul K (2003). Anti-inflammatory activity of (E)-1-(3,4-dimethoxyphenyl)butadiene from *Zingiber cassumunar* Roxb. J. Ethnopharmacol. 87:143–148.
Kiatyingungsulee N, Wangmud M, Swasdimongkol S, Mokkhasmit M (1979). Some pharmacological studies of active constituents in Plai (*Zingiber cassumunar* Roxb.). Bull. Dept. Med. Sci. 21:13-24.
Krisanaprakornkit S, Chotjumlong P, Ong-chai S, Kongtawelert P, Reutrakul V., 2005. An anti-inflammatory effect *Zingiber cassumunar* Roxb. in oral cells. Proceeding of 1th the Research Path of Chiangmai University conference, December 8-10. Chiangmai, Thailand p. 44.
Kuroyanagi M, Fukushima S, Yoshihira K, Natori S, Dechatiwongse T, Mihashi K, Nishi MSH (1980). Further characterization of the constituents of a thai medicinal plant, *Zingiber cassumunar* Roxb. Chem. Pharm. Bull. 28:2948-2959.
Lana MA, Ribeiro-Sobrinho AP, Stehling R, Garcia GD, Silva BK, Hamdan JS, Nicoli JR, Carvalho MA, Farias M (2001). Micro-organisms isolated from root canals presenting necrotic pulp and their drug susceptibility *in vitro*. Oral Microbiol. Immunol. 16:100-105.
Masuda T, Jitoe A, Mabry TJ (1995). Isolation and structure determination of Cassumunarins A, B, C: New anti-inflammatory antioxidants from a tropical ginger, *Zingiber cassumunar*. J. Am. Oil Chem. Soc. 72:1053-1057.
Nagano T, Oyama Y, Kajita N, Chikahisa L, Nakata M, Okazaki E, Masuda T (1997). New curcuminoids isolated from *Zingiber cassumunar* protect cells suffering from oxidative stress: A flow-cytometric study using rat thymocytes and H_2O_2. Jpn. J. Phamacol. 75:363-370.
Nakanishi T, Shimizu H, Hosokawa Y, Matsuo T (2001). An immunohistological study on cyclooxygenase-2 in human dental pulp. J. Endod. 27: 85-388.
Ozaki Y, Kawahara N, Harada M (1991). Anti-inflammatory effect of *Zingiber cassumunar* Roxb. and its active principles. Chem. Pharm. Bull. 39:2353-2356.
Panthong A, Kanjanapothi D, Niwatananun V, Tuntiwachwutikul P, Reutrakul V (1990). Antiinflammatory activity of compounds isolated from *Zingiber cassumunar* . Planta Med. 56:60.
Pongprayoon U, Soonthornsaratune P, Jarikasem S, Sematong T, Wasuwat S, Claeson P (1997a). Topical antiinflammatory activity of the major lipophilic constituents of the rhizome of *Zingiber cassumunar*. Part I: The essential oil. Phytomedicine 3:319-322.
Pongprayoon U, Tuchinda P, Claeson P, Sematong T, Reutrakul V, Soonthornsaratune P (1997b). Topical antiinflammatory activity of the major lipophilic constituents of the rhizome of *Zingiber cassumunar*. Part II: Haxane extractives. Phytomedicine 3:323-326.
Sundqvist G (1992). Associations between microbial species in dental root canal infections. Oral Microbiol. Immunol. 7:257-262.
Tewtrakul S, Subhadhirasakul S (2007). Anti-allergic activity of some selected plants in the Zingiberaceae family. J. Ethnopharmacol. 109:535-538.
Thai Pharmacopoeia Committee (1995). Thai Herbal Pharmacopoeia. Department of Medical Sciences, Ministry of Public Health, Nonthaburi, Thailand 1:51-56, 113.
Tokuda M, Sakuta T, Fushuku A, Torii M, Nagaoka S (2001). Regulation of interleukin-6 expression in human dental pulp cell cultures stimulated with *Prevotella intermedia* lipopolysaccharide. J. Endod. 27:273-277.
Yun KJ, Kim JY, Kim JB, Lee KW, Jeong SY, Park HJ, Jung HJ, Cho YW, Yun K, Lee KT (2008). Inhibition of LPS-induced NO and PGE2 production by asiatic acid via NF-[kappa]B inactivation in RAW 264.7 macrophages: Possible involvement of the IKK and MAPK pathways. Int. Immunopharmacol. 8:431-441.