The Journal of Applied Pharmacology, 14, 160-165(2006)

Terpene-Strengthened *Ginkgo biloba* Extract as a Platelet-Activating Factor Antagonist

Zhejiu QUAN[1], Tae Chul MOON[1], Ju Hye YANG[1], Hyeun Wook CHANG[1*], Young-Hyun PARK[2], Young Ha KIM[3], Kyung Hee LEE[3], Yeon Sook CHI[4], Hyun LIM[4], Hyoung Chun KIM[4] and Hyun Pyo KIM[4*]

[1]*College of Pharmacy, Yeungnam University, Gyeongsan 712-749, Korea*

[2]*College of Natural Sciences, Soonchunhyang University, Asan 336-745, Korea*

[3]*Research Center, Yuyu Inc., Anyang 430-804, Korea*

[4]*College of Pharmacy, Kangwon National University, Chunchon 200-701, Korea*

(Received July 26, 2006; Accepted August 29, 2006)

Abstract – Since platelet-activating factor (PAF) is involved in inflammation, allergic response and anaphylactic shock, PAF receptor antagonists may have potential for controlling these disease conditions. The extract of the leaves of *Ginkgo biloba* having a higher content of terpenoids (12%) with flavonoids (24%) (YY1224) was prepared in order to obtain the increasing PAF antagonistic activity. As expected, YY1224 showed a higher PAF antagonistic binding affinity (IC_{50} = 0.09 μg/ml) using [^{3}H]PAF and rabbit platelets as ligand and receptor source, compared with an IC_{50} of > 100 μg/ml by Egb 761, a standardized extract. YY1224 also showed a higher inhibitory activity against PAF-induced platelet aggregation and NO production from lipopolysaccharide-treated RAW 264.7 cells. In addition, it protected PAF-induced death in mice by oral administration at 15 mg/kg. All these results suggest that YY1224 may show favorable effects on PAF-related disorders.

Key words □ *Ginkgo biloba*, YY1224, Egb 761, PAF antagonist, Platelet aggregation, NO

INTRODUCTION

Platelet-activating factor (PAF) (1-*O*-alkyl-2-acetyl-sn-glycero-3-phosphocholine) exerts a number of biological actions, including platelet aggregation, chemotaxis of inflammatory cells, provoking inflammation, allergic response and anaphylactic shock in mice (Hanahan *et al.*, 1987; Prescott *et al.*, 1991; Koltai *et al.*, 1996). Therefore, PAF receptor antagonists may show some beneficial effects on these pathologic reactions. According to this background information, many PAF antagonists have been developed and they show anti-inflammation, antiallergy and prevention of PAF-induced anaphylactic shock.

The standardized extract of *Ginkgo biloba* leaves (Egb 761) contains 24% flavonoid glycosides and 6% terpenoids like ginkgolides and bilobalide (DeFeudis, 1991). Although ginkgolide B among these constituents is found to be a powerful PAF receptor antagonist (Bourgain et al., 1992), Egb 761 has only a weak PAF antagonistic activity. Since enhancing PAF antagonistic activity may be favorable against some PAF-related diseases, *G biloba* extract containing a higher amount of terpenoids has been prepared by Yuyu Inc. (Korea). This extract (YY1224) comprises 12% terpenoids with same amount of flavonoids (24%). Here in this investigation, the pharmacological properties of this newly prepared extract were evaluated based on PAF antagonistic activity.

*Corresponding author
Tel: +82-53-810-2811, Fax: +82-33-250-6915
E-mail: hwchang@yu.ac.kr, hpkim@kangwon.ac.kr

MATERIALS AND METHODS

Materials

PAF (C_{16}), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and lipopolysaccharide (LPS, *Escherichia coli* 0127:B8) were purchased from Sigma Chem. (St. Louis, MO). Ginkgolide B was a product of Roth (Germany). CV-6209 was from Biomol. (Plymouth Meeting, PA). ABT491 was obtained from Takara (Japan). [^{3}H]PAF (1-*O*-[^{3}H]-octadecyl, 200 Ci/mmol) was from Amersham Chem. Egb 761 and YY1224 were provided from Yuyu Inc. LipofecAMINE PLUS, DMEM and other cell culture reagents including FBS were products of Gibco BRL (Grand Island, NY). Protein assay kit was purchased from Bio-Rad Lab. (Hercules, CA).

Animals

Male New Zealand white rabbits and ICR mice were purchased from Orient Co. (Seoul, Korea). Animals were maintained in a specific pathogen-free facility under the conditions of 22±1°C, 40 - 60% relative humidity and 12 h/12 h (light/dark) cycle feeding with pellet chow and water *ad libitum*. Animals were used after acclimatization at least 7 days.

PAF receptor binding assay

From the neck vein of rabbits, blood was withdrawn in sodium citrate solution (5:1). After centrifugation at 230 g for 10 min, platelet rich plasma (PRP) was obtained. PRP was centrifuged again at 800 g for 15 min and platelet pellet was mixed with Tris-tyroide buffer (pH 7.3, 10 mM). After centrifugation, platelets were diluted to 2×10^8 cells/ml with Tris-tyroide buffer containing 0.25% BSA. The binding assay was essentially followed by the slightly modified procedure of the original method of Valone *et al.* (1982). In brief, [^{3}H]PAF was dried under N_2 and 10 mM Tris-tyroide buffer with 0.25% BSA was added, followed by sonification for 2 min. From the preliminary experiments with different concentrations of [^{3}H]PAF for various incubation periods, 1.5 nM [^{3}H]PAF and 50 min incubation time were found to be optimum. Thus, the following experiments were carried out under this condition. For a total binding experiment, test samples including reference compounds (25 μl) were added to platelet suspension (200 μl) and pre-incubated for 5 min. Then, [^{3}H]PAF (1.5 nM) was added and the tubes were incubated further for 50 min. For a nonspecific binding, [^{3}H]PAF and cold PAF (300 nM) were added to platelet suspension in the presence or absence of test samples, and the tubes were incubated for 50 min. The solution was filtered on GF-C glass fiber filters using Skatron cell harvester. The filters were dried and radioactivity was counted in toluene-based cocktail. Specific binding and % inhibition were calculated as below:

Specific binding = total binding – nonspecific binding

$$\%\ \text{inhibition} = \frac{Sc - Ss}{Sc} \times 100 = \frac{(Tc - Nc) - (Ts - Ns)}{(Tc - Nc)} \times 100$$

Sc: specific binding of control, Ss: specific binding of sample, Tc: total binding of control, Ts: total binding of sample, Nc: nonspecific binding of control, Ns: nonspecific binding of sample

All experiments were triplicated and arithmetic means were represented. The same experiments were carried out three times in the measurement of antagonistic activity of *G. biloba* extracts.

Measurement of platelet aggregation

The platelet aggregation in vitro was performed as previously described (Akiba *et al.*, 2000). In brief, PRP from rabbit blood was centrifuged at 800 g for 15 min, and then washed twice with HEPES buffer (137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 5.6 mM glucose and 3.8 mM HEPES, pH 6.5) containing 0.35% BSA and 0.4 mM EGTA. The washed platelets were resuspended in the same buffer (pH 7.4) without EGTA to 3×10^8 cells/ml. Platelets were incubated at 37°C for 3 min with test samples in the presence of 1 mM $CaCl_2$ in an aggrometer (470-vs, Chrono-log Co., PA, USA), and platelet aggregation was induced by an addition of PAF (2 nM). The resulting aggregation, measured as the change of light transmission, was recorded for 10 min. Each inhibition rate was obtained from the maximal aggregation induced by respective PAF at the concentrations using the following equation. Inhibition rate = (maximal aggregation rate (MAR) of vehicle-treated platelets - MAR of sample-treated platelets/MAR of vehicle-treated platelets)× 100.

RAW 264.7 cell culture and measurement of NO concentration

RAW 264.7 cells (mouse macrophage cell line, American Type Culture Collection) were routinely cultured with DMEM supplemented with 10% FBS and 1% antibiotics under 5% CO_2 at 37°C according to the previously described procedure (Chi *et al.*, 2001). Briefly, cells were plated in 96-well plates (2×10^5 cells/200 ml/well). After pre-incubation for 2 h, various concentrations of test samples and LPS (1 μg/ml) were added and incubated for 24 h, unless otherwise specified. Test samples were dissolved in DMSO and diluted with serum-free DMEM into appropriate concentrations. Final concentrations of DMSO in the culture never exceeded 0.1% (v/v). Cell viability was assessed with MTT assay. For determination of NO concentration, the stable conversion product of NO, nitrite (NO_2^-), was measured using Griess reagent [1:1 mixture (v/v) of 1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 5% H_3PO_4]. Optical density was measured with a microplate reader (Spectra Max, Molecular Devices) at 550 nm.

Western blot of inducible nitric oxide synthase (iNOS)

For measuring the protein level of iNOS, Western blotting

technique was used (Chi *et al.*, 2001). RAW cells were cultured in 6-well plates (5×10^6 cells/well) in the presence or absence of LPS (1 μg/ml) with/without test samples for 20 h. After preparing cell homogenate, the supernatant was obtained by centrifugation at 15,000 g for 30 min. Using Tris-glycine gel (4-15%, Novex Lab.), electrophoresis was carried out and the bands were blotted to PVDF membranes. iNOS antibody (N32030, Transduction Lab.) was incubated and the bands were visualized by the treatment of secondary antibody and DAB reagent (Vector Lab.).

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

RAW cells were cultured in 6-well plates (5×10^6 cells/well) in the presence or absence of LPS with/without test samples for 5 h. After preparing cell homogenate, total RNA was extracted using RNeasy mini kit (Quiagen) according to the supplier's protocol. The concentration of RNA content was determined at 260 nm and 280 nm. cDNAs were synthesized using RT reaction at 42°C, 5 min and 99°C, 5 min in Gene Cycler thermal cycler (Bio-Rad). Primers were synthesized on the basis of the repeated mouse cDNA sequence for iNOS and glyceraldehyde-3-phosphate dehydrogenase (G3PDH). The primer sequences used for PCR were as follows: iNOS: sense, 5'-CCC TTC CGA AGT TTC TGG CAG CAG C-3', antisense, 5'-GGC TGT CAG AGC CTC GTG GCT TTG G-3', 469 bp; G3PDH: sense, 5'-TGA AGG TCG GTG TGA ACG GAT TTG GC-3', antisense, 5'-CAT GTA GGC CAT GAG GTC CAC CAC-3', 983 bp. The amplification was performed at 94°C for 15 - 60 sec, 50 - 68°C for 30 - 60 sec, and 72°C for 45 - 90 sec with 25 -30 cycles under saturation, in 25 μl reaction mixture. After amplification, 5 μl of reaction mixture was analyzed on 1.5% agarose gel electrophoresis and the bands were visualized by ethidium bromide staining for 10 min.

PAF-induced death in mice

For measuring the protective effect against PAF-induced toxicity, PAF-induced death model was used as previously described (Terashita *et al.*, 1992). Test samples were dissolved in 0.1% carboxymethyl cellulose (CMC)/0.9% NaCl solution. PAF (1 mg/ml ethanol) was dissolved in Tris-tyroide (5 mM Tris-HCl, pH 7.4) containing 0.8% NaCl/0.019% KCl/1% glucose/0.1% BSA without Ca^{++} and Mg^{++}. Test samples were orally administered to ICR mice. Thirty minutes later, PAF (10 μg/kg) was injected via tail vein. And % survival was calculated from survival mice/total mice within each group.

RESULTS

The PAF antagonistic activity was measured using [^{3}H]PAF as a ligand and rabbit platelets as a receptor source. As shown in Fig. 1, each test compound showed antagonistic receptor binding activity. CV-6209, a synthetic PAF antagonist, most

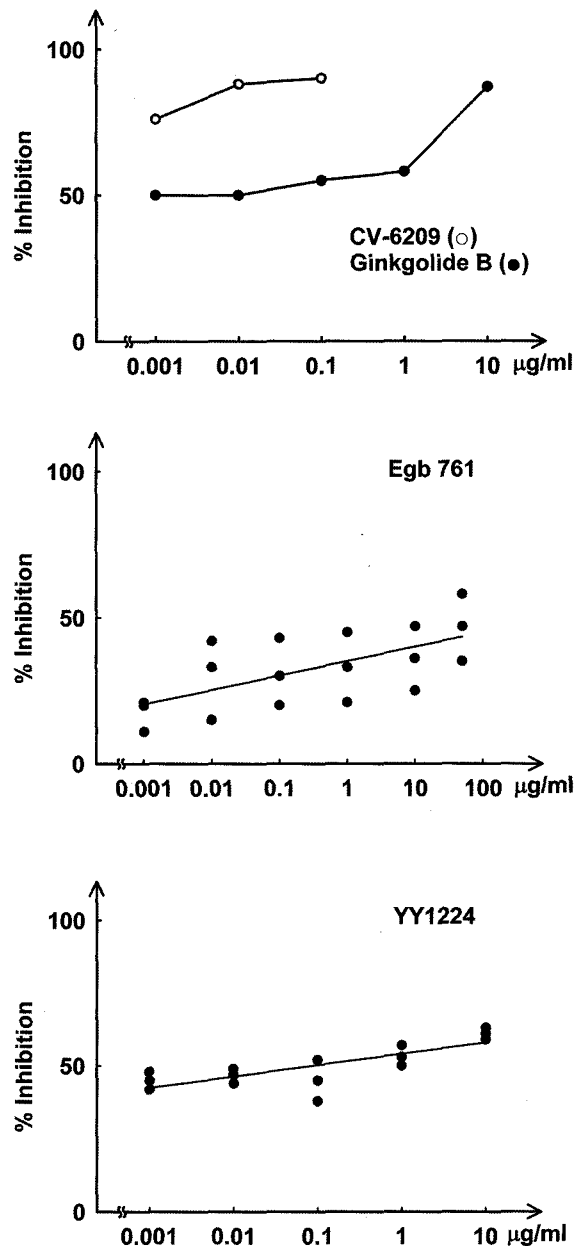

Fig. 1. Inhibition of PAF receptor binding. Rabbit platelets and [^{3}H]PAF were used for PAF receptor source and agonist, respectively. The cpm values for total and nonspecific binding were 7,765 ± 120 and 4,361 ± 102 cpm, respectively (n = 3). Each data point represented the arithmetic mean of each experiment.

potently inhibited PAF binding (IC_{50} < 0.001 μg/ml), showing 78% inhibition at 0.001 mg/ml. Ginkgolide B also showed a potent inhibition (IC_{50} = 0.06 μg/ml). When *G. biloba* extracts were compared, YY1224 showed a higher antagonistic activity than Egb 761 as expected. The IC_{50} values for YY1224 and Egb761 were found to be 0.09 and > 100 μg/ml, respectively. When the inhibitory activity against platelet aggregation was examined, the similar responses were found. Against PAF-induced aggregation, YY1224 showed a stronger inhibitory activity than Egb 761 as shown in Fig. 2. The IC_{50} values were 10.1 and 17.35 μg/ml for YY1224 and Egb 761, respectively. In this experiment, the IC_{50} of the reference molecule (ginkgolide B) was 0.16 μg/ml. These two experiments clearly demonstrated that YY1224 possessed a higher PAF antagonistic activity than Egb 761.

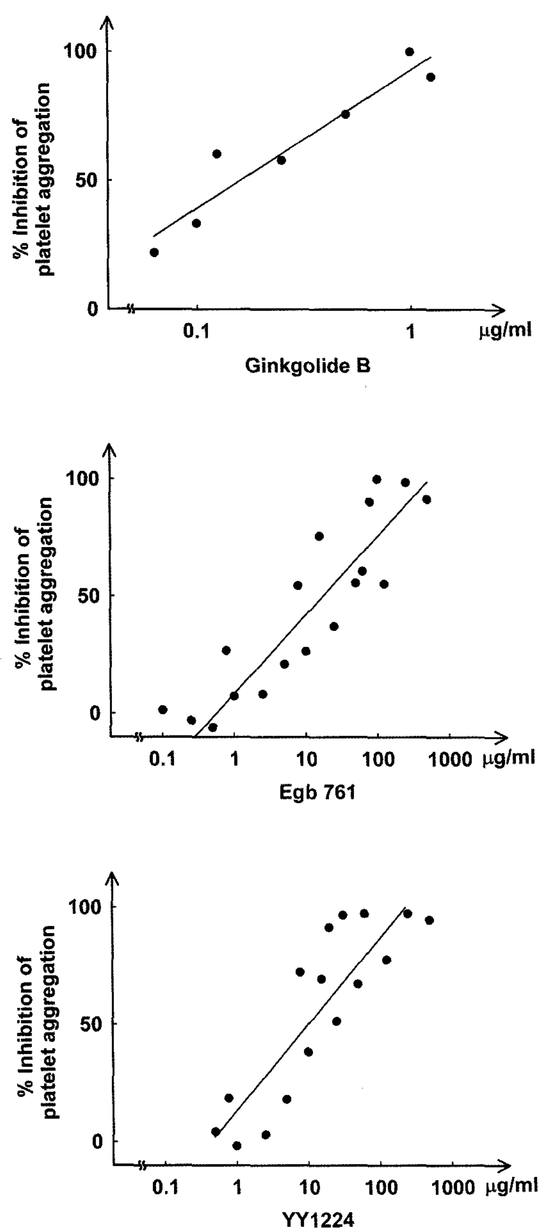

Fig. 2. Inhibition of PAF-induced platelet aggregation. Rabbit platelets were used for platelet source and 2 nM PAF was used to induce aggregation. Each inhibition rate was obtained from the maximal aggregation induced by respective PAF using the formula described in experimental section. Each data point represented the arithmetic mean of each experiment.

To extend the PAF antagonistic action to other cellular system, the effect on iNOS-mediated NO production from LPS-treated macrophage cell line, RAW 264.7, was examined. As shown in Fig. 3A, these extracts inhibited iNOS-induced NO production in somewhat different sensitivities. The IC_{50} values were found to be 64.0 and 478.9 μg/ml for YY1224 and Egb 761, respectively. In this experiment, approximately 8-fold higher potency was observed in YY sample, compared with that of Egb 761. To unveil the cellular mechanism of inhibition, iNOS mRNA and protein levels were checked. Fig. 3B and 3C have clearly shown that iNOS down-regulation at transcriptional as well as translational levels was at least in part the cellular mechanism of NO reduction by YY1224. In these experiments, YY1224 showed a higher inhibitory activity of iNOS mRNA expression, compared to Egb 761. In iNOS protein level, Egb 761 showed the comparable inhibitory activity with YY sample.

Finally, in vivo effect was examined using PAF-induced death model in mice. Table I demonstrated that PAF treatment to ICR mice gave approximately 76% lethality, while an orally active synthetic PAF antagonist (ABT491) completely protected lethal effect of PAF at 0.3 - 0.4 mg/kg. YY1224 showed some protection against PAF-triggered death at 15 mg/kg. Egb 761, however, did not show any protective activity at 5-30 mg/kg. Treatment of YY1224 and Egb 761 at 30 mg/kg exerted toxic effect (instead of protection), showing increase of lethality.

DISCUSSION

This study has proven that the terpene-strengthened *G. biloba* extract (YY1224) possesses a higher PAF-antagonistic activity in vitro and in vivo, compared with the activity of Egb 761. The IC_{50} value of Egb 761 on PAF receptor binding inhibition could not be obtained since maximum inhibition did not exceed 50%. On the other hand, Egb 761 inhibited PAF-induced platelet aggregation in comparable potency with YY sample, suggesting that some other constituents such as flavonoids may also participate in interfering this biological

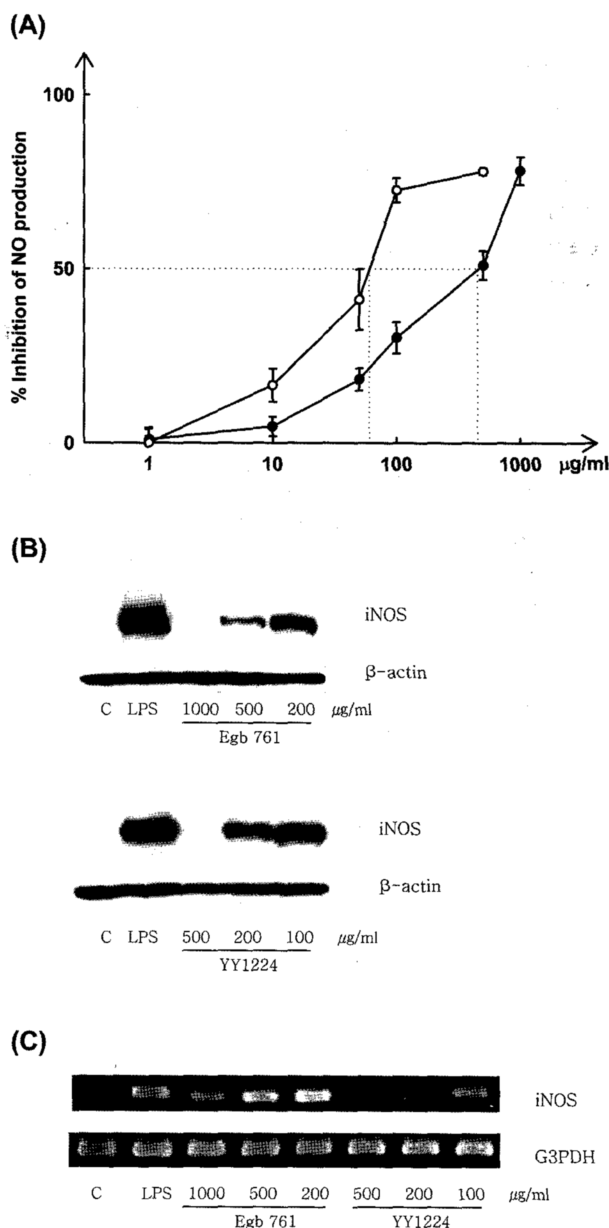

Fig. 3. Inhibition of iNOS-mediates NO production. RAW 264.7 cells were incubated with LPS (1 μg/ml) and test materials. (A) Inhibition of NO production from LPS-treated RAW 264.7 cells. YY1224 showed the significant cytotoxic effects by MTT assay, 17.9 ± 2.6% at 1000 μM. Data points and bars represented the arithmetic means ± S.D. (n = 2). YY1224 (○), Egb 761 (●). (B) Western blotting. (C) RT-PCR analysis.

response.

Previously, Egb 761 and its terpenoids such as ginkgolide A, B and bilobalide were reported to inhibit iNOS-mediated NO production from macrophage cell line, THP-1 (Cheung *et al.*, 2001). Our results were in accordance with this previous observation in that Egb 761 showed inhibition and the terpene-

Table I. Inhibition of PAF-induced death

	Dose (mg/kg)	Survival/total animals	% Survival rate
Control(PAF)	-	17/50	34
Egb 761	5	2/10	20
	15	3/10	30
	30	0/10	0
YY1224	5	3/9	33
	15	5/10	50
	30	2/10	20
ABT491	0.3	10/10	100
	0.4	10/10	100

PAF (10 μg/kg) was injected via tail vein. Mortality was determined 24 h after PAF injection.

strengthened extract had a higher inhibitory activity than Egb 761. And they inhibited NO production at least in part by down-regulation of iNOS expression.

Enhancing PAF-antagonistic activity may be favorable to treat several disease conditions including septic shock, inflammation, allergy, etc. (Tesch and Konig, 1979; Myers *et al.*, 1988; Redl *et al.*, 1990). However, the results of the present investigation indicate that the newly prepared extract (YY1224) may not be beneficial against septic shock since they showed toxic effects at the higher dose used in PAF-induced death model, instead of protection. It is not understood at present why these extracts including Egb 761 showed toxic effect in this model, but the concomitant administration of PAF and *G. biloba* extracts gave deleterious effect. Nonetheless, YY1224 showed the significant protection of PAF-induced lethality at an appropriate dose (15 mg/kg), while Egb 761 did not exert any protection at all doses tested.

In conclusion, it is found that YY1224 possesses PAF antagonistic activity in vitro and in vivo. Its potency of PAF antagonistic action is substantially higher than that of Egb 761. Therefore, it is suggested that YY1224 may show favorable property against PAF-related diseases.

ACKNOWLEDGEMENTS

This study was supported by the research fund from Ministry of Commerce, Industry and Energy, Korea (2005) and BK21 project.

REFERENCES

Akiba, S., Murata, K., Kitatani, K. and Sato, T. (2000). Involve-

ment of lipoxygenase pathway in docosapentaenoic acid-induced inhibition of platelet aggregation. *Biol. Pharm. Bull.* **23**, 1293-1297.

Bourgain, R. H., Andries, R., Esanu, A. and Braquet, P. (1992). PAF-aceter induced arterial thrombosis and the effect of specific antagonists. *Adv. Exp. Med. Biol.* **316**, 427-440.

Cheung, F., Siow, Y. L. and O, K. (2001). Inhibition by ginkgolides and bilobalides of the production of nitric oxide in macrophages (THP-1) but not in endothelial cells (HUVEC). *Biochem. Pharmacol.* **61**, 503-510.

Chi, Y. S., Cheon, B. S. and Kim, H. P. (2001). Effect of wogonin, a plant flavone from Scutellaria radix, on the suppression of cyclooxygenase and the induction of inducible nitric oxide synthase in lipopolysaccharide-treated RAW 264.7 cells. *Biochem. Pharmacol.* **61**, 1195-1203.

DeFeudis, F. V. (1991). Ginkgo biloba extract (Egb761): pharmacological activities and clinical applications. Elsevier, Paris.

Hanahan, D. J. (1987). Platelet activating factor: A biologically active phosphoglyceride. *Ann. Rev. Biochem.* **55**, 483-509.

Koltai, M., Hosford, D. and Braquet, P. G. (1996). Platelet-activating factor and its antagonists in allergic and inflammatory disorders. *Clin. Allergy Immunol.* **8**, 463-489.

Myers, A. K., Nakanishi, T. and Ramwell, P. (1988). Antagonism of PAF-induced death in mice. *Prostaglandins* **35**, 447-458.

Prescott, S. M., Zimmerman, G. A. and McIntyre, T. M. (1991). Platelet-activating factor. *J. Biol. Chem.*, 17381-17384.

Redl, H., Vogl, C., Schiesser, A., Paul, E., Thurnher, M., Bahrami, S. and Schlag, G. (1990). Effect of the PAF antagonist BN 52021 in ovine endotoxin shock. *J. Lipid Mediat.* **2 Suppl**, S195-S201.

Terashita, Z., Kawamura, M., Takatani, M., Tsushima, S., Imura, Y. and Nishikawa, K. (1992). Beneficial effects of TCV-309, a novel potent and selective platelet activating factor antagonist in endotoxin and anaphylactic shock in rodents. *J. Pharmacol. Exp. Ther.* **260**, 748-755.

Tesch, H. and Konig, W. (1979). Origin and biological role of lipid mediators during inflammation. *Immun Infekt.* **7**, 157-164.

Valone, F. H., Coles, E., Reinhold, V. R. and Goetzl, E. J. (1982). Specific binding of phospholipid platelet-activating factor by human platelets. *J. Immunol.* **129**, 1637-1641.