# Inhibition of HMGB1 Translocation by Green Tea Extract in Rats Exposed to Environmental Tobacco Smoke

Sirintip Chaichalotornkul [a], Wisuda Suvitayavat [b], Vanida Sangalangkarn [c], Yuko Nawa [d], Kiyoshi Kikuchi [e],
Koichi Kawahara [f], Tawee Saiwichai [g], Somphong Narkpinit [a], Pratap Singhasivanon [h], Ikuro Maruyama [f]
and Salunya Tancharoen [c]

[a] Department of Pathobiology, Faculty of Science, Mahidol University
[b] Department of Physiology, Faculty of Pharmacy, Mahidol University
[c] Department of Pharmacology, Faculty of Dentistry, Mahidol University
[d] Department of Anesthesiology, Sapporo Medical University School of Medicine, Sapporo, Japan
[e] Department of Neurosurgery, Yame Public General Hospital, 540-2 Takatsuka, Yame 834-0034, Japan
[f] Department of Laboratory and Vascular Medicine, Kagoshima University Graduate School of Medical and
Dental Science, Kagoshima, Japan
[g] Department of Parasitology and Entomology, Faculty of Public Health, Mahidol University
[h] Department of Tropical Hygiene, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand

## Abstract

Environmental tobacco smoke (ETS) exposure is linked to carcinogenic, oxidative and inflammatory cellular reactions. Green tea polyphenol reportedly plays a role in the prevention of inflammation-related diseases. To evaluate the effects of green tea extract (GTE) on cellular location of High Mobility Group Box-1 (HMGB1) protein, we studied the lung tissue in rats exposed to cigarette smoke (CS). Rats were divided into three groups; CS, CSG, and C, which were groups of CS-treated only, CS-treated with GTE dietary supplement, and the control, respectively. Our findings by immunocytochemistry showed that abundant HMGB1 translocated from the nucleus to the cytoplasm in the lung tissues of rats that were exposed to CS, whereas HMGB1 was localized to the nuclei of CSG and C group. For *in vitro* studies, cotinine stimulated the secretion of HMGB1 in a dose and time dependent manner and the HMGB1 level was suppressed by GTE in murine macrophage cell lines. Our results could suggest that GTE supplementation which could suppress HMGB1 may offer a beneficial effect against diseases.

*Keywords:* green tea; HMGB1; environmental tobacco smoke (ETS); nuclear translocation

## 1. Introduction

Environmental tobacco smoke (ETS) or second-hand smoke, the combination of two forms of sidestream and mainstream smoke from burning tobacco products contains thousands of chemicals, and among these ten are known carcinogens (Moir *et al*., 2008). The main cause of death in 2000 was tobacco smoke; 18.1% of 435,000 US deaths (Mokdad *et al*., 2004). Exposure to ETS impairs the function of various organs and may result in respiratory illness through inflammatory processes and oxidative stress leading to cell injury. Moreover, this can worsen existing pulmonary symptoms in people with asthma and chronic bronchitis (Rona *et al*., 1985; Budhiraja *et al*., 2004; Eisner *et al*., 2005; Bruske-Hohlfeld, 2009). Involuntary smoking of ETS causes lung cancer in never-smokers with an excess risk in the order of 20% for women and 30% for men (Husgafvel-Pursiainen, 2004).

HMGB1 is a highly conserved chromosomal protein, an omnipresent protein ubiquitously expressed in the nuclei and cytoplasm of nearly all cell types (Yang *et al*., 2010). HMGB1 is involved in many biological processes including chromatin remodeling, transcription, cell signaling of inflammation, DNA damage repair and others (Liu *et al*., 2010a). Oxidative stress induces the presence of HMGB1 as nuclear translocation (Hamada *et al*., 2008) and is released in the extracellular medium as a response to various stimuli implicated in cancerogenesis (Kostova *et al*., 2010). Several recent reports indicate that HMGB1 has a role in pro-inflammatory activity and is found in various cell types, including inflammatory cells, smooth muscle cells, endothelial cells and squamous-cell carcinoma (Inoue *et al*., 2007; Liu *et al*., 2010b; Luan *et al*., 2010). Recent findings identify HMGB1 as a cytokine-like mediator of endotoxin lethality and present in response to lung injury (Lotze *et al*., 2005).

Green tea contains a polyphenolic rich compound such as epigallocatechin gallate (EGCC). Polyphenolic compounds, particularly phenolic acids, have powerful biological activities *in vitro* and *in vivo* such as anti-adherence, anti-oxidant and anti-inflammation (Ofek *et al*., 2003; Tipoe *et al*., 2007). Epigallocatechin-3-gallate inhibits interleukin-1 beta (IL-1β), an important mediator of the inflammatory response, is involved in various cellular activities *e.g*., cell proliferation, differentiation, and apoptosis (Kim *et al*., 2008). Moreover, it can reduce vascular inflammation by increasing the NO synthesis, which blocks endothelial exocytosis. It also has a high potential to impair the UVB-induced infiltration of leukocytes and the subsequent ROS generated in human skin (Katiyar *et al*., 1999).

We hypothesized that CS leads to HMGB1 shuttling from the nuclei to the cytoplasm of lung tissue and subsequent induce tissue injury. GTE as a naturally occurring compound with known anti-inflammatory properties may have therapeutic potential against the effects of CS, involving inflammation and oxidative stress through the nuclear protein HMGB1.

## 2. Materials and Methods

### 2.1. Animal model and green tea extract

The animal model and green tea extract processes were conducted as described previously (Saiwichai *et al*., 2010). Briefly, 14-week-old male rats (*Rattus norvegicus*) were kept on a 12-h light/dark cycle under specific pathogen-free conditions, with 22°C controlled temperature and *ad libitum* food and water provided. The acclimatization time for rats was provided 1 wk before treatment was started. All animal procedures were approved by the Animal Care and Use Committee, Mahidol University. Fifteen rats were randomized to 3 groups and treated with: (1) CS only, (2) dietary supplement with GTE 4.5 mg/day and CS (CSG), and (3) the control group (C), rats with neither GTE nor CS provided. The investigators were blinded to specific treatment.

Fresh green tea leaves originating from Indonesia were prepared with a slight modification (Saiwichai *et al*., 2010). The extract contained 39% (w/w) epigallocatechin-3-gallate (EGCC), 4.5% epigallocatechin (EGC), 17.52% epicatechin gallate (ECG), 3.7% epicatechin (EC), and 1.4% catechin. Standard catechin derivatives were from Sigma Biochemicals (St. Louis, MO USA).

### 2.2. Rats' green tea consumption and environmental tobacco smoke exposure protocol

Rats of the treated group (CSG) fasted for 2 h; then received diet supplemented with GTE 4.5 mg/day for the entire 8-wk experiment. The same diet without GTE was given for control group rats. Normal food chunks and water were provided *ad libitum* after rats finished the restricted GTE food, and halted again before the restricted GTE-food was provided. Rats of the treatment group were exposed to CS from 4 cigarettes twice daily 5 days/wk (Mon-Fri) 4 wk (weeks 5 to 8). The smoke was produced by introducing ignited cigarettes into sterile whole-body exposure chambers with airflow ventilation (4 rats/container). The control group rats were placed into the chamber and exposed to laboratory air. These two processes were performed as previously described (Saiwichai *et al*., 2010).

### 2.3. Immunocytochemistry

We investigated whether ETS exposure influenced HMGB1 cellular location on lung tissue of rats. The immunocytochemistry assay was conducted as described previously (Nawa *et al*., 2009). After tissues were embedded in paraffin, 6-microns thick sections were cut, deparaffinized and rehydrated. The slides were blocked with 1% BSA in PBS containing 0.1% Triton X-100. After washing, the slides were incubated with primary antibody (anti-HMGB1 rabbit polyclonal antibody) at room temperature for 1 h. After further washing, the slides were incubated with Alexa Fluor 488-labeled goat anti-rabbit IgG (1:200), washed again, and stained with DAPI. Cells were visualized by immunofluorscence under an Axioskop microscope (Carl Zeiss, Oberkochen, Germany).

### 2.4. Cell culture and treatment

RAW264.7 cells were maintained in RPMI 1640 medium (GibCo BRL, Grand Island, NY) with 2 mmol/L glutamine (Hyclone Logan, UT) and 10% fetal bovine serum supplementation. At 80 to 90% confluence, cells were washed twice, then cultured in serum-free OPTI-MEM I medium (GibCo BRL) and stimulated by cotinine (Sigma; St. Louis, MO, USA) with or without GTE at various concentrations from 0.025 to 25 μg/ml.

### 2.5. Western blotting

HMGB1 levels in culture supernatants were analyzed by Western blot analysis as described previously (Kawahara *et al*., 2008). In brief, each culture supernatant was incubated with 50 μl heparin-sepharose 6B beads for 4 h. The heparin beads were then washed

with 10 mM PBS (pH 7.0), mixed with 50 μl sample buffer (62.5 mM Tris-HCl, pH 6.8), 2% sodium dodecyl sulfate (SDS), 10% glycerol, and 0.002% bromophenol blue, and boiled for 5 min. These HMGB1 samples (40 μl) were subjected to 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE); separated proteins were subsequently transferred onto a nitrocellulose membrane (GE Healthcare Bio-Sciences KK, Piscataway, NJ). The membrane was blocked with 5% nonfat dry milk in TBST at room temperature (RT) for 1 h and was then incubated with 2 μg/ml of the anti-HMGB1 antibody (Shino-Test, Kanagawa, Japan) in TBST. The membrane was washed and incubated with HRP-conjugated anti-rabbit IgG polyclonal antibody (Invitrogen) in TBST containing 2.5% nonfat dry milk at RT. The membrane was washed once more, and immunoreactive bands formed were visualized using an enhanced chemiluminescence detection system (GE Healthcare Bio-Sciences KK).

## 2.6. Statistical analysis

In Western blot analysis, bands corresponding to HMGB1 amounts were measured using NIH Image 1.61 software. Results of the densitometric analysis of protein signal differences were evaluated by non-parametric statistical test. Significant differences in inhibition-study tests were analyzed by Mann–Whitney U test procedure.

## 3. Results and Discussion

### 3.1. Inhibitory effect of GTE on HMGB1 release in the lung tissues of rats exposed to CS

Our findings showed a distinct translocation of nuclear HMGB1 to the cytoplasm of alveolar cells in the lung tissues of rats which were exposed to CS (arrowhead, Fig. 1(D-F)), whereas HMGB1 was localized to the nuclei of C group [(Fig. 1 (A-C)]. GTE inhibited HMGB1 translocalization to the cytosol [Fig. 1 (G-I)]. These results demonstrated that CS-induced HMGB1 expression in the cytoplasm and GTE could inhibit its transportation. Our study correlates well with prior studies indicating that HMGB1 is translocated from the nucleus to the cytosol and released into the serum following tissue damage and immune response (Oppenheim and Yang, 2005; El Gazzar, 2007; Sivasubramaniyan *et al.*, 2008). These results imply that the polyphenol compounds which contain in the GTE may have a therapeutic effect over HMGB1 and protect against inflammation.

### 3.2. Cotinine effects on HMGB1 in murine macrophage-like cells line (RAW264.7 cells)

Cotinine is a major metabolite of nicotine. It has been recently used as a biochemical marker of ETS (Benowitz, 1996; Jarvis, 1987). HMGB1 released from murine macrophage-like cells has a well documented effect on inflammatory responses (Wang *et al.*, 1999). We investigated whether cotinine could induce HMGB1 release from RAW264.7 cells. For this purpose, cells were stimulated with cotinine in concentrations similar to those observed in serum of CS exposure rats (Saiwichai *et al.*, 2010). Cotinine-induced HMGB1 released into the supernatant in a dose dependent fashion, reached a peak at 1 μg/ml as determined by western blot analysis (Fig. 2). Cotinine stimulation for 10 ng/ml significantly increased HMGB1 release compared with the control ($p < 0.05$), while stimulation with 100 and 1,000 ng/ml resulted in an even higher significance of difference ($p < 0.01$).

### 3.3. GTE suppresses cotinine-induced HMGB1 RAW264.7 cells in a dose-dependent manner.

RAW264.7 cells were pre-treated with various concentrations of GTE (0.025, 0.25, 2.5 and 25 μg/ml) and then stimulated with cotinine. HMGB1 levels were determined by Western blotting (Fig. 3). The results showed that GTE concentration ranging from 0.025 to 25 μg/ml significantly suppressed cotinine-induced HMGB1 release ($p<0.05$) in a dose dependent manner. Corresponding to the western blot analysis, HMGB1 reduced by ~77% in the presence of 25 μg/ml GTE and cotinine compared with the cotinine stimulated cells alone as previously investigated by enzyme-linked immunosorbent assay (ELISA) technique (Saiwichai *et al.*, 2010). These *in vitro* studies supported our results from the rat model in which GTE suppresses HMGB1 release, suggesting that CS induces an important role in chronic inflammation and GTE may have a therapeutic potential for ETS-related diseases. Therefore, a greater understanding of its role and signaling pathway should be further investigated.

## 4. Conclusion

It can be hypothesized that the translocation of HMGB1 from the nucleus to the cytoplasm in the lung tissues of rat model and its release into the cell supernatant of macrophage cell lines may play an important in the pathophysiology of lung injury and that modulation of this pro-inflammatory mediator by GTE might reveal new therapeutic modalities.
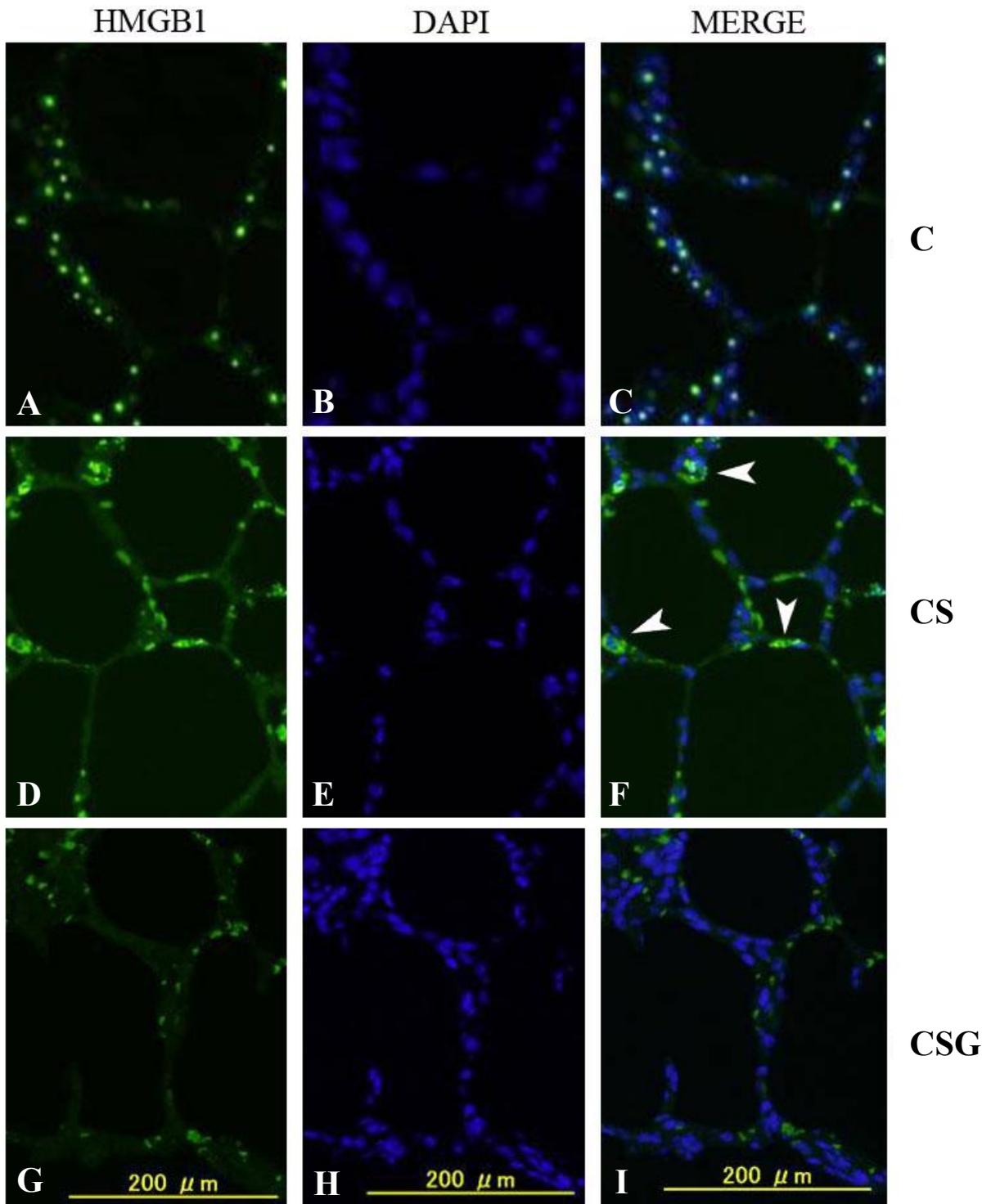
Figure 1. Inhibitory effect of GTE on HMGB1 release in the lung tissues of rats exposed to CS. Lung tissues from the control (C) group: A-C, Cigarette smoke (CS) group: D-F, Cigarette smoke with green tea supplement (CSG) group: G-I were incubated with anti-HMGB1 rabbit polyclonal antibody (green). Nuclei were labeled with 4', 6-diamidino-2-phenylindole (DAPI), blue.
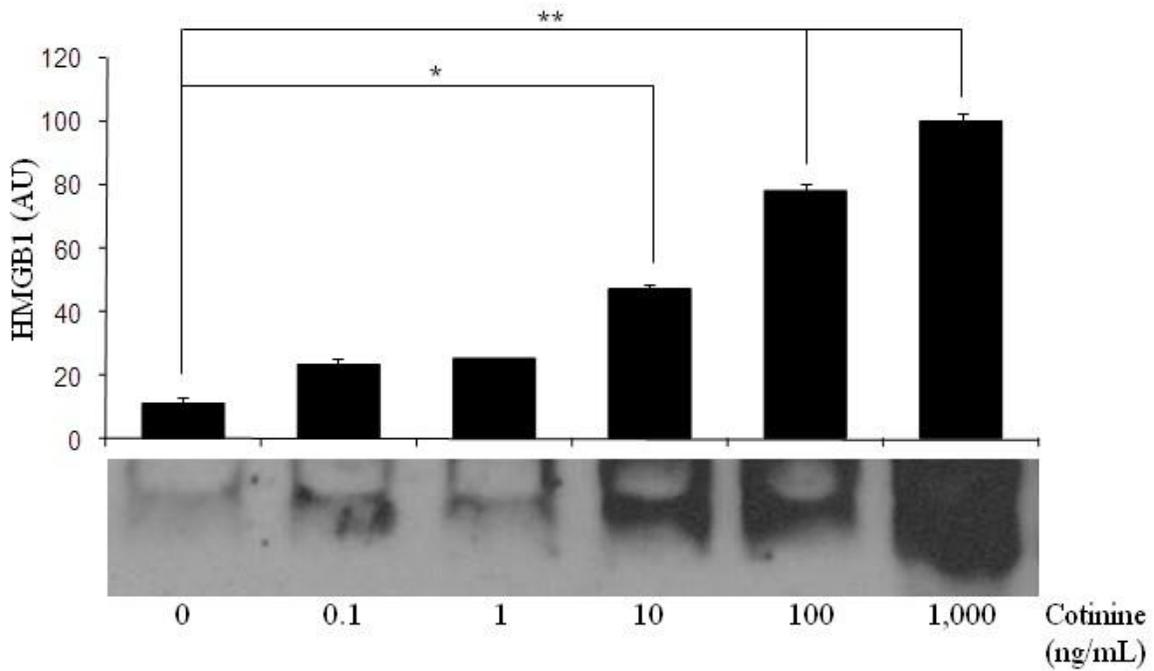
Figure 2. Evaluation of HMGB1 levels in the RAW264.7 cells supernatant. Cells were exposed to various concentrations of cotinine (0.1, 1, 10, 100 and 1,000 ng/ml) for 16 h, the supernatant was collected and evaluated for HMGB1 level by Western blotting. The results are shown as means ± SD, experiments were performed in triplicate. *$p < 0.05$, **$p < 0.01$.
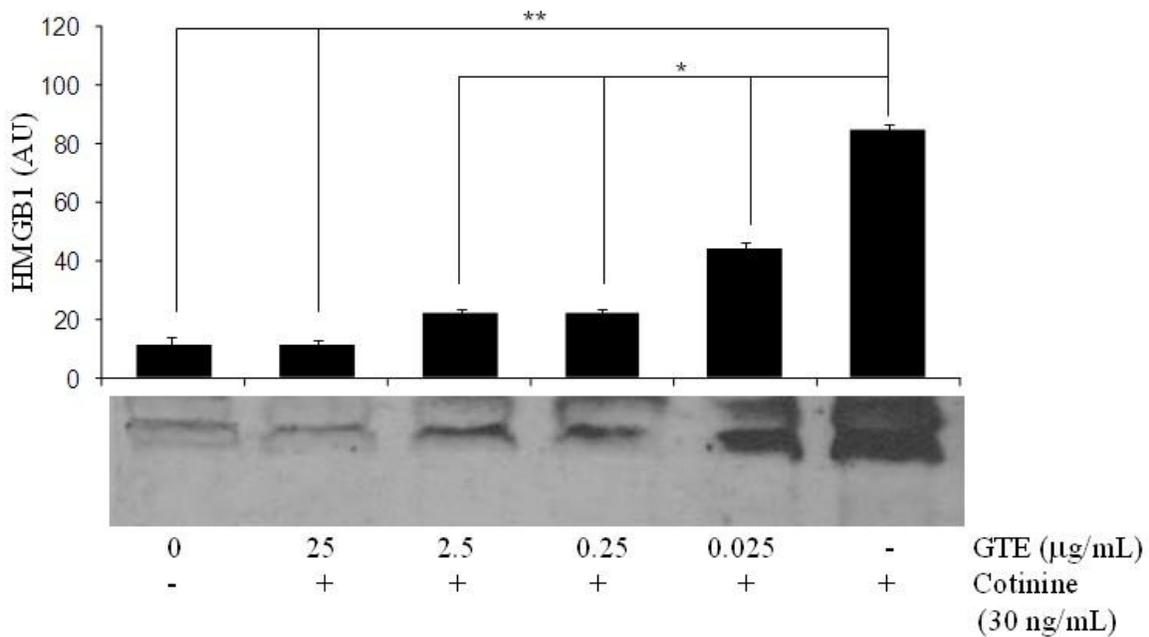


Figure 3. GTE suppresses cotinine-induced HMGB1 in RAW264.7 cell culture determined by Western blotting. RAW264.7 cells were exposed to various concentrations of GTE at 0.025, 0.25, 2.5 and 25 μg/ml with and without cotinine (30 ng/ml). The results are shown as means ± SD, condition performed in triplicate. *$p < 0.05$, **$p < 0.01$.

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**Correspondence to**
Dr. Salunya Tancharoen
Department of Pharmacology,
Faculty of Dentistry,
Mahidol University,
Bangkok 10400,
Thailand.
Tel: 66 (0) 2203-6481 ext. 18
Fax: 66 (0) 2203-6484
E-mail address: dtsta@mahidol.ac.th