

Full Length Research Paper

Protective effect of piperine from *Piper chaba* fruits on LPS-induced inflammation in human intestinal cell line

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Received 6 June, 2020; Accepted 6 July, 2020

***Piper chaba* is a flowering vine in the family of piperaceae. It is a spicy plant widely used as food and herbal medicine in the South and Southeast Asian countries. The study evaluated anti-inflammatory and immunomodulatory activities of a major alkaloid extract, piperine, from *P. chaba* fruits on human colonic epithelial cells. The effects of piperine extract on cell viability, pro-inflammatory cytokine inhibition, and immunomodulation on inflammatory genes were evaluated *in vitro* using lipopolysaccharide (LPS)-induced inflammatory model in Caco-2 cells. *T*-test and ANOVA were used for statistical analysis. Piperine, in the doses of non-cytotoxicity (1, 10, 50 µg/ml), reversed effect of LPS on pro-inflammatory cytokines; tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and interleukin (IL)-6 production. In addition, it also confirmed the activity on mediating IκB-α/NF-κB signaling proteins by activating IκB-α and suppressing NF-κB expression leading to attenuate pro-inflammatory cytokine release during the inflammatory process. The study concludes that piperine from *P. chaba* inhibits LPS-induced inflammation in intestinal epithelial cells by modulating cytokine gene expression via down regulation of IκB-α/NF-κB signaling pathway.**

Key words: Piperine, Pro-Inflammatory Cytokine, Ikb-A, Nf-Kb, Caco-2 Cells.

INTRODUCTION

Inflammation is part of the body reaction to invasive stimuli. The immune cells are responsible for inflammatory response by such as macrophages, neutrophils, lymphocytes and epithelial cells (Cheng et al., 2018). When the host is stimulated by micro-organisms or tissue injury, the guard cells react on acute-phase response by releasing acute-phase proteins including serum amyloid, chemokines, mediators, or inflammatory cytokines, to play a concert in inflammatory

process leading to several systemic responses (Buamann and Gauldie, 1994; Laskin and Pendino, 1995; Gabay and Kushner, 1999). The significant pro-inflammatory cytokines or mediators participating at the beginning of inflammatory process are tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and interleukin (IL)-6, nitric oxide (NO) or prostaglandin (PG) E₂ (Buamann and Gauldie, 1994; Gabay and Kushner, 1999).

Gastrointestinal (GI) epithelia also play an important

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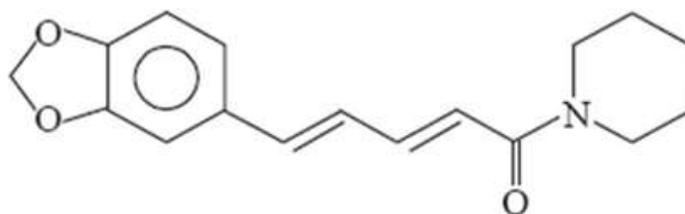


Figure 1. Structure of piperine.

role in immunity because they are the first line of barrier against external environment, the GI content, located in our body. The ingested foods, as an external environment, contain invasive pathogens, chemicals, and toxins that can produce inflammation (Chen et al., 2006). These inflammatory stimulators activate innate and acquired immune response from immune cells residing in the gut wall, or intestinal epithelial cells lining intestinal mucosa, resulting in mucosal inflammation. If an inflammation is not removed, it will bring the situation into inflammatory disease such as inflammatory bowel diseases (IBD) which is caused by pathogens, non-pathogens, or autoimmune disorders (Chakravorty and Kuma, 1999; Panja et al., 1998). For bacterial cause, lipopolysaccharides (LPS) are inflammatory factors derived from outer part of gram-negative bacteria and are active parts to stimulate inflammation. Therefore, They are usually used in the study of inflammatory mechanism in *in vitro* model (Choi et al., 2014).

Plants are naturally potential sources of medicinal agents nowadays. Previous studies showed many beneficial effects of plants as herbal medicine and as natural sources of pharmaceutical active compounds. For herbal medicine, they can be potentially used as crude of both a single plant or incorporated into herbal recipe. *Piper chaba* is a flowering vine in the family of piperaceae. It is a spicy plant of pepper family which is widely used as food and herbal medicine in South and Southeast Asian countries. It is a creeper plant spreading on the ground or growing around the large trees. The fruit looks similar to long pepper. It turns red when ripe and turns dark brown or black when dry. In Thailand, *P. chaba* is called 'Dee Plee'. The stem, root, and fruit of Dee Plee, are commonly used in the recipe of folk medicine (Islam et al., 2020).

Piperine (Figure 1) is a major compound derived from natural herbs such as curcumin, *P. chaba* and *Piper nigrum* (black pepper) (Naz et al., 2012). These pepper plants are usually used as spice in foods and as phytomedicine. As a dietary medicine, piperine extract has been shown to exhibit many pharmaceutical effects such as anti-oxidant (Prakash and Srinivasan, 2010; Rauscher et al., 2000) anti-inflammation (Srinivasan, 2007; Tasleem et al., 2014), antispasmodic activity (Doucette et al., 2013; Mehmood and Gilani, 2010),

anti-angiogenesis (Lu et al., 2012), antimalarial activity (Thiengsusuk et al., 2018), anti-diarrhea by inhibiting cAMP-mediated Cl⁻ secretion in human colonic epithelial cells (Pongkorpsakol et al., 2015), and prevention of gallstones (Li et al., 2015a). However, the role of piperine on anti-inflammation on human intestinal epithelial cells has not been reported. The study evaluated an effect of piperine extracted from *P. chaba* fruits on LPS-induced pro-inflammatory cytokine release and the immunomodulation in Caco-2 cells (Figure 1).

MATERIALS AND METHODS

Plant extraction

P. chaba fruits were collected, identified, and extracted by the Thai Medicine Development Center, National Science and Technology Development Agency (NSTDA), Pathum Thani, Thailand, 12120. Piperine extraction was done using high performance liquid chromatography (HPLC). The yellow crystals of piperine extract were done for quantitative analysis and the purification obtained is 95%.

Chemicals

Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich (St. Louis, MO, USA). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was purchased from Invitrogen Thermo Fisher Scientific (Waltham, MA, USA). Materials for cell culture were purchased from Gibco-BRL (Grand Island, NY, USA). Enzyme-linked immunosorbent assay (ELISA) kits for TNF- α , IL-1 β , and IL-6 quantification were brought from Merck Millipore (Etobicoke, Ontario, Canada). InstantOne™ ELISA kits for inhibitory kappaB (I κ B)- α and nuclear factor-kappaB (NF- κ B) (p65) analysis were purchased from Affymetrix eBioscience (Waltham, MA, USA).

Cell culture

Caco-2 cells were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). They were cultured in dulbecco's modified eagle medium (DMEM)/F12 containing 10% fetal bovine serum (FBS) (Gibco-BRL, Grand Island, NY, USA), penicillin (100 U/ml) and streptomycin (100 μ g/ml) at 37°C in a humidified atmosphere of 5% CO₂. Piperine extract was dissolved in DMSO and was diluted to stock concentrations with free FBS culture medium. Cells were incubated with piperine in various concentrations and were stimulated with LPS for the incubation time.

Table 1. Effect of piperine extract on cell culture viability.

Parameter	Concentration of piperine ($\mu\text{g/ml}$)					
	Control	0.1	1	10	50	100
%Cell viability	100	101.4 \pm 3.4	100.3 \pm 2.5	99.8 \pm 2.8	99.8 \pm 3.1	84.6 \pm 5.8*

*Statistical significance as $p < 0.05$ when compared with the control group.

Cell viability assay

The cell viability was measured based on MTT colorimetric assay. Caco-2 cells of 1×10^4 cells/ml were grown in 96-well plates until confluence. Fresh media were added and the cells were further incubated at 37°C with piperine (0.1, 1, 10, 50 and 100 $\mu\text{g/ml}$) for 8 h in a humidified atmosphere of 5% CO_2 . At the end of the incubation time, fresh medium and tetrazolium dye (MTT) solution (5 mg/ml) were added. The cells were further incubated for 4 h at 37°C in a humidified atmosphere of 5% CO_2 . A 200 μl aliquot of DMSO was added and the absorbance at 540 nm was measured in a Bio Tex Power Wave XS automatic microplate reader (Bio Tex Instrument Inc., Winooski, VT, USA).

Pro-inflammatory cytokine determination

Caco-2 cells of 5×10^5 cells/ml were grown in 24-well plates until they reached confluence. After that, they were incubated with various concentration of piperine extract (1, 10, and 50 $\mu\text{g/ml}$) in an absence or presence of LPS (1 $\mu\text{g/ml}$) for 24 h at 37°C in a humidified atmosphere of 5% CO_2 . Supernatant fluids were collected and stored at -80°C until cytokines were analyzed. The pro-inflammatory cytokines were determined by using commercial human TNF- α , IL-1 β and IL-6 ELISA kits according to the manufacturer's instructions.

Signaling protein determination

Caco-2 cells of 1×10^4 cells/ml were seeded in 96-well plate and were incubated with only LPS (1 $\mu\text{g/ml}$) and LPS plus various concentrations of piperine (1, 10, and 50 $\mu\text{g/ml}$) for 24 h at 37°C in a humidified atmosphere of 5% CO_2 . The cells were washed with phosphate buffer saline (PBS) and then lysed with lysis buffer included with InstantOne TM ELISA kits. The signaling proteins of I κ B- α and NF- κ B (p65) were determined by using commercial human InstantOne TM ELISA kits according to the manufacturer's instructions. The absorbance at 450 nm for O.D. values was measured in a Bio Tex Power Wave XS automatic microplate reader (Bio Tex Instrument Inc., Winooski, VT, USA).

Statistical analysis

Data from at least three individual experiments in each protocol were analyzed, and are presented as mean \pm SEM. Statistical significance was determined using one-way ANOVA and student *t*-test. P value less than 0.05 is statistically significant.

RESULTS

Cytotoxic effect of piperine

To study the cytotoxicity of piperine extract on culture

cells, the cells were treated with progressively increase in piperine concentration (0.1, 1, 10, 50, 100 $\mu\text{g/ml}$). Cell survival was evaluated by MTT calorimetric assay. The results are shown in Table 1. Piperine extract in the doses of 0.1 to 50 $\mu\text{g/ml}$ shows survival rate of nearly 100% compared to non-treated control group, while the concentration of 100 $\mu\text{g/ml}$ reveals significantly decreased cell viability.

Effect of piperine on LPS-induced pro-inflammatory cytokine production

Piperine extract, in the concentration of non-cytotoxicity (0.1, 1, 10, 50 $\mu\text{g/ml}$), is further used to evaluate the effect on pro-inflammatory cytokine production. The concentrations of TNF- α , IL-1 β , and IL-6 were measured in cell culture media of LPS-treated and non-treated groups after 24 h incubation by ELISA method. The cells were divided into four groups: Group 1, growing without both LPS and piperine; Group 2, growing with only LPS (1 $\mu\text{g/ml}$); Group 3, growing with only piperine in various concentrations; and Group 4, growing with LPS plus piperine of non-cytotoxic concentrations. The results demonstrate that piperine alone has no effect on pro-inflammatory cytokine stimulation; however, LPS alone acts as a potent inflammatory stimulator by activating pro-inflammatory cytokine release. Piperine extract clearly reveals a protective effect on inflammation by suppressing pro-inflammatory cytokine release during LPS stimulation in a dose-dependent manner (Figure 2).

Effect of piperine on LPS-induced signaling protein, expression

LPS stimulates inflammatory response via NF- κ B/Rel family signaling proteins. In the resting state, NF- κ B forms dimer with I κ B- α and locates in the cytosol. During LPS activation, I κ B- α dissociates from NF- κ B and is degraded in the cytosol while NF- κ B moves into the nucleus to stimulate various inflammatory genes. To investigate the role of piperine extract on cellular mechanism of pro-inflammatory cytokine production, I κ B- α and NF- κ B expression was evaluated using instantOne ELISA which the principle is close to semi-quantitative analysis of western blot. The levels of signaling proteins, I κ B- α and NF- κ B, in various groups of study are shown

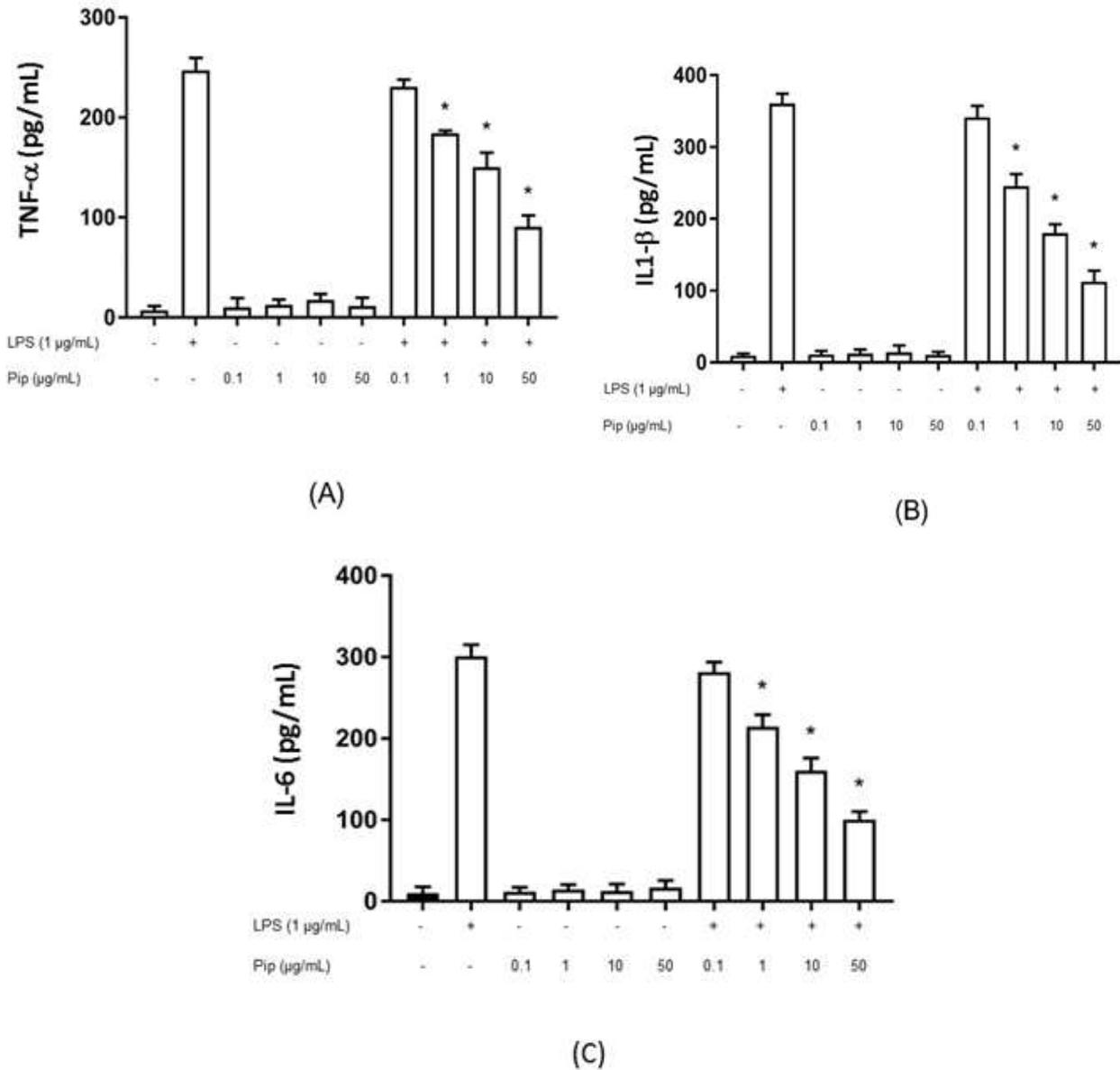


Figure 2. Effect of piperine on TNF- α (A), IL-1 β (B), and IL-6 (C), production in Caco-2 cells. The cells were treated with and without LPS (1 μ g/ml) plus piperine at indicated doses. Culture media were evaluated for pro-inflammatory cytokine levels by ELISA method. Data are depicted as mean \pm SEM with ANOVA analysis. * Statistical significance as $p < 0.05$ when compared with LPS alone. LPS: lipopolysaccharide; Pip: piperine.

in Figure 3 as O.D. values compared with those of positive control of instantOne ELISA.

LPS alone significantly suppressed I κ B- α expression in Caco-2 cells. However, the concentrations of cytosolic I κ B- α were significantly resumed in a concentration-dependent manner when the cells were added with piperine extract. An expression of NF- κ B, on the other hand, was exponentially increased when the cells were incubated with only LPS. This protein levels were significantly reduced in a concentration-dependent manner when the cells were co-cultured with piperine

extract.

DISCUSSION

Our body has defensive mechanisms to protect ourselves from aggressive invaders such as pathogens, toxins, and chemicals. This is an innate immunity that attempts to remove attackers and to initiate healing process known as inflammation which is characterized by pain, swelling, redness, and hot. In the GI tract, the innate immunity is

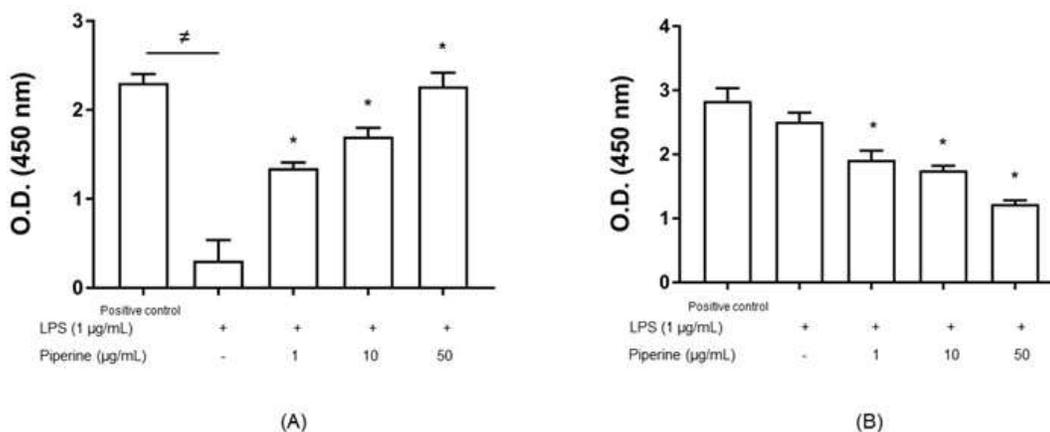


Figure 3. Effect of piperine on activation of IκB-α (A) and suppression of NF-κB (B) in Caco-2 cells. The cells were treated with LPS (1 μg/ml) alone and LPS plus piperine at indicated concentrations. IκB-α and NF-κB levels in cell lysate were evaluated using instantOne ELISA. *Statistical significance as $p < 0.05$ when compared with LPS-treated group, and # as $p < 0.05$ when compared with the positive control group. LPS: lipopolysaccharide; Pip: piperine.

responsible by immune cells as well as epithelial cells lining the gut (Neurath et al., 1998). When the body is stimulated by pathogenic stimuli, these guard cells release chemokines such as leukotriene and histamine, inflammatory mediators such as prostaglandins (PGEs) and (NO), or inflammatory cytokines e.g. TNF-α, IL-1β, or IL-6 to act on inflammatory process. Interleukin-1β stimulates the expression of adhesive molecules on endothelial cells to allow the migratory action of leukocytes to an inflammatory site and also involves with hyperalgesia and fever (Jeong et al., 2014). TNF-α promotes the production of other inflammatory cytokines to join an inflammation that can lead to autoimmune disorders (Guadagni et al., 2007). The intestinal mucosa, as the first line of bodily protection, encounters toxins from pathogens which commonly cause inflammation. Chronic or recurrent inflammation, can cause inflammatory diseases.

There are documents supporting that the important pathways regulating inflammatory response in several cell types are MAPKs and IκB-α/NF-κB (Ying et al., 2013a, b). LPS, provokes an immune response in host cells via mediating membrane receptor protein such as Toll-like receptor 4 (TLR4) (Palsson-McDermott and O'Neill, 2004). This protein receptor passes the signal through cytosolic signaling proteins such as NF-κB/Rel family to the nucleus and activates several genes of inflammation and necrosis (Hayden et al., 2006; Beinke and Ley, 2004). Suppression on these genes has been accepted to become a good way for curing inflammatory diseases (Salminen et al., 2012; Nam, 2006). The nuclear protein NF-κB modulates the inflammatory genes by coordinating with cytosolic inhibitory protein, IκB-α. During unstimulated state, NF-κB protein forms dimer to IκB-α in the cytosol. When LPS binds to the membrane

receptor and the cell is stimulated, IκB-α is phosphorylated and dissociates from NF-κB to the process of degradation (Hayden et al., 2006; Choi et al., 2014). The free NF-κB moves into the nucleus as transcription factor, binds to the target genes, and stimulates inflammatory cytokine release. Therefore during inflammation, the level of IκB-α could decrease and the level of NF-κB could increase.

Piperine is an alkaloid substance found in several medicinal plants such as, curcumin, black pepper, and long pepper (Yaffe et al., 2015). It has been proposed to have many therapeutic effects in traditional medicine especially anti-inflammation (Li et al., 2015b). The effect of piperine extract from *P. chaba* fruits on LPS-stimulated inflammation was investigated and its activity on the expression of inflammatory signaling proteins was evaluated. The results show that piperine extract attenuated LPS-induced pro-inflammatory cytokine releases in a concentration-dependent manner. In addition, during an inflammatory process (stimulated by LPS), piperine extract modulated the signaling proteins by elevating the levels of IκB-α, which is suppressed when LPS presents, and decreases levels of and decreasing the levels of NF-κB, which is stimulated by LPS.

These findings confirm that LPS stimulates inflammatory gene expression as shown in increased pro-inflammatory cytokine levels, compared to LPS-untreated cells. Piperine extract can inhibit LPS action by modulating IκB-α level during inflammatory process. This allows less number of free NF-κB to stimulate inflammatory genes as shown in low levels of pro-inflammatory cytokine expression. The results of the present study are supported by the studies on murine macrophage models showing that piperine inhibited

LPS-induced PGE₂, NO, TNF- α , and cox II gene expression by inhibiting NF- κ B nuclear translocation (Ying et al., 2013a; Kim et al., 2012).

In Caco2 cells, piperine shows reverse effect of LPS on I κ B- α and NF- κ B expression. This may be due to piperine, has inhibitory action on the process of I κ B- α phosphorylation and degradation. This leads to sustain a high level of this protein in the cytosol and restrains NF- κ B transcription factor in an inactive form. As stated by the previous studies, piperine inhibited I κ B- α phosphorylation and NF- κ B nuclear localization (Jeong et al., 2014; Ying et al., 2013b). Taken together, in Caco-2 cells, piperine shows the same effects as in murine macrophage because it may also have an inhibitory effect on I κ B- α phosphorylation. This makes the two signaling proteins stay as dimer in the cytosol. Therefore, the inflammatory genes are not activated if piperine is present. The present study gives scientific support to medicinal plants containing piperine on curing inflammatory diseases, especially the inflammatory diseases in the GI tract such as peptic ulcer and IBD. In IBD, an inhibition of NF- κ B activation has been suggested as a treatment strategy (Dijkstra et al., 2002). The results of the present study could support the natural source of active compound for serving that treatment.

Conclusion

The findings of the study confirm that piperine from *P. chaba* fruits can inhibits LPS-induced gastrointestinal epithelial cell inflammation by reversing LPS effect on inflammatory gene expression. This would suggest that *P. chaba* fruits could be further developed as a biomedicine for curing inflammatory diseases of the GI tract. It could benefit supplementary natural product to reduce the use of synthetic steroid drugs that have several side effects. However, further study as a use in medicine is still needed.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

The author appreciates Thammasat University, Pathum thani, Thailand, for funding the research.

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