Full Length Research Paper

Inflammatory suppressive effect of Benjakul, a Thai traditional medicine on intestinal epithelial cell line

Anyanee Burodom1* and Arunporn Itharat2

1Department of Preclinical Science, Faculty of Medicine, Thammasat University, Paholyothin Road Pathum Thani, 12120, Thailand.
2Department of Applied Thai Traditional Medicine, Faculty of Medicine, Thammasat University, Pathum Thani, Thailand.

Accepted 7 November, 2013

Plants are important sources of new therapeutic agents. Benjakul is a Thai traditional plant preparation composes of Piper chaba Linn., Piper samentosum Roxb., Piper interruptum Opiz., Plumbago indica Linn. and Zingiber officinale Roscoe. It is commonly used as an adaptogen for element balancing and immunity support. In this study, we evaluated the activities of its ethanolic extract on pro-inflammatory cytokine production in lipopolysaccharide (LPS)-induced inflammatory process in colonic epithelial, Caco-2, cells. In 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) experiment, the selected doses of the herbal extract used in this study showed no cytotoxicity on culture cells. An evaluation of inflammatory cytokine releases, using enzyme-linked immunosorbent (ELISA) method, indicated that this medicinal plant mixture potentially attenuated tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and IL-6 releases. These results suggest that Benjakul extract contains effective anti-inflammatory agents. This would give a scientific support in using Benjakul as a traditional medicine in curing inflammatory diseases.

Key words: Benjakul extract, inflammatory cytokine, tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6, Caco-2 cells.

INTRODUCTION

Recently, many attentions have been focused on using natural products to make new potential drugs for disease treatments. Benjakul is a herbal preparation of Thai traditional medicine. According to the name “Benja” that means five, it constitutes five medicinal plants that are Piper chaba Linn. (fruit), Piper samentosum Roxb. (root), Piper interruptum Opiz. (stem), Plumbago indica Linn. (root) and Zingiber officinale Roscoe. (rhizome) (Itharat et al., 2010). A fruit of P. chaba is generally called “Dee Plee” and is used as antitussive, antifungal and uterus-contracting agent in Thailand (Matsuda et al., 2008). The plant of P. samentosum or Thai name “Chaplu” is usually used as an expectorant, carminative, antitussive and muscle pain relief (Peungvicha et al., 1998). P. interruptum, mostly found in the Northern and North-eastern parts of Thailand, is used as a carminative and antiflatulent (Seewaboone et al., 2012). The root of P. indica is the traditional herb used as appetite promoting, diuretic, uterine-contracting and antibacterial agents. The rhizome of Z. officinale (ginger) is usually used to reduce bloatedness, sore throats, diarrhea and to improve loss of appetite (Sabina et al., 2011).
Benjakul recipe is traditionally used as an adaptogen by folk doctors to serve balanced health in patients and healthy people (Itharat et al., 2010). Adaptogens are plants or herbal formula pharmacologically used in traditional medicine to serve the balance of the body elements. In folk medicine, it is believed that human body composes of four elements that are earth, water, wind and fire or heat elements. Healthy people are ordinarily in the balance stage of those elements. If there is a balance shift, the person will get into the disease stage. The idea of balanced health is fit into the body homeostasis in modern science. At present, Thai government promotes applications of traditional medicines in treatment of diseases in coupling with synthetic drugs. It has been reported that a Benjakul remedy was applied to cancer patients for two to three weeks in order to give the internal body balance and to improve immunity before chemotherapy. The results showed significantly beneficial effects to the patients as they had fewer side effects of chemical drugs such as less pain and hair falling and more stable white cell count (Itharat et al., 1998; Prince of Songkla University, Thailand, research report).

Several studies have been done on Benjakul remedy to find some specific potentials. The crude extract of this preparation was shown to inhibit the growth of bacteria associated with diarrheal diseases (Kondo et al., 2010) and toxic to lung cancer cell line (Ruangnoo et al., 2012). Additionally, the extracts of some plant elements of Benjakul present anti-inflammatory properties in vivo studies such as P. chaba and Z. officinale (Sabina et al., 2011; Matsuda et al., 2008). However, the effect of Benjakul on pro-inflammatory cytokine release has not been reported.

Inflammation is a part of biological response of an organism to hurtful stimuli. An inflammatory process is triggered mainly by immune cells such as macrophages, neutrophils and lymphocytes, and is involved with cytokine, chemokine and mediator releases (Buamann and Gauldie, 1994; Laskin and Pendino, 1995). Epithelial cells lining the wall of gastrointestinal (GI) tract act as the first barrier against invasive corers from foods such as chemicals, toxins and micro-organisms, and have important roles in immunity (Chen et al., 2006). Bacterial invasion causes intestinal epithelial cells to readily stimulate innate and acquired immune response by releasing the chemokine and cytokine mediators (Panja et al., 1998; Chakravortty and Kumar, 1999). These factors usually activate an acute immune response and also work in concert with the onset of inflammatory process (Chen et al., 2006). Bacterial lipopolysaccharide (LPS) which is a part of Gram-negative bacterial cell wall was clearly shown to stimulate pro-inflammatory cytokine releases from immune cells such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and IL-6 (Wang et al., 2012). These cytokines are excellent biomarkers for determining inflammation (Chiang et al., 2012). This study aimed to investigate an anti-inflammatory activity of the Benjakul extract on LPS-induced cytokine release in colonic epithelial cell line.

MATERIALS AND METHODS

Plant

Materials of five plant ingredients were collected from all parts of Thailand. Authentication of plant materials was done by the herbarium of the Department of Forestry, Bangkok, Thailand.

Plant materials were dried in oven at 50°C then underwent ethanolic extraction process as previously mentioned by Ruangnoo et al. (2012). In short, 100 g of each dried-plant component were mixed together and ground into rough powder then macerated with 95% ethanol. The solution was filtrated and concentrated to become dry under reduced-pressure equipment to obtain an ethanolic extract. The herbal extract was kept at -20°C until the experiments were performed. Stock solutions of various concentrations were prepared by dissolving the mixture-plant extract in dimethyl sulfoxide (DMSO) and diluted with culture medium to obtain the final concentrations.

Cell culture

The colonic epithelial cell line, Caco-2, used in this research was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). They were grown in DMEM/F-12 medium containing 10% heat-inactivated fetal calf serum (Gibco-BRL, Grand Island, NY, USA), penicillin (100 U/ml), and streptomycin (100 µg/ml). The cells were incubated at 37°C in a humidified atmosphere of 5% CO₂.

Cell viability assay

The method of Mosmann using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay was used to determine cell viability (Mosmann, 1983). Caco-2 cells were grown in 96-well plates until they reached confluence. Fresh media were added and the cells were further incubated at 37°C with varying concentrations of Benjakul extract for 8 h in a humidified atmosphere of 5% CO₂. At the end of the incubation period, fresh medium and MTT solution (5 mg/ml) were added and the cells were further incubated at 37°C for 4 h in a humidified atmosphere of 5% CO₂. A 200 µl aliquot of DMSO was added and the absorbance of each well was measured at 540 nm in a BioTek Power Wave XS automatic microplate reader (Bio-tex Instrument Inc., Winooski, VT, USA).

Pro-inflammatory cytokine determination

Caco-2 cells were incubated with different concentrations of the alcoholic extract in the absence or presence of lipopolysaccharide (LPS) (1 µg/ml) at 37°C for 24 h in a humidified atmosphere of 5% CO₂. Supernatant fluids were collected and stored at -80°C until cytokines were analyzed. The pro-inflammatory cytokine productions were determined by using commercial human TNF-α, IL-1β and IL-6 enzyme-linked immunosorbert assay (ELISA) kits (BD Bioscience Pharmingen, San Diego, CA, USA) according to the...
 Statistical analysis

Data from three individual experiments were analysed and each were done in triplicate and are presented as mean ± standard error of mean (SEM). Statistical significance was determined by using one-way analysis of variance (ANOVA) and student Newman-Keuls. P-value less than 0.05 is statistically significant.

RESULTS

Effect of Benjakul extract on cell viability

Cytotoxic effects of the Benjakul preparation on human colonic epithelial, Caco-2 cells prior to evaluation of its activity on inflammatory cytokine production to confirm the non-toxic doses of the extract to be used in assessment of cytokine release was examined. Cell viability was assessed by tetrazolium dye (MTT) calorimetric assay. The results are shown in Table 1. Benjakul extract in the doses of 0.1 to 100 µg/ml had no toxicity to Caco-2 cells. Cell survival was about 100% compared to the non-treated control group. However, a high dose of 1000 µg/ml expressed cytotoxicity to epithelial cells (P < 0.05). The non-cytotoxic doses were selected for further study.

Effect on TNF-α release

In the absence of LPS stimulation, the levels of TNF-α in the culture medium of Caco-2 cells were very low of almost negligible amount (Figure 1). When the cells were challenged with LPS alone, they drastically released TNF-α to nearly 250 µg/ml. Benjakul extract (1 to 100 µg/ml) had no stimulatory effects on TNF-α production. On the other hand, it had inhibitory effect on LPS-stimulated groups by significantly decreased TNF-α levels in Benjakul plus LPS groups as compared to the LPS-treated group (P < 0.05).

Effect on IL-1β release

The level of IL-1β release in the control experiment was very low as shown in Figure 2; the value was 5.1 ± 0.2 pg/ml. LPS significantly elevated IL-1β production. Conversely, the medicinal-plant extract (1 to 100 µg/ml) drastically reversed cytokine expression in LPS plus groups in dose-dependent manner (P < 0.05).

Effect on IL-6 release

In the experiment of IL-6 cytokine measurement, the botanical extract (1 to 100 µg/ml) distinctly abolished the effect of LPS-stimulated IL-6 release down to one-third of the maximum level of LPS stimulation. This is in accordance with the observations as regards TNF-α and IL-1β expression (Figure 3).

DISCUSSION

Inflammation is a part of biological responses of our body to invasive organisms or hurtful stimuli. It is a protective mechanism of the host in attempt to remove injurious stimuli and to initiate healing process, and is classified as innate immunity. LPS is recognized by the host membrane protein, Toll-like receptor 4 (TLR4), during the process of immune response (Palsson-McDermott and O’Neill, 2004) and is associated to innate immunity (Dziarski et al., 2000). The immune cells as well as endothelial or epithelial cells directly trigger immunological

Table 1. Effect of Benjakul extract on cell culture viability. Data are shown as mean ± SEM. *Statistical significance (P < 0.05) compared to the control group.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Concentration of Benjakul extract (µg/ml)</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>0.1</td>
<td>1</td>
<td>10</td>
<td>100</td>
<td>1000</td>
</tr>
<tr>
<td>% cell viability</td>
<td>100</td>
<td>100.6±0.7</td>
<td>101.2±0.7</td>
<td>102.5±0.8</td>
<td>100.7±0.8</td>
<td>62.9±0.7*</td>
</tr>
</tbody>
</table>
events such as release of cytokines, chemokines and inflammatory mediators (Yang et al., 2012). The mucosal cells of the gut act as the first line of bodily protection and are found to release inflammatory cytokines responding to mediators from immune cells or pathogenic stimuli (Baumann and Gauldie, 1994). These cytokines work in concert with inflammatory cytokines secreted by immune cells in inflammatory process. Moderate levels of these
cytokines support usual function and growth of those cells in the intestine. However, high levels during inflammatory process may give damaging effects to them (Boonkaewwan et al., 2006). Consequently, the role to lessen cytokine production can be occupied as criteria for assessment of anti-inflammatory effect of natural products.

Medicinal plants are plentiful sources of pharmacological molecules for curing diseases. The study of herbal plant properties progress very quickly. A number of plant species are fallen into several medicinal research areas to determine their potentials for therapeutic applications. Many ideas show that using plants as medicines have less deteriorating effects, low cost and less toxicity (França et al., 2010; Yayeh et al., 2012). In traditional medicine, several kinds of herbal plants are used as sophisticated mixtures rather than individual plants (Bussmann et al., 2010). The healing properties are derived from many active substances containing in the preparation. In this study, we found that Benjakul mixture potentially inhibited cytokine releases during inflammatory process through intestinal epithelial cells.

In some inflammatory diseases, such as inflammatory bowel disease (IBD), the pathogenesis is affected by several factors that are decreased in immunity, oxidative stress, or microbial content in the GI tract (Rahimi et al., 2010). Recent studies have given more scientific information of a Benjakul formula, for example, the chemical constituents, bioactive compounds and therapeutic property. The chemical analysis of the alcoholic extract revealed that piperine, plumbagin and 6-gingerol are major active compounds of Benjakul preparation (Ruangnou et al., 2012). These bioactive substances exhibit identical behaviors to authentic compounds which are available by commercial companies (Itharat et al., 2010). In addition, in *in vitro* study, Benjakul expresses cytotoxic effect against lung cancer cell line, COR-L23 (IC_{50} = 19.8 ug/ml). However, this effect varies in a single plant component of the preparation (Ruangnou et al., 2012). The studies of some plants composing Benjakul show distinguished therapeutic activities.

It has been proposed that the methanolic extract of *P. chaba* possesses a potential protective effect on liver injury induced by D-galactosamine (D-GalN) and LPS in mice by reducing serum aspartate aminotransferase (AST) and serum alanine aminotransferase (ALT). Piperine, a major active compound of this plant, has cytoprotective effect on cell death in TNF-α-induced cytotoxicity in primary culture of mouse hepatocytes (Matsuda et al., 2008). Moreover, piperine was also found to promote immunity by increasing the number of white blood cells in Balb/c mice immunized with sheep red blood cells (SRBC) (Sunila and Kuttan, 2004). Ginger, another constituent of Benjakul remedy containing 6-gingerol as a major active substance, exhibits protective effect on acetaminophen-induced hepatic injury in mice (Sabina et al., 2011). These all together would support our findings on Benjakul extract that can attenuate LPS-induced TNF-α, IL-1β and IL-6 production. However, additional information on mechanism of action in molecular level is needed for further elucidation.

**Conclusion**

The results of this study suggest that Benjakul contains...
anti-inflammatory activity by inhibition of cytokine releases in an inflammatory process induced by LPS in vitro study. This valuably provides the scientifically medical support in using Benjakul for treating inflammatory diseases in traditional medicine. In addition, this would give the background knowledge for further studies of herbal plants in pharmaceutical area.

ACKNOWLEDGEMENTS

The authors thank Dr. Prasert Saichua for his helpful assistance in data analysis. This work was granted by The National Research University Project of Thailand Higher Education Commission.

ABBREVIATIONS

ALT, Alanine aminotransferase; AST, aspartate aminotransferase; Ben, benjakul; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; D-GalN, D-galactosamine; Gl, gastrointestinal; IBD, inflammatory bowel disease; IL-1β, interleukin one beta; IL-6, interleukin six; LPS, lipopolysaccharide; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); SRBC, sheep red blood cells; TLR4, toll-like receptor 4; TNF-α, tumor necrosis factor alpha.

REFERENCES


