EFFECT OF SENNA (Senna alexandrina Mill.) AND POMEGRANATE (Punica granatum L.) LEAVES EXTRACTS AND ITS FRACTIONS ON PRO-INFLAMMATORY CYTOKINES OF THE OBESE ZEBRAFISH (Danio rerio)

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ABSTRACT

Obesity-associated with several chronic diseases. Based on this condition, natural products use should be considered as an alternative treatment. The objectives of this research are to evaluate the effect of senna (Senna alexandrina Mill.) and pomegranate (Punica granatum L.) leaves extracts and its fractions on pro-inflammatory cytokines suppression of the obese zebrafish. Methods: acclimatization period was performed for two weeks. All zebrafish was given by standard diet intake. Post acclimatization, zebrafish were divided into 10 groups (n = 15 in each group) including normal group; obese group; senna extract group (SE) 100µg/ml; pomegranate extract group (PE) 100µg/ml; senna fractions groups [(n-hexane fraction (HFS) 50µg/ml, ethyl-acetate fraction (EFS) 50µg/ml, and water fraction (WFS) 50µg/ml], and pomegranate fractions groups [(n-hexane fraction (HFP) 50µg/ml, ethyl-acetate fraction (EFP) 50µg/ml, water fraction (WFP) 50µg/ml]. For a period of 4 weeks, the normal group was given by a normal diet and other groups by a high-fat diet. Obese group was given by 240 mg/group/fish of the experimental diet. Gene expression analysis was performed using quantitative Real-Time Polymerase Chain Reaction method. Results: The results of this study showed that both senna and pomegranate leaves extracts and its fractions have ability to suppress pro-inflammatory cytokines (such as TNF-α, IL-1β, leptin and increased anti-inflammatory cytokine (such as IL-10) of high-fat diet-induced obese zebrafish. Conclusion: senna and pomegranate leave extracts and its fractions have a potential effect and very promising to suppress pro-inflammatory cytokines and increased anti-inflammatory cytokines of high-fat diet-induced obese zebrafish.

Keywords: Senna, Pomegranate, Cytokines, Obese, Zebrafish

INTRODUCTION

Obesity prevalence has increased, a growing epidemic, and becoming major public health worldwide.¹ Etiology of obesity is an imbalance between energy intake and energy expenditure.² Obesity associated with chronic diseases involving dyslipidemia, hypertension, nonalcoholic fatty liver disease (NAFLD), atherosclerosis, cardiovascular disease (CVD), type-2 diabetes mellitus (T2DM), and cancers.³, ⁴ Obesity condition occurs caused by excess and abnormal fat accumulation in adipose tissue.⁵ Adipose tissue is an endocrine organ released several adipokines involving metabolic regulation and inflammatory process. In lean individuals, adipose tissue released anti-inflammatory cytokines, but in obese individual adipose tissue released pro-inflammatory cytokines.⁶ The important problem of obesity is the induction of chronic low-grade inflammation and increase inflammatory factors. Numerous studies demonstrated an
association between a high-fat diet consumption with the release of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and leptin. Released of pro-inflammatory adipokines and decreased of anti-inflammatory adipokines caused by excess adipose tissue has a contribution to the development of obesity-related metabolic diseases. Association between excess dietary intake and obesity condition have been studied. In laboratory animal, such as rodents (rats or mice), an obese model was acquired by genetic mutations and the use of excessive diet intake. Although rats and mice have a significant contribution against the understanding of human obesity process, however, experiment using rodents need several supports including laboratory staff and good infrastructural system. Therefore, the options of simple and inexpensive animal models should be considered. As vertebrate, zebrafish (Danio rerio) is a good animal model of obesity, due to its similarities of genome and physiology to humans. Zebrafish is a tropical fish which can be found in India and South Asia. Some studies showed that zebrafish was an ideal model for obese which caused by a high-fat diet. These reports explained that zebrafish could be useful as the animal model for obesity research.

Senna (Senna alexandrina Mill.) and pomegranate (Punica granatum L.) are widely used as traditional medicine for several purposes. All of the parts from these plants have beneficial pharmacological activities. Senna can be found in Africa and Asia. The functional parts of senna are the leaves, pods, and fruits. Pharmacological activities of senna including antipyretic, laxative, purgative, and diuretic. Pomegranate (Punica granatum L.) was known to the Punicaceae family, can be found in America, Europe, and Asia. The functional parts of pomegranate are the roots, barks, fruits, peels, and leaves to treat several diseases involved cancer, infections, obesity, and inflammation. Based on this background, the objective of this study is to evaluate the effect of senna and pomegranate leaves extracts and its fractions on pro-inflammatory cytokines of obese zebrafish.

**EXPERIMENTAL**

**Plant Materials and Identification**

Senna leaves and pomegranate leaves were collected from Bandung, Indonesia. Furthermore, senna and pomegranate were identified in Tropical Biopharmaca Research Center, Bogor Agricultural University and Herbarium Bandungense, School of Life Sciences and Technology, Bandung Institute of Technology, Indonesia, respectively.

**Extraction and Fractionation**

Senna and pomegranate leaves were dried and followed by ground into powder. The powder of senna and pomegranate leaves were extracted with 96% ethanol by reflux and evaporated by rotary evaporator at 50°C, speed at 50 rpm. The obtained extract was fractionated by liquid-liquid extraction using n-hexane and ethyl-acetate. Furthermore, extracts and its fractions were used for in vivo study using zebrafish.

**Animals**

Adult zebrafish were obtained from Tropical Biopharmaca Research Center, Bogor Agricultural University (IPB), Bogor, Indonesia. Adult zebrafish in this study were maintained under a controlled environment involving 12:12-h light: dark cycle (26°C), pH 7.5, and water quality conditions were maintained according to the zebrafish guidance. The zebrafish which was used in this study 4 months ages post-fertilization, male fish, and with body weight 0.1 - 0.2 g. All procedure in this study has been accepted by Animal Ethic Committee of Biopharmaca Research Center IPB (No.96-2018 IPB).

**Experimental Design**

Acclimatization period was performed for two weeks. All zebrafish was given by standard diet intake. Post acclimatization, zebrafish were divided into 10 groups (n = 15 in each group) including normal group; obese group; senna extract group (SE) 100µg/ml; pomegranate extract group (PE) 100µg/ml; senna fractions groups [(n-hexane fraction (HFS) 50µg/ml, ethyl-acetate fraction (EFS) 50µg/ml, and water fraction (WFS) 50µg/ml], and pomegranate fractions groups [(n-hexane fraction (HFP) 50µg/ml, ethyl-acetate (EFP) 50µg/ml, water fraction (WFP) 50µg/ml]. For a period of 4 weeks, the normal group
was given by a normal diet and other groups by a high-fat diet. Obese group was given by 240 mg/group/fish of the experimental diet. High-fat diet for this research was prepared with ingredient: fat and oils (50%), protein (12%), fiber (1.4%), and other compounds (8.5%).

RNA Extraction and Quantitative RT PCR
After treated with extract and fractions of senna and pomegranate, zebrafish were sacrificed. Zebrafish anesthetized using cold shock method. Amount of 20 mg of ventral adipose tissue of zebrafish was isolated for each treatment. Total RNA was extracted using Genezol reagent (Geneaid, Taiwan). The RNA concentration was measured by spectrophotometry method. The cDNA synthesis and purification were conducted from 1 µg RNA using Revert Ace® qPCR RT Master Mix with gDNA Remover (Toyobo, Japan) following the manual instruction. The qPCR reactions were performed at Rotor-Gene 6000 qPCR machine (Corbett, USA) using SensiFAST™ SYBR® NO-ROX Kit (Bioline, UK) in a total volume of 20 µl. Amplification program was set at 95°C 2 min, and 40 cycles of 95°C for 5 s, and 60°C for 15 s. The genes expression was normalized to β-actin gene and stated as fold change relative to the control group. Gene expression was analyzed using the 2−ΔΔCt methods. All primer for qPCR analysis from this study was presented in Table-1.

<table>
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<tr>
<th>Gene Symbol</th>
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<th>Antisense 5′-3′</th>
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Statistical Analysis
The data were expressed as means ± standard deviation (SD) with three replications at each treatment. Statistical comparisons were performed using the one-way ANOVA followed by post hoc test Tukey HSD (p<0.05). All statistical analysis was conducted in the SPSS v17 software (SPSS Inc., USA).

RESULTS AND DISCUSSION
Obesity is a chronic condition caused by an imbalance between energy intake and energy expenditure. Excessive fat accumulation in adipose tissues of obese condition leads to chronic low-grade inflammation. Chronic low-grade inflammation has an important role in the pathogenesis of insulin resistance, type 2 diabetes mellitus, and cardiovascular disease. Adipose tissue is an endocrine organ released several adipokines involving metabolic regulation and inflammatory process. In lean individuals, adipose tissue released anti-inflammatory cytokines such as adiponectin, IL-10, and SFRP5, but in obese individuals adipose tissue released pro-inflammatory cytokines such as IL-1, IL-6, IL-18, MCP-1, TNF-α, leptin, resistin, PAI-1, and RBP4 (Fig.-1). In this study, not all pro-inflammatory and anti-inflammatory were analyzed.

The recent study, obese zebrafish model had been developed using high-fat diet administration for 4 weeks. Based on the results, high-fat diet-induced obese zebrafish showed increased of pro-inflammatory cytokines (such as TNF-α, IL-1β, leptin) and decreased of anti-inflammatory cytokines (such as IL-10). These results consistent with other studies using obese zebrafish were induced by high-fat diet. Increased of pro-inflammatory cytokines and decreased of anti-inflammatory cytokines in obese condition has responsible against the presence of the metabolic syndrome. Fig. 2 showed that high-fat diet consumption associated with increased TNF-α expression on the obese group. SE at the dose 100 µg/ml has a low activity to suppress TNF-α expression (4.492 ± 1.634) if compared to PE dose 100µg/ml (1.776 ± 0.871). EFS dose 50 µg/ml and WFS dose 50 µg/ml exhibited higher activities to decrease TNF-α expression than SE dose 100µg/ml. In addition, pomegranate fractions also showed the ability to inhibit TNF-α expression of obese zebrafish. Figure-2 shows TNF-α relative expression on obese zebrafish after treating with different extracts and fractions of senna and pomegranate. Expression data were normalized...
to the β-actin gene and compared to the expression in the non-obese control for the relative expression. Data represent the mean ± SD (n=3). Senna extract (SE); pomegranate extract (PE), n-hexane senna fraction (HFS), ethyl-acetate senna fraction (EFS), and water senna fraction (WFS); n-hexane pomegranate fraction (HFP), ethyl acetate pomegranate fraction (EFP), water pomegranate fraction (WFP).

Fig.-1: Association between Pro-inflammatory Cytokines and Metabolic Syndrome.

TNF-α is a pro-inflammatory cytokine, was expressed and released by adipose tissue. Several types of research showed that high expression of TNF-α in adipose tissue caused by obesity has associated with insulin resistance and type-2 diabetes mellitus (T2DM) in humans and experimental animal model. The result of this study consistent with the previous study that high-fat diet-induced obese zebrafish showed increased TNF-α expression. TNF-α has an effect to induce insulin resistance by reducing insulin receptor substrates-1 (IRS-1) phosphorylation and decreasing NO releases via the PI3K/Akt/eNOS pathway.23, 24 Treatment strategies to blockade TNF-α may be reducing the incidence of insulin resistance and the development of T2DM.25

Figure-3 shows IL-1β relative expression on obese zebrafish after treating with different extracts and fractions of senna and pomegranate. Expression data were normalized to the β-actin gene and compared...
to the expression in the non-obese control for the relative expression. Data represent the mean ± SD (n=3). Senna extract (SE); pomegranate extract (PE), n-hexane senna fraction (HFS), ethyl-acetate senna fraction (EFS), and water senna fraction (WFS); n-hexane pomegranate fraction (HFP), ethyl acetate pomegranate fraction (EFP), water pomegranate fraction (WFP).

Figure-3 demonstrated that high-fat diet consumption has contribution against increased IL-1β expression on the obese group. Both extract and fractions of senna and pomegranate have the ability to inhibit IL-1β expression. SE dose 100 µg/ml showed higher activity to decrease IL-1β (0.00166 ± 0.00151) compared to PE dose 100 µg/ml (1.118 ± 0.640). IL-1β is a pro-inflammatory cytokine that is critical against host-defense response and injury. This cytokine associated with metabolic syndromes such as T2DM, atherosclerosis, chronic heart failure (CHF), and autoimmune diseases (such as type 1 diabetes mellitus, rheumatoid arthritis, inflammatory bowel diseases). IL-1β expressed in many cells including macrophage, natural killer cells (NKC), monocytes, and neutrophils. Other study reported that IL-1β has also been produced and secreted by pancreatic islets. The presence of IL-1β in pancreatic islets leads the acceleration of inflammation and induction of insulin resistance in obesity.26

![Fig.-3: IL-1β Relative Expression on Obese Zebrafish after Treating with Different Extracts and Fractions of Senna and Pomegranate.](image)

![Fig.-4: IL-10 Relative Expression on Obese Zebrafish after Treating with Different Extracts and Fractions of Senna and Pomegranate.](image)

Figure-4 shows IL-10 relative expression on obese zebrafish after treating with different extracts and fractions of senna and pomegranate. Expression data were normalized to the β-actin gene and compared to the expression in the non-obese control for the relative expression. Data represent the mean ± SD (n=3). Senna extract (SE); pomegranate extract (PE), n-hexane senna fraction (HFS), ethyl-acetate senna fraction (EFS), and water senna fraction (WFS); n-hexane pomegranate fraction (HFP), ethyl acetate pomegranate fraction (EFP), water pomegranate fraction (WFP).

Fig. 4 showed that decreased IL-10 expression occurred in the obese group. PE dose 100µg/ml gave a stronger effect to increase IL-10 expression compared to SE dose 100 µg/ml. In contrast, HFS dose 50µg/ml showed the best activity to increase IL-10 expression if compared to another group. IL-10 is a cytokine with anti-inflammatory properties that plays an important role against immune response.27

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Lower IL-10 was found in obese condition and associated with the presence of the metabolic syndrome. The results of this study consistent with other studies that diet-induced obesity-related to increased IL-1β and decreased IL-10 expressions.

Figure 5 shows Leptin relative expression on obese zebrafish after treating with different extracts and fractions of senna and pomegranate. Expression data were normalized to the β-actin gene and compared to the expression in the non-obese control for the relative expression. Data represent the mean ± SD (n=3). Senna extract (SE); pomegranate extract (PE), n-hexane senna fraction (HFS), ethyl-acetate senna fraction (EFS), and water senna fraction (WFS); n-hexane pomegranate fraction (HFP), ethyl acetate pomegranate fraction (EFP), water pomegranate fraction (WFP).

Fig. 5 revealed that PE dose 100 µg/ml has a higher ability to inhibit leptin expression compared to SE dose 100 µg/ml. In contrast, WFS dose 50 µg/ml and HFP dose 50 µg/ml gave a higher effect to inhibit leptin expression compared to another group. Leptin is an adipocytes-secreted hormone, abundantly expressed in adipose tissue with several effects in the body such as appetite control, metabolism, and body weight regulation. Recent studies showed that leptin has an important role in obesity, metabolic syndrome, and cardiovascular disease. In the obese condition, hyperleptinemia is proportional to the amount of body fat stores. Consistent with our result, high-fat diet-induced zebrafish showed increased leptin expression. The results of this study showed that administration of senna and pomegranate leaves extract and its fractions showed a reduction of TNF-α, IL-1β, and leptin expression in obese zebrafish. Furthermore, senna and pomegranate leave extracts and its fractions also showed activity to increase IL-10. Therefore, senna and pomegranate administration has an important role to improve metabolic syndrome in obese condition by pro-inflammatory cytokines suppression.

CONCLUSION
The recent study found that senna and pomegranate very promising to reduce metabolic syndrome-associated obesity via regulation of pro-inflammatory as well as anti-inflammatory cytokines, which senna leaves extract and ethyl-acetate fractions specifically inhibited releasing pro-inflammatory cytokines (such as TNF-α, IL-1β, leptin) and increased anti-inflammatory cytokines (IL-10).

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