Nutmeg extract modulates triglyceride levels and gene expressions of CD36, FATP1, and CPT1B in rat skeletal muscle

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Obesity has become a global health problem. It affects all organs in the human body including skeletal muscle as fat deposits in skeletal muscle. \textit{Myristica fragrans} (nutmeg), which is a native spice from Indonesia, has been used in daily basis and have potential effect to hyperlipidemia. Various \textit{in vivo} studies have been proven that nutmeg extract can reduce body weight and lipid deposition. However, there is limited information about the effects of nutmeg on lipid transport regulation, particularly in skeletal muscle cells. We investigated the effects of nutmeg extract on blood lipid profile and lipid metabolism in skeletal muscles. Male Wistar rats were divided into two groups: control and nutmeg extract-treated group. The treatment group consumed nutmeg extract for 12 weeks. Blood samples were collected for lipid profile examination. In addition, the soleus muscles of all groups were isolated and collected for mRNA examination. The expression of lipid transporter in the cell membrane (CD36) and mitochondrial membrane (FATP1 and CPT1B) were analyzed by semi-quantitative PCR. Although the daily food intake increased, there was no weight gain in the nutmeg-treated group. Additionally, nutmeg extract has been shown to decrease triglyceride blood levels in the treatment group. Interestingly, the CD36, FATP1 and CPT1B mRNA expressions in the soleus muscles increased with nutmeg treatment. The results of this study suggest that nutmeg extract modulates lipid utilization in skeletal muscle through CD36, FATP1 and CPT1B lipid transporter.

**Keywords**: CD36, CPT1B, FATP1, Lipid, Nutmeg, Soleus muscle

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Worldwide prevalence for metabolic syndromes is gradually increasing. The current estimation is at around 20-25% globally and almost similar with the United States of America (USA)\textsuperscript{1}. One of the causes of metabolic syndrome is an energy imbalance between calorie intake and low physical activity that leads to the accumulation of subcutaneous, peripheral, and intravisceral fat in the body. In addition, a high-fat diet since an early age has also contributed to early metabolic syndrome as fat deposition will occur in all systems including skeletal muscles. One of the key functions of skeletal muscles is to regulate the energy homeostasis of fatty acid and glucose oxidation and adjust fuel supply for energy consumption\textsuperscript{2}. Consequently, fat deposition in skeletal muscles can reduce muscle mass.

Adipose cells secrete cytokines which can lead to proinflammatory, prothrombotic and insulin resistant conditions. Insulin resistance is a risk factor for pancreatic beta-cell destruction, impaired glucose metabolism, hypertension, hypertriglyceridemia, hyperglycemia and dyslipidemia. In chronic conditions, metabolic syndrome will induce cardiovascular disease, diabetes mellitus type 2 and stroke.

Herbal medicines are potential candidates to treat obesity. Nutmeg, a local herb from Maluku,
Indonesia, has been extensively used as food spice and herbal medicine. However, its effect on lipid modulation is unknown. Recent studies reported that nutmeg extract reduced fat stored in the muscles as macelignan found in nutmeg extract may have a role in modulating peroxisome proliferator-activated receptor gamma (PPAR-γ) activity. PPAR-γ can be activated by endogenous ligands, such as fatty acids and prostanooids, and exogenous ligands, such as thiazolidinediones (TZD) drugs. PPAR-γ exogenous ligands can be found in various food ingredients and medicinal plants. The nuclear receptor PPAR-γ plays a key role in regulating the energy balance in the body. Furthermore, this gene is expressed extensively in the body especially in adipose tissues, cardiac muscles, and skeletal muscles. PPAR-γ may have interactions with lipid transporter CD36, which has facilitated long-chain fatty acid uptake by cardiac and skeletal muscles. Previous studies revealed that activation of PPAR-γ increased CD36 protein expression. Conversely, CD36 downregulation is correlated with insulin resistance. Another study using ligands from nutmeg extract (nectandrin B) showed lipogenic gene suppression due to nutmeg treatment in the liver. However, its effects on lipid metabolism skeletal muscles have not been extensively studied.

In this study, we investigated the lipid metabolism modulation by nutmeg extract and studied the expression level of lipid transporters in skeletal muscles in vivo. Our results suggested that nutmeg extract may change lipid metabolism through skeletal muscle lipid transporters.

Materials and Methods

Nutmeg extract
Dried nutmeg seeds were collected during the dry season from Maluku and West Java Island in Indonesia. Thirty kilograms of nutmeg seeds were powdered using a pilot-scale extractor with a circulator rate of 150-200 rpm for 30 min. The nutmeg powder was then evaporated at a temperature range of 40-60°C and a pressure range of 400-500 mmHg to produce the extract. Safrrole and myristicin compounds were eliminated from the final extract.

Food intake and body weight
Pre-weighed food was provided in standard steel hoppers to the rats. On a daily basis, the rats were briefly removed from their cages and weighed. In addition, the amount of remaining food, including any at the bottom of the cages or that spilled onto the plastic sheets under each cage, was recorded. The values obtained from the food intake and body weights were analyzed on a weekly basis, by comparing the mean total food intake and the mean total body weight from all groups.

Animals
Animal handling, maintenance, and euthanasia procedures have been approved by the Ethics Committee, Faculty of Medicine, Universitas Padjadjaran.

Animal treatment
Twenty-eight Wistar male rats, 8 weeks old, that weighed around 180-200 g were randomly divided into control and nutmeg-treated groups. Nutmeg extract was given to the nutmeg-treated group, while water was given to the control group by gavage every morning at the same time for 12 weeks. The dose administered to the treatment group was a conversion of the human dose (300 mg/day) 0.54 mg/day/kg body weight). The specific dose was recorded for each rat. In addition, Pulvs Gummi Arabicum (PGA) 2% was given to the control group. The rats were sacrificed using isoflurane anesthesia, with the soleus muscles then removed, weighed, frozen in liquid nitrogen and stored at -80°C until use.

Colorimetry test
Serial standards were made with concentrations of 0, 25, 50, 100, 200, and 400 mg/dL, then adjusted with sample serum. A mixture of 200 mL of trig reagent (Biorex and Labkit) and 2 mL serum samples or blanks were added to the microplates. The mixture was then incubated for 5 min at 37°C. Absorbance was read at a wavelength of 546 nm for triglycerides and 505 nm for cholesterol. The sample concentration was then removed, weighed, frozen in liquid nitrogen and stored at -80°C until use.

RNA extraction and semi-quantitative reverse transcription-PCR
Total ribonucleic acid (RNA) from muscle tissues was isolated with Trizol reagent from Invitrogen, USA, and used according to the manufacturer’s instructions. One-step Polymerase Chain Reaction (PCR) kit (Bioline, USA) was used in this study. Specific primers for CD36, fatty acid transport protein...
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1 (FATP1), and carnitine palmitoyltransferase 1B (CPT1B) were used, with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) being used as an internal control. Primer sequences and annealing temperatures are shown in Supplementary Table 1. The PCR results for each sample were normalized with GAPDH messenger-RNA levels that acted as the control.

Statistical analyses
Data were analysed with the One-Way Analysis of Variance (ANOVA) test using SPSS V.13. and Bonferroni test to analyse the statistical difference. Statistical significance was designated at $p<0.05$. Data were expressed as mean ± standard error minimum (SEM).

Result
Food consumption and body weight between four groups of rats
The observation of food consumption and body weight were conducted weekly. The weekly consumption of the rats was observed to be higher in the nutmeg-treated group as shown in Figure 1. The nutmeg-treated group consumption was 1.5 folds higher than the control group. Despite the higher intake during the treatment period, no bodyweight differences were observed between both groups by the end of the 12 weeks (Fig. 2, 3).

Nutmeg treatment decreased the lipid profile in blood
Lipid was absorbed in the small intestine to form triglycerides and enter the blood circulation. In order to examine the effects of nutmeg extract to blood lipid profile, the cholesterol and triglyceride levels were measured (Fig. 4). The results showed that triglyceride levels of the nutmeg-treated group were 0.3 folds lower than the control (Fig. 4a). However, no difference was observed in blood cholesterol levels (Fig. 4b) and glucose levels (Fig. 4c).

Gene-regulated lipid transporter is increased by nutmeg treatment in the soleus muscles
To study the lipid metabolism in the skeletal muscles, the lipid transporters in the soleus muscles were examined. Semi-quantitative RT-PCR was used to analyse CD36, FATP1 and CPT1B gene expressions (Fig. 5, 6). In the nutmeg-treated group, CD36 gene expressions were significantly higher (4.5 fold, $p<0.01$). Consistently, FATP1 and CPT1B gene expressions were upregulated in the same group. These results suggest that nutmeg extract modulated mRNA expressions of the lipid membrane and mitochondria transporters in the skeletal muscles.

Discussion
Brain function as a center of appetite will affect the consumption of daily food. A prior study showed that the effects of nutmeg could increase one's appetite
due to the increase of dopamine 1A expressions in the brain. Consistently, we observed that nutmeg extract consumption for 12 weeks as shown in Figure 7 increased food consumption. While fixed weight with nutmeg was consistent with our previous findings that nutmeg extract increased muscle mass in older animal models, nutmeg extract may modulate muscle myogenesis through the insulin-like growth factor 1-protein kinase B-mammalian target of rapamycin (IGF1-Akt-mTOR) pathway. Furthermore, nutmeg extract has PPAR-γ agonist activities. PPAR-γ agonist affects insulin sensitivity and lipid metabolism through the regulation of adipogenesis and secretion of adipocytokines. PPAR-γ agonists as transcription factors play a key role in muscle and fat cell differentiation.

Fig. 5 — Increased expression of the lipid transport gene in muscle cells shows that lipid metabolism increases in the cells of the soleus muscle. Nutmeg treatment increases gene expression of FATP1, CD36, and CPT1B in soleus muscle. Data represent ratio in mean ± SEM of experiment. **p<0.01 compared with the control group. Increasing FATP1 and CD36 gene expression indicated that there is fatty acid entering the soleus muscle, and then fatty acid goes into the mitochondria for oxidation.

Fig. 6 — Fatty acid entered cell muscle with transporter lipid CD36 & FATP1 in membrane cell. In the cytoplasm, fatty acid-binding with fatty acid binding protein (FABP), and then fatty acid united with CoA. If the body needs energy, fatty acid-CoA enters the mitochondria with lipid transporter CPT1B for the oxidative process.
During starvation, the body stimulates lipid metabolism in various organs including skeletal muscles. Consequently, lipid accumulation in skeletal muscles is broken down to produce fatty acids. Fatty acids enter skeletal muscle cells through the gate of the muscle cell membranes. Muscle cell membranes have lipid transporters such as CD36 and FATP1 to allow easier movements of fatty acids into the cells. Inside the muscle cells, fatty acids bind to Coenzyme A (CoA). Then, the fatty acids and CoA enter the mitochondria through CPT1B lipid transporter to undergo the β-oxidation process. Fatty acid oxidation occurs in three stages: activation, transport into the mitochondria and oxidation to acetyl-CoA. Thus, fatty acid oxidation produced more energy which can be used for whole body metabolic processes in low glucose conditions.

Our results showed that nutmeg extract enhanced lipid transporter expressions in skeletal muscle cells. The increase of CD36 and FATP1 lipid transporters shows that the entry of fatty acids into the soleus muscle cells will stimulate PPAR-γ activities to induce lipoprotein lipase (LPL), FATP1 and acyl-coenzyme A synthetase in adipose tissues selectively to free the fatty clearance and adipogenesis that occurs in the skeletal muscles. After activation, PPAR-γ will bind to retinoid X receptor (RXR) so that a PPAR-RXR heterodimer complex will be formed before binding to the hormone response element (HRE) target cell deoxyribonucleic acid (DNA) to form peroxisome proliferative response element (PPRE) ions to initiate the transcription process. PPAR-γ expressions are the main muscle tissue for decreased insulin resistance as compared to adipose tissues. PPAR activation makes remodeling of large fat tissues into small adipose tissues, in order to suppress tumor necrosis factor-alpha (TNF-α) cytokines and other compounds to stimulate the synthesis and translocation of glucose transporter-4 (GLUT-4) to the cell plasma membranes. Nutmeg extract has a positive effect on insulin sensitivity as a PPAR-γ agonist.

**Conclusion**

Nutmeg may play a role in regulating blood triglyceride levels and lipid metabolism in skeletal muscles through the modulation of CD36, FATP1 and CPT1B gene expressions.

**Supplementary Data**

Supplementary data associated with this article is available in the electronic form at http://nopr.niscpr.res.in/jinfo/ijtk/IJTK_21(03)(2022)531-536_SupplData.pdf

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All the authors have agreed on the paper’s contents. The authors have no conflicts of interest

**Authors’ Contributions**

RL, SH, and AP participated in the design of the research, supervised the experiment and provided mentorship support. SS and NA performed biochemistry experiment and gene expression, YSP, Mas RAS and US supervised and advised the experiments. HG and KL carried out the experiment, helped to draft the article and provided mentorship support. RL, KL and RA participated in the design of the research and conducted the experiment. All author read and approved the final version of the article.

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