Short communication:
Effects of Canarium indicum oil in enhancing brain-derived neurotrophic factor in rats

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Abstract. Rahman H, Anggadiredja K, Gusdinar T, Sitompul JP, Tursino. 2020. Short communication: Effects of Canarium indicum oil in enhancing brain-derived neurotrophic factor in rats. Nusantara Bioscience 12: 168-174. The brain-derived neurotrophic factor (BDNF) is one of the neurotrophins family and plays an essential role during the development of neurons called neurogenesis. Since BDNF is critical for the function and survival of neurons, thus it represents the neuroprotective agent which useful in preventing neurodegenerative diseases. Here, we investigated BDNF serum concentrations after feeding the canarium oil (CO) and Canarium oil-based structured triglyceride (COST) in rats. Analysis of the BDNF serum was conducted by sandwich enzyme-linked immunosorbent assay (ELISA). The results demonstrated that the CO and the COST with the main composition of unsaturated fatty acids at the sn-2 position as much as 94.92% mol may enhance BDNF serum concentrations of up to 2.66% and 21.96% compared to the normal group of rats, respectively. This finding proves that the COST has prospects as an inducer of BDNF. However, further studies should be conducted to identify the precise dosage and safety products.

Keywords: Brain-derived neurotrophic factor, Canarium indicum oil, Canarium oil-based structured triglyceride, neurogenesis, unsaturated fatty acids

Abbreviations: BDNF: brain-derived neurotrophic factor; CO: Canarium indicum oil; COST: canarium oil-based structured triglyceride; ELISA: enzyme-linked immunosorbent assay; MUFA: monounsaturated fatty acid; PUFA: poly-unsaturated fatty acid; SFA: saturated fatty acid; sn-: stereospecific numbering; UFA: unsaturated fatty acid

INTRODUCTION

BDNF is a unique member of the neurotrophin family in the brain and is crucially involved in the brain neurons during development, neurogenesis, neural circuit formation, and synaptic plasticity (Mercado et al. 2017; Taj et al. 2017; Tharmaratnam et al. 2018). BDNF is also involved in processes of brain aging and cognitive function, thus it may serve as an intermediate biomarker for investigating subclinical vascular disease and also have biological potential as a therapeutic target for prevention of brain diseases (Libman-Sokolowska et al. 2015; Jasinska et al. 2016; Lin et al. 2017; Razavi et al. 2017). Moreover, several clinical reports suggest that BDNF plays a crucial role in neurodegenerative diseases, such as Alzheimer’s, Parkinson’s and psychiatric disorders like schizophrenia and depression (Jeon et al. 2014; Choi et al. 2018). The low BDNF levels have been observed in patients with Alzheimer’s disease (Borba et al. 2016), patients with coronary artery disease, type 2 diabetes mellitus and syndrome metabolic (Motamedi et al. 2017; Jin et al. 2018) and patients with depressive disorders and schizophrenia (Sakata 2014; Ihara et al. 2016; Vilibic et al. 2018). The Framingham study conducted by Pikula et al. (2013) concluded that the low levels of the BDNF serum were associated with increased risk of incident stroke. Another function of BDNF also reported by Spagnuolo et al. (2018) that BDNF act as a regulator for biosynthesis of cholesterol, including the biosynthesis of cholesterol on the brain.

BDNF is synthesized in the brain, among others in the hippocampus, the frontal cortex, and amygdala (Miranda et al. 2019), and it can be transported to the blood across the Blood-Brain Barrier or BBB (Khaliq et al. 2016; Sugasini et al. 2020). A number of studies have reported that the serum BDNF levels in the blood may reflect the levels of BDNF in the brain (Klein et al. 2011; Naegelin et al. 2018; Gejl et al. 2019). It consequently making it easier to measure the levels of BDNF in the blood if compared to measure BDNF in the brain, so it possible applied to humans.
Meanwhile, many studies have reported the role of fatty acids especially the essential fatty acids that contribute to the development of the brain, involved to control the structure and function of biological membranes, including membranes on the central nervous system (Janssen and Kiliaan 2014; Witte et al. 2014; Hadjighassem et al. 2015). Polyunsaturated fatty acids (PUFAs) are essential fatty acids and classified mainly into omega 3 and omega 6 groups that also essential nutrition in the development and functioning of the brain as well as visual system (Janssen and Kiliaan 2014; Kulzow et al. 2016). Study on animals indicated that dietary PUFAs deficiency may alter brain chemistry composition and demonstrate inhibition of the development and neurotransmission processes. The deficiency of dietary omega 3 may influence the specific neurotransmitter systems, particularly the dopaminergic system of the frontal cortex, which is related to cognition (Tsuchimine et al. 2015). Another role of the essential fatty acids is the precursor for the formation of eicosanoids compound through desaturation and elongation reactions. The products of eicosanoids resulted in effects that arrange the physiological processes of the body (Janssen and Kiliaan 2014).

*Canarium indicum* (C. indicum) is a species of canarium that belongs to Burseraceae family. *C indicum* is a native plant of eastern part of Indonesia, Papua New Guinea, Solomon Island, and Vanuatu (Mogana and Wiart 2011). The kernel of *C indicum* (Figure 1) may contain up to 75% oil (Rahman et al. 2015), therefore it can be used as a source of vegetable oil. According to epidemiological report, the emulsion of well-ripe seeds of *C indicum* may be used as milk substitute for infants (Rahman et al. 2019), which means that *C. indicum* seed is non-toxic and secure to be utilized in food applications. In addition, as shown in Figure 2 that the fatty acids on canarium oil have good nutritive value with predominantly of MUFA (oleic and palmitoleic acids), PUFA (linoleic and linolenic acids), and SFA (myristic, palmitic and stearic acids) (Rahman et al. 2015).

This study was conducted to measure the BDNF levels on rats’ serum after feeding of canarium oil (CO). In addition, we make modifications to the structure of canarium oil triglyceride as canarium oil-based structured triglyceride (COST). Both CO and COST contain 94.92% mol unsaturated fatty acids in the sn-2 position in structured triglycerides. Based on the fatty acids composition of CO and COST, hence we hypothesize that CO and COST may act as an inducer substance to enhance the BDNF serum. To the best of our knowledge, this is the first in-vivo study to measure BDNF on blood serum after feeding CO and COST.

**MATERIALS AND METHODS**

**Preparation of the CO and the COST**

The canarium oil (CO) was obtained through extraction using a mechanical pressing as in the method given in detail by Rahman et al. (2015), whereas COST made as reported in our previous study (Sitompul et al. 2018). CO is a raw material for making COST through an enzymatic reaction, namely by incorporating medium-chain fatty acid (caprylic acid) onto positions of sn-1 and sn-3 of triglycerides of CO. Hereafter, analysis of the composition and distribution of fatty acids in CO and COST was described also in our previous study (Rahman et al. 2015; Sitompul et al. 2018).
Animals and diet
In this study, we used male rats of Wistar strain with age 8-12 weeks (n=16), weighing 200-300 g which derive from the animal laboratory in School of Pharmacy of Institute of Technology Bandung. The rats were cage-housed in pairs at 20°C in light/dark cycle. Drinking water and food pellet were available ad libitum. All animal procedures were approved by the Health Research Ethics Committee of the Medical Faculty of University Padjadjaran of Indonesia (No. 249/UN6.C10/PN/2017). The animals were divided randomly into 4 groups; with each group consisting of 4 rats. Group 1. standard diet, group 2. hypercholesterolemic diet, group 3. standard diet and the CO 90 mg/kg body weight, while group 4. standard diet and the COST 90 mg/kg body weight. The product was administrated orally for 28 consecutive days. The composition of diet normal containing 59% carbohydrate, 16% crude protein, 10% crude fat, 14% crude fiber, and 1% vitamin, while the diet for hypercholesterolemia containing 1% pure cholesterol.

Preparation of serum for in vivo analysis
The serum preparation for all groups of rats performed based on the methods by Elfving et al. (2010) and Klein et al. (2011). Blood sampling conducted after a fasting period of 10-12 hours, and collected between 10: 30 to 11: 30 A.M because the distribution of BDNF is circadian rhythm (Elfving et al. 2010; Laske et al. 2011). Serum was diluted with standard/sample diluent (1: 20 v/v) prior to use. Serum was prepared by taking rat blood through a vein in the tail with standard/sample diluent (1 20 v/v) prior to use. Serum was prepared by taking rat blood through a vein in the tail with standard/sample diluent (1: 20 v/v) prior to use. Serum was prepared by taking rat blood through a vein in the tail with standard/sample diluent (1: 20 v/v). The mixture was then pipetted each of 100 mL and incubated at 4°C. The sample was pipetted of 10 mL and diluted with standard sample diluent 200 mL (1: 20 v/v). The mixture was then pipetted of 100 mL and incorporated into wells on the plate. The plate was immediately placed into the ELISA reader to measure the absorbance of the samples at λ=450 nm.

Preparation of the sandwich ELISA system
The BDNF levels were measured in rats’ serum using the Chemokine BDNF ELISA kit, according to the manufacturer’s instructions (Millipore-Germany, Cat. CYT306). In general, the preparation of a sandwich ELISA performed with 5 stages, (1) coating the microtiter plate with an antigen, (2) blocking all unbound sites to prevent false-positive results, (3) adding primary antibodies to the wells on the plate, (4) adding secondary conjugated antibodies to the enzyme, and (5) generating a reaction between the substrate and the enzyme to produce a colored product that can be measured at λ=450 nm.

Data analysis
Data were analyzed using Kolmogorov-Smirnov’s test to find out normality and homogeneity of data. As our data were normally distributed and homogeneous, then data analysis was done using analysis of variance followed by the Least Significant Difference (LSD) tests for post hoc comparisons. The difference was considered significant at p<0.05.

RESULTS AND DISCUSSION
Composition and distribution of fatty acids in CO and COST
The composition of fatty acids in CO has previously been measured as reported in Sitompul et al. (2018), which is the three highest fatty acids respectively were oleic acid (51.71 mol%), linoleic acid (32.40%) and palmitic acid (10.31 mol%). The three fatty acids were from the MUFA, PUFA and SFA. Furthermore, to improve the triglyceride structure of CO, the modification of the triglyceride structure was carried out using an enzymatic reaction to produce structured triglyceride that we called COST. Structured triglycerides are triglycerides that have been modified and or restructured by changing the composition and or distribution of fatty acid positions in the glycerol backbone of a triglyceride (Kim and Akoh 2015). The purpose of improving the triglyceride structured of CO is to increase the nutritional value reduce the SFAs in the sn-1 and sn-3 positions by replacing their positions with caprylic acid. Caprylic acid is a group of medium-chain saturated fatty acids. The presence of SFAs is not beneficial in terms of health.

The difference between the CO and the COST is in the composition and distribution of the fatty acids in triglyceride both CO and COST. CO triglycerides having fatty acid composition and distribution of the random, whereas COST mostly fatty acids at position sn-1 and sn-3 have been occupied by caprylic acid. Because caprylic acid has a medium chain length, it provides quick delivery of energy and has lower tendencies to be deposited in the adipose tissue (Kim and Akoh 2015). The term sn-1, sn-2, and sn-3 are used to indicate the position of fatty acids in a triglyceride. Sn-1 and sn-3 show fatty acids in the outer position of triglyceride, while sn-2 is the middle position occupied by fatty acids. Distribution of fatty acid positions in triglycerides as illustrated in Figure 3.

The composition of UFAs in the sn-2 position on both CO and COST amounted to 94.92%, by which the contents of the UFAs were palmitoleic acid, oleic acid, linoleic acid, and α-linolenic acid. Based on the data reported in Sitompul et al. (2018), we hypothesize that the presence of UFAs in sn-2 position may enhance the levels of the BDNF serum. To prove the effect of CO and COST in rats on neurogenesis, then the study continued by measuring BDNF activities.

Figure 3. The relative positions of the fatty acid in the sn-1, sn-2 and sn-3 in structure triglyceride
Standard curve for measuring BDNF concentrations

Next, we examined the effects of concentration BDNF after feeding CO and COST to normal rats, respectively. In this study, we measured the level of the BDNF serum using sandwich ELISA method. Before measuring the levels of blood serum BDNF, a standard curve created using the BDNF standards that made in various concentration ranges. The standard curve was useful to find out the linearity of the measurement and then the linear regression equation will be obtained to be used in establishing the BDNF concentration of the samples. The standard curve as shown in Figure 4.A demonstrated that fairly good linearity by the correlation coefficient was $R^2=0.9983$, hence the method for BDNF measurement using ELISA can be applied in this study. Determination of BDNF concentration using the equation $y=0.0028x+0.1427$. ELISA preparation results are shown in Figure 4.B.

Effect of CO and COST on BDNF serum

The mean values of the BDNF concentration in serum (pg/mL) for group 1, group 2, group 3, and group 4 were 7600.76±565.10, 5043.62±901.67, 7802.11±395.61, and 9269.11±1089.86, respectively. Group 4 which fed with the COST significantly different (p=0.020) and may enhance BDNF concentration as much as 21.96% compared to group 1 as shown in Table 1. The mechanism of the rising of BDNF level after feeding the COST may be explained due to the process of metabolism of the structured triglycerides. As mentioned above, COST is a structured triglyceride which has a triglyceride structure that is better than the triglyceride structure of CO, based on distribution analysis of fatty acids composition using HPLC. COST has a good triglyceride structure because the fatty acids in the $sn$-1 and $sn$-3 positions are occupied by short-chain fatty acids, namely caprylic acid. Because caprylic acid has a short carbon chain (C8: 0), its metabolic process produces energy quickly. The result of this study indicates that administration of COST may increase serum BDNF levels significantly compared with the provision of normal diet (as shown in Table 1), because COST is metabolized rapidly so that the fatty acid at the $sn$-2 position undergo desaturation and elongation quickly. Desaturation is the reaction of adding double bonds to fatty acids while the elongation reaction is the reaction of increasing the amount of carbon which causes the extension of the fatty acid chain. The sequence of desaturation and elongation reactions causes the formation of PUFAs which can increase BDNF levels (Janssen and Kiliaan 2014; Hadjighassem et al. 2015). Meanwhile, CO triglycerides have a random composition and distribution of fatty acids. Fatty acids at $sn$-1 and $sn$-3 positions from CO still consist of long-chain saturated fatty acids (including palmitic acid and stearic acid) hence the triglyceride metabolism process will run slowly and produce a lot of long-chain saturated fatty acids. The existence of excessive long-chain saturated fatty acids is not beneficial from a health point of view and also such fatty acids are converted into energy rather slowly in lipid metabolism, resulting in the formation of PUFAs will also be slow (Kim and Akoh 2015).

Table 1. The mean of BDNF concentrations in the treatment groups

| Treatment  | Serum concentration of BDNF (pg/mL) |
|------------|------------------------------------|
| Group 1    | 7600.76±565.10 a                    |
| Group 2    | 5043.62±901.67 c                    |
| Group 3    | 7802.11±395.61 a                    |
| Group 4    | 9269.11±1089.86 b                   |

Note: BDNF concentrations were compared among groups after receiving diets (p<0.05). Group 1. normal diet; group 2. cholesterol diet; group 3. normal diet and CO; group 4. normal diet and the COST. Note: different letters in the same column indicate significant differences (p<0.05).

Figure 4. The standard curve for BDNF (A) and sandwich ELISA preparation (B)
The essential fatty acid in the sn-2 position will be metabolized by the activities of enzymes of desaturase and elongase. The metabolic pathways of essential fatty acid by the elongation and desaturation process as discussed by Zhang et al. (2016) and Grag et al. (2017). Elongase is a group of enzymes that catalyze the addition of the number of carbon atoms, while desaturase is an enzyme that catalyzes the addition of the double bond in the fatty acid structure. The results of some studies suggested that the intake of linoleic acid (C18: 2n-6), palmitic acid, and oleic acid which esterified in the sn-2 position on triglycerides correlating to the levels of arachidonic acid (C20: 4n-6), palmitoleic acid (C16: 1n-7) and eicosatrienoic acid (C20: 3n-9) as a result of successive metabolism of linoleic acid, palmitic acid, and oleic acid. It can be seen from the reduced concentration of linoleic acid which is accompanied by increased concentration of arachidonic acid in plasma (Zhang et al. 2016; Czumaj and Sledzisizki 2020). A similar case also occurred in the metabolism of fatty acids in the COST.

The most abundant PUFAs in the brain are docosahexaenoic acid (DHA) and arachidonic acid (ARA). DHA and ARA may be generated from α-linolenic acid (ALA, 18: 2n-3) and linoleic acid (LA, 18: 2n-6), respectively, via desaturation and elongation reactions. ARA has a role as the precursor for the formation of prostacyclin (PGI2) (Pisani et al. 2015). On the wall of a cerebral artery, BDNF activates ARA metabolism, hence it will increase production of PGI2. PGI2 is the main mediator which facilitates the vasodilating properties of cerebral artery walls and protective effect of the stimulus that can cause vasoconstriction. In addition, PGI2 level in the arterial wall acts to enable signaling Peroxisome Proliferator-Activated Receptors γ (PPARγ). All of these effects can increase the resistance toward the cerebral circulation on the injury or trauma to the artery wall (Santhanam et al. 2010). Meanwhile, according to Kumar et al. (2014), that the intake of omega 3 fatty acids can normalize levels of BDNF in mice that have been made Traumatic Brain Injury (TBI). The decrease of BDNF level may be caused by TBI that can interfere with cognitive function. BDNF acts as synaptic transmission and can enhance learning by enabling synapsin I and cAMP-Responsive Element Binding (CREB). DHA levels are lacking in brain also associated with Alzheimer’s disease, schizophrenia, and depression (Janssen and Kiliaan 2014; Witte et al. 2014). Among omega 3 fatty acids, α-linolenic acid (ALA), can significantly increase the plasma levels of BDNF after each individuals receiving ALA capsules in the study of Hadjighassem et al. (2015), so they conclude that ALA treatment could be used in adjunction with routine stroke therapies. All the studies concluded that there was a direct correlation between omega 3 fatty acids consumption and the increase of serum BDNF.

As a conclusion, in the evaluation in vivo, the results are promising concerning the future use of the COST to induce the BDNF, due to as shown by the ability in inducing BDNF serum concentration in amount of 21.69%. Consequently, it may be used in the prevention of diseases due to the activities and expression of BDNF, for instance, Alzheimer's, Parkinson's and other neurodegenerative diseases, vascular brain disease, and cardiovascular disorders. In our study, BDNF measurement was conducted using a blood sample, although BDNF is given by the term "brain-derived". Several animal studies have shown that levels of BDNF in the blood may reflect levels of BDNF in the brain, because BDNF may circulate through the BBB as mentioned before. Klein et al. (2011) conducted research that concluded there was a positive correlation between the levels of BDNF in the brain and BDNF levels in the blood for all the species of animals used. Although measurements of BDNF in this study were very favorable to be applied to the measurement of BDNF in humans, but to ensure maximum BDNF measurement results, it is advisable to measure BDNF in the brain using other BDNF measurement methods, such as Western blotting, Elisa and histochemical staining. Measurement of BDNF in the brain of course only be applied to test animals and with more complex sample preparation methods.
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