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Anti-HMG-CoA reductase and antioxidant activities of Sacha inchi (*Plukenetia volubilis* L.) nutshell extract

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ABSTRACT

Background: Hypercholesterolemia is one of the major risks of cardiovascular diseases (CVDs). Hypercholesterolemia and oxidative stress are involved in the pathogenesis of atherosclerosis. Thailand has high percentage of unawareness of hypercholesterolemia, with low percentage of treatment and control. HMG-CoA reductase is a rate limiting step enzyme in cholesterol biosynthesis. Synthetic drugs such as statin are normally used to lower cholesterol level, however it causes adverse effects on the liver and muscle. Thus, HMG-CoA reductase inhibitors of plant origin are needed. *Plukenetia volubilis* Linneo, commonly known as Sacha inchi or inca peanut is a potential oilseed crop. The seeds of this plant are rich in omega-3 fatty acid. Some studies have shown that consuming Sacha inchi could reduce blood cholesterol. However, the exact mechanism of their lipid lowering activity is still unknown.

Objectives: This study aimed to investigate the anti-HMG-CoA reductase, anti-cholesterol esterase and antioxidant activities of different parts of Sacha inchi extracts.

Materials and methods: Hot water extracts of 3 different parts of Sacha inchi (nutshell, baby nut and leaf) and Sacha inchi nut oil were evaluated the anti-HMG-CoA reductase and antioxidant activities. HMG-CoA reductase inhibitory activity was determined spectrophotometrically by NADPH oxidation, using HMG-CoA as a substrate. HMG-CoA reductase inhibitory mechanism was also analyzed by using Lineweaver-Burk plot. Antioxidant activity was determined by ABTS and DPPH radical scavenging assay. Total phenolic content was also measured by Folin-Ciocalteu reagent. Moreover, cholesterol esterase inhibition assay was also performed.

Results: Sacha inchi nutshell extract revealed the highest anti-HMG-CoA reductase activities at about 99% at concentration of 250 µg/mL, with uncompetitive inhibition in Lineweaver-Burk plot analysis. It also showed the highest cholesterol esterase inhibitory activity around 38% at concentration of 125 µg/mL. Moreover, Sacha inchi nutshell extract also showed the highest antioxidant activity by both ABTS and DPPH assay. Antioxidant activity was correlated to total phenolic content.

Conclusion: The experimental data suggested that Sacha inchi nutshell extract is a source of antioxidant compound and may lower cholesterol level by inhibiting HMG-CoA reductase and cholesterol esterase enzymes. Investigation in an *in vivo* model could further confirm the potential use of Sacha inchi nutshell extract as a supplement for treatment of hypercholesterolemia.

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Introduction

Cardiovascular diseases (CVDs) are becoming a leading cause of death in developing countries due to demographic transition and lifestyle changes.[1] The prevalence of CVDs has increased rapidly. According to WHO fact sheet in 2016, there are 17.9 million people died from CVDs around the world. In Thailand, the prevalence and mortality rate have also increased and most of CVDs patients are adults.[2] A lack of income, reduced productivity, and increased health care costs lead to overall economic losses. Thus, the prevention of CVDs can save not only billions for the economy but also many lives. Major risk factors of CVDs include dyslipidemia, diabetes, smoking, high blood pressure, and family history of atherosclerotic cardiovascular diseases (ASCVD). Moreover, additional risks of CVDs have also been reported such as alcohol consumption, unhealthy diet, sedentary lifestyle, obesity, stress, and air pollution.[3] Pathogenesis of CVDs begins with the development of atherosclerosis causing by an accumulation of cholesterol in the intima layer of blood vessel. Low density lipoprotein (LDL) contains the highest amount of cholesterol compare to other lipoprotein particles. Accumulation of LDL in intima layer of blood vessel and modification of LDL particle by free radical and carbohydrate trigger the development of atherosclerosis. Thus, hypercholesterolemia and oxidative stress status directly involved in atherosclerosis. Moreover, excess adipose tissue in obesity can cause inflammation, induce oxidative stress by releasing of pro-inflammatory cytokines and induce insulin resistant which leads to type 2 diabetes mellitus (T2DM).[4,5] Lowering cholesterol level and improving oxidative stress status can prevent CVDs and T2DM.

The 3-hydroxy-3-methylglutaryl-coenzyme A (HMG CoA) reductase is a rate limiting step enzyme in cholesterol biosynthesis pathway. Inhibition of this enzyme can lower cholesterol level in blood. Statin, HMG-CoA reductase inhibitor, is effectively used for treatment of hypercholesterolemia. However, statins increased risk of liver and muscle-related adverse effects in long-term use such as myalgia, myopathy and rhabdomyolysis.[6] Thus, HMG-CoA reductase inhibitors of plant origin are needed. Medicinal plant that contains flavonoids showed anti-atherosclerotic effect.[7] Another way to reduce cholesterol level is inhibition of cholesterol esterase (CE). The hydrolysis of dietary cholesterol ester into cholesterol by CE in the intestinal lumen is an essential process for absorption. Therefore, inhibition of cholesterol esterase enzyme activity could inhibit absorption of dietary cholesterol esters.[8-10]

Sacha inchi (*Plukenetia volubilis* L.) belongs to the family Euphorbiaceae. It is also known as inca peanut. It is a tropical rain forest Amazonian plant. Sacha inchi becomes an economic crop in Southeast Asia especially in Thailand. Sacha inchi seeds contain mainly oil and protein. Commercially available Sacha inchi oil is beneficial for health. Sacha inchi oil contains high amount of long-chain n-3 fatty acids and antioxidant compounds such as tocopherol and vitamin A.[11-13] In addition, dried Sacha inchi leaves and nutshell are available as tea. Many customers who consumed Sacha inchi products experienced good controlled of blood glucose, decreased cholesterol level especially LDL-cholesterol and increased high-density lipoprotein cholesterol (HDL-cholesterol).[14] Sacha inchi seeds showed antioxidant activity[15] and immunomodulatory activity,[16] however other parts of plant have never been studied before. This study aimed to investigate anti-HMG-CoA reductase, anti-cholesterol esterase and antioxidant effects of Sacha inchi extracts (dried leaves, nutshells, and baby nuts) as well as Sacha inchi oil

Materials and methods

Chemicals

The 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS), gallic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 4-nitrophenyl butyrate (pNPB), cholesterol esterase from porcine pancreas and HMG-CoA reductase assay kit were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethanol and isopropanol were purchased from MERCK (Darmstadt, Germany). L-ascorbic acid (vitamin C), and sodium hydrogen phosphate were obtained from Ajax Finechem Pty Limited (Taren Point, New South Wales, Australia). Acetonitrile and sodium chloride were purchased from RCI Labscan Limited (Bangkok, Thailand). Sodium fluoride (NF) was acquired from BDH Laboratory Supplied (Poole, England). Triton X-100 was purchased from Amresco (Solon, Ohio, USA) All reagents were analytical or HPLC grade. Distilled water and ultrapure water were used for all experiments.

Plant materials and extracts

Sacha inchi leaves, nutshells, and baby nuts were provided by Oil Star Tak Limited Partnership, Tak province, Thailand. Each part of plant was dried and ground to powder. One hundred grams of Sacha inchi leaf, nutshell, and baby nut powder was extracted by boiling with 1 liter of distilled water for 15 minutes and further incubated for 4 hours. Solution was filtered through straining cloth and centrifuged at 3,500 rpm for 5 minutes. After centrifugation, supernatant was filtered through Whatman paper filter no.1. The filtrate was completely dried in a freeze-dryer and stored at -20°C until further use. The total yield percentage of the Sacha inchi extract was calculated by the formula, yield percentage (%) = (weight of extract obtained)/ (total weight of sample loaded)×100.

Sacha inchi nuts were mainly used to produced sacha inchi oil which contain high amount of polyunsaturated fatty acid and antioxidant compounds such as tocopherol and vitamin A. Cold pressed Sacha inchi oil was received from Oil Star Tak limited partnership, Tak province, Thailand. The Sacha inchi oil was stored at room temperature. The oil was dissolved in isopropanol into the desired concentration before performing any experiments or added directly into the reaction mix in the volume that meet the desired concentration.

Total phenolic content (TPC)

The TPC of Sacha inchi extracts were analyzed spectrophotometrically using modified Folin-Ciocalteau colorimetric method.[17] Sacha inchi extracts were dissolved to the concentration of 1 mg/mL with deionized (DI) water. Samples (100 µL)

were mixed with 100 µL of Folin-Ciocalteau's phenol reagent (Sigma-Aldrich, MO). Then, 300 µL of 20% sodium carbonate solution were added into the reaction and incubated at room temperature for 15 min. After incubation, 100 µL of DI water were added into the reaction and centrifuged at 1,250 ×g at 25˚C for 5 min. Supernatant were collected and measured an absorbance at 765 nm by using UV-Visible spectrophotometer (SPECORD PLUS 250, Germany). Diluted extracts were used as sample blank. Gallic acid (0-300 µg/ml) was used as a standard. Calibration curve was plot against concentration of gallic acid and absorbance at 765 nm. TPC of extracts were expressed as mg of gallic acid equivalents/gram dried weight (mg of GAE/g).

2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) assay

This study aimed to evaluate the free radical scavenging capacity of each Sacha inchi extracts, to reduce the radical cation $ABTS^{+}$ to ABTS according to the method described by Kokina *et al*.[18] Briefly, ABTS stock solution was prepared by mixing ammonium persulfate (4.9 mM) and ABTS (7 mM) in equal ratio and incubating at RT in dark for 12-16 hrs. The working $ABTS^{+}$ solution was prepared by dilution of the stock solution with 80% methanol to absorbance at 734 equals to 0.700±0.020. Different concentrations (1-20 mg/mL) of Sacha inchi extract (10 µL) were added to 990 µL of $ABTS^{+}$ working solution. An absorbance (734 nm) was measured at 1 min after incubation by using UV-Visible spectrophotometer. L-ascorbic acid was used as a standard. Calibration curve was plot against concentration of ascorbic acid and absorbance at 734 nm. The results were expressed as mg of ascorbic acid equivalent/gram dried weight.

2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay

Free radical scavenging activity of Sacha inchi extract was investigated using DPPH assay as described by Hajlaoui *et al*.[19] Sacha inchi extracts (40 µL) at various concentration (1-20 mg/mL) were mixed with 1.2 mL of DPPH reagent and incubated at room temperature for 15 min. Samples were centrifuged at 1,250 ×g at 25˚C for 5 min after incubation. The supernatants were collected and measured absorbance at 517 nm by using UV-Visible spectrophotometer. Diluted extracts were used as sample blank. Gallic acid was used as a standard. Calibration curve was plot against concentration of gallic acid and absorbance at 517 nm. Results were expressed as mg of gallic acid equivalents/gram dried weight (mg of GAE/g).

HMG-CoA reductase activity assay

This study aimed to investigate inhibitory effect of Sacha inchi extracts on HMG-CoA reductase enzyme activity. The HMG-CoA reductase catalyzed HMG-CoA into mevalonate using NADPH. The HMG-CoA reductase activity was measured by the reduction of NADPH at 340 nm. Briefly, 4 µL of NADPH, 1 µL of samples, 12 µL of HMG-CoA substrate, and 181 µL of assay buffer were mixed. Pravastatin at final concentration of 0.2 µg/mL was used as inhibition control. To initiate the reaction, HMG-CoA reductase catalytic domain was added into reaction mixture. The absorbance (340 nm) was measured at 37 °C every 15 sec for 5 min by UV-Visible spectrophotometer. For blank, the assay buffer was added into reaction tube instead of HMG-CoA reductase. The enzyme activity was calculated using the following equation:

$$\text{Units/mgP} = \frac{(\Delta A_{340}/\text{min}_{\text{sample}} - \Delta A_{340}/\text{min}_{\text{blank}}) \times TV}{12.44 \times V \times 0.6 \times LP}$$

where ΔA: change of absorbance, TV: total volume of the reaction in mL, 12.44: coefficient of NADPH, V: volume of enzyme used in the assay, 0.6: enzyme concentration in mg-protein, LP: light path in cm.

Cholesterol esterase activity assay

Cholesterol esterase inhibitory activity of Sacha inchi extracts was evaluated spectrophotometrically at 25 °C using the method of Asmaa and Ream[20]. The cholesterol esterase from porcine pancreas (CEase) was solubilized in 1 mL of 0.1 M sodium phosphate buffer pH 7.0 and stored at -80˚C as a stock CEase. Prior to use, working CEase was prepared by diluting a stock CEase to 5 µg/mL with the same buffer. The reaction mixture consisted of 500 µL of Triton X-100 (5% w/w), 20 µL of p-nitrophenyl butyrate (0.05 M in acetonitrile), 40 µL of 2% acetonitrile in 400 µL of assay buffer (100 mM sodium phosphate, 100 mM sodium chloride, pH7.0) and 20 µL samples. The mixture was mixed and incubate at 25 °C for 5 min. The reaction was initiated by adding of 20 µL of working CEase (5 µg/mL). After 15 min of incubation at 25°C, the absorbance was measured by spectrophotometer at 405 nm. NaF was used as inhibition control. The percentage of inhibition were calculated using the following formula:

$$\%\text{Inhibition} = \frac{\text{Abs}_{\text{Activity}} - \text{Abs}_{\text{Sample}}}{\text{Abs}_{\text{Activity}}} \times 100$$

Statistical analysis

Data were expressed as mean±standard deviation (SD) of three independent experiments. The data were analyzed by one-way ANOVA. $p<0.05$ was considered to be statistically significant.

Results

Total phenolic content and antioxidant activity of Sacha inchi extracts

Leaves, baby nuts and nutshells of Sacha inchi were extracted with hot water. Total yield percentage of the Sacha inchi extracts were calculated. Leaf extract showed the highest yield percentage followed by baby nuts and nutshells, respectively (Table 1). The characteristic of leaves, nutshells and baby nuts hot water extracts were shown in Figure 1.

Table 1 Total yield percentage of hot water extracts of each part of Sacha inchi.

Sacha inchi	Yield of extract (%)
Leaves	19.73
Nutshells	12.64
Baby nuts	14.57

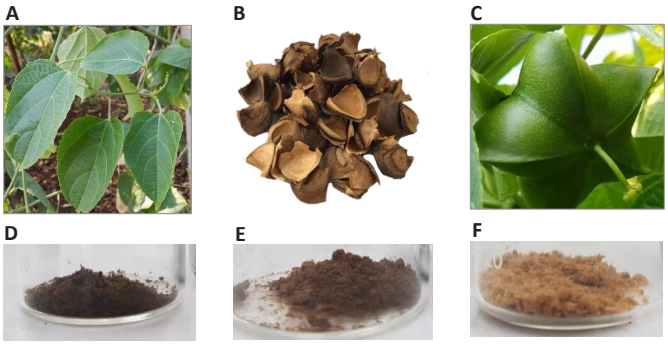

Figure 1. *Characteristic of tested materials. A: leaves, B: nutshells, C: baby nuts of Sacha inchi, D-F: corresponding dried hot water extracts (D-F).*

TPC was determined using Folin-Ciocalteu calorimetric assays. Sacha inchi nutshell and baby nut extracts showed much higher TPC compared to others, which were about 74.8 mg and 63.6 GAE/100 g dried weight, respectively. Sacha inchi leaf extracts and oil showed lower TPC which were 18.2 and 10.0 mg GAE/100 g dried weight (Figure 2).

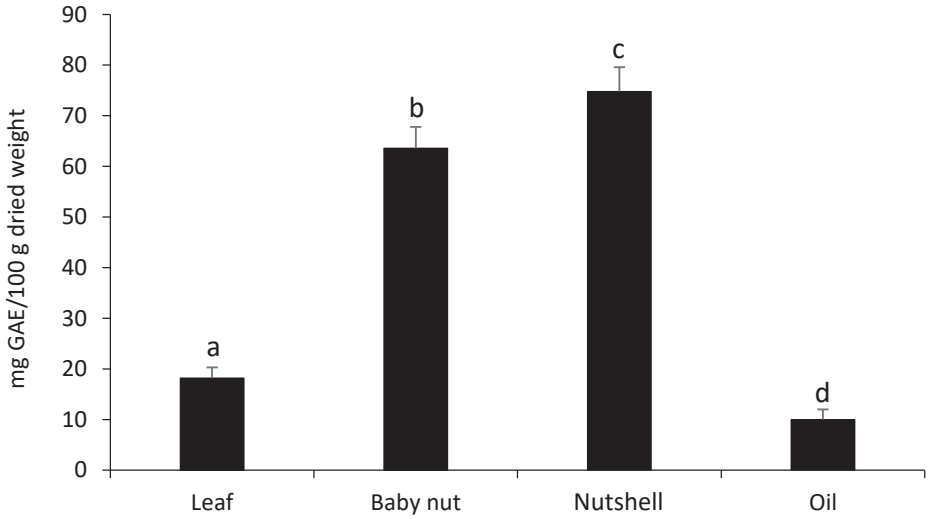

Figure 2. *Total phenolic content of different parts of Sacha inchi extracts and Sacha inchi oil at concentration of 1 mg/mL. The data were expressed as the mean±SD of sample tested in 3 independent experiments. Values having different letters differ significantly (p<0.05).*

The antioxidant activity was determined by DPPH and ABTS radical scavenging assay. Results from antioxidant activity by both methods were similar to the results from TPC. Sacha inchi nutshell extracts presented the highest DPPH and ABTS radical scavenging activity which were 78 mg GAE/100 g dried weight and 450 mg ascorbic acid equivalent/ 100 g dried weight, respectively. In contrast, Sacha inchi oil had the lowest antioxidant activity (Figure 3 and Figure 4).

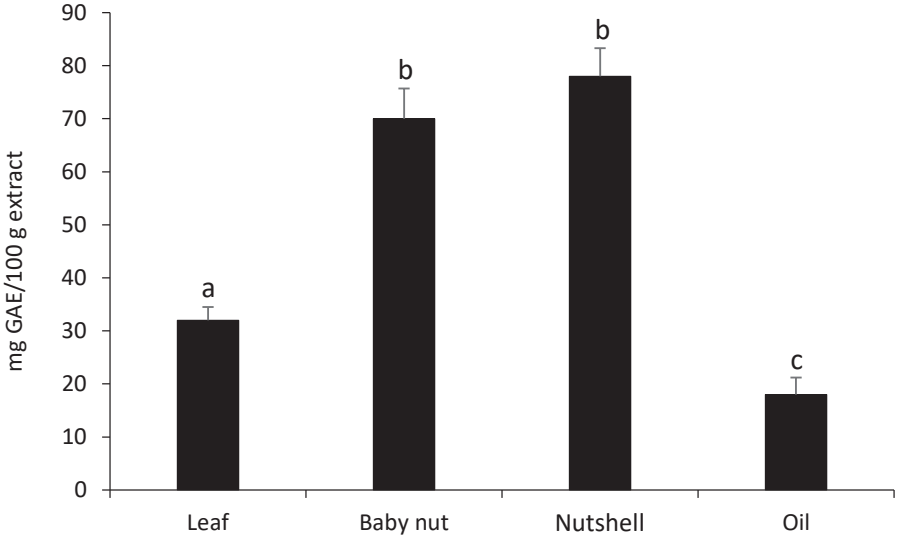

Figure 3. *DPPH radical scavenging activity of different parts of Sacha inchi extracts and Sacha inchi oil at concentration of 1 mg/mL. The data were expressed as the mean±SD of sample tested in 3 independent experiments. Values having different letters differ significantly (p<0.05).*

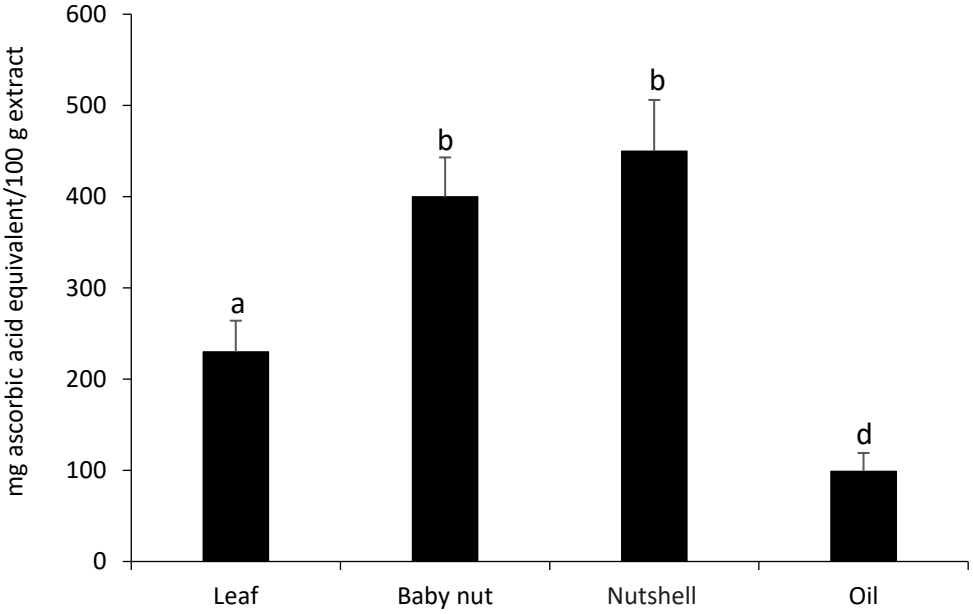

Figure 4. *ABTS radical scavenging activity of different parts of Sacha inchi extracts and Sacha inchi oil at concentration of 1 mg/mL. The data were expressed as the mean±SD of sample tested in 3 independent experiments. Values having different letters differ significantly (p<0.05).*

HMG-CoA reductase inhibitory activity

The HMG-CoA reductase inhibitory effect of Sacha inchi extracts and Sacha inchi oil were evaluated. Pravastatin was used as an inhibitory control which can inhibit HMG-CoA reductase activity for 75%. Only, Sacha inchi extract from nutshells presented HMG-CoA reductase inhibitory activity about 65% at the concentration of 125 µg/mL (Figure 5). The rest of the Sacha inchi extracts and Sacha inchi oil had no effect on HMG-CoA reductase activity. Inhibition of HMG-CoA reductase activity by Sacha inchi nutshell extract was dose-dependent manner. It inhibited HMG-CoA reductase activity up to 99% at the concentration of 250 µg/mL (Figure 6).

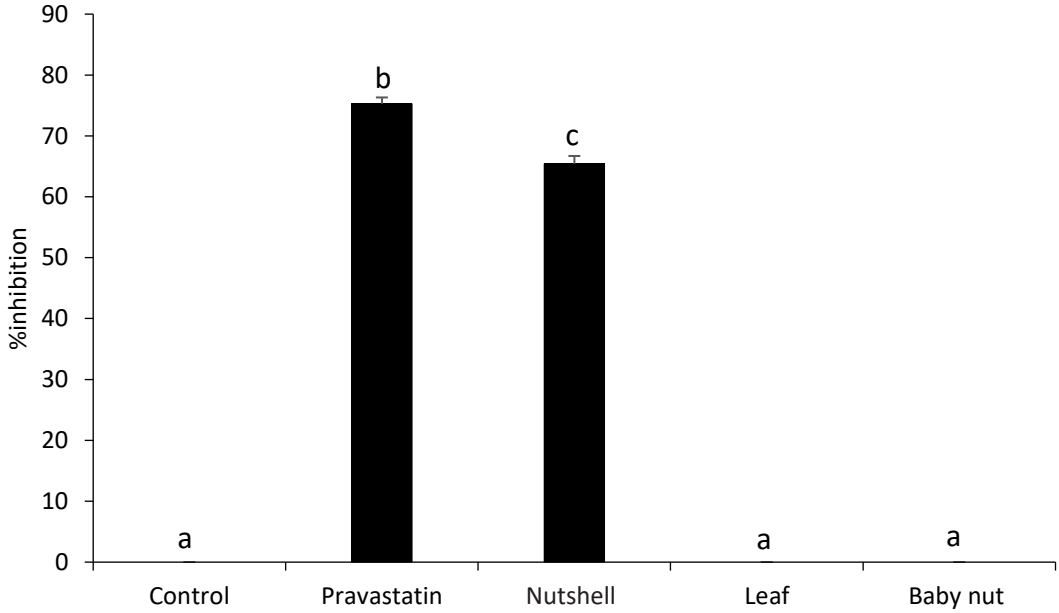

Figure 5. *HMG-CoA reductase inhibitory activity of different parts of Sacha inchi extracts (125 µg/mL). Distilled water was used as a negative control and pravastatin (0.2 µg/mL) was used as a positive control. All data were expressed as the mean±SD of sample tested in 3 independent experiments. Values having different letters differ significantly (p<0.05).*

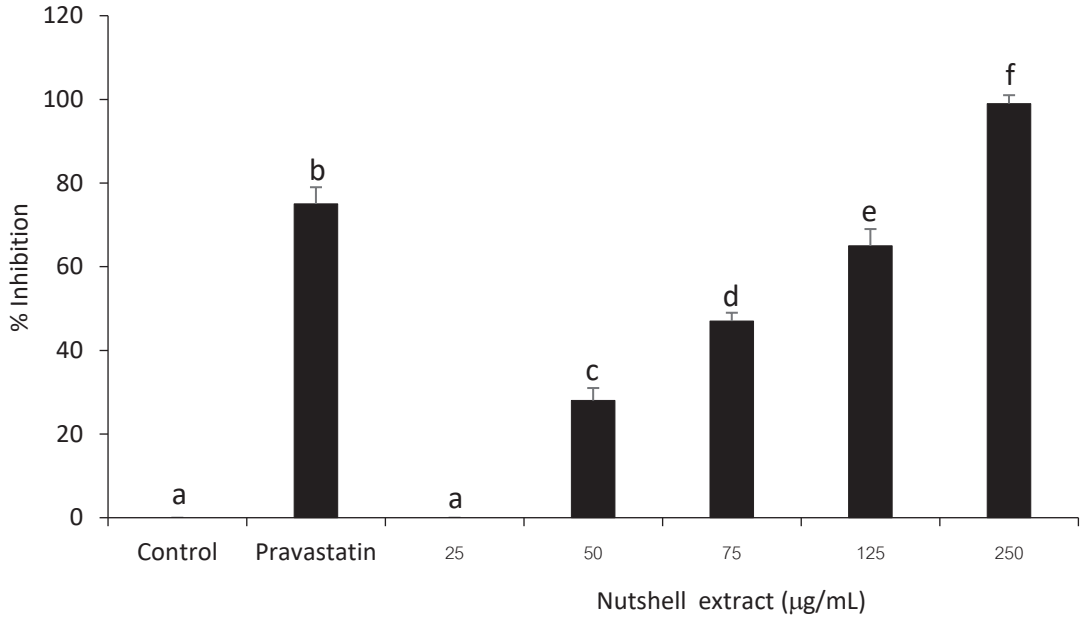

Figure 6. *Inhibition of HMG-CoA reductase activity by various concentrations of Sacha inchi nutshell extracts. Distilled water was used as a negative control and pravastatin was used as a positive control. All data are presented as the mean±SD of samples tested in 3 independent experiments. Values having different letters differ significantly (p<0.05).*

Sacha inchi nutshell extract was further analyzed the type of enzymatic inhibition by Lineweaver-Burk plot analysis. The Lineweaver-Burk plot analysis showed parallel line pattern (Figure 7), indicating the uncompetitive inhibition.

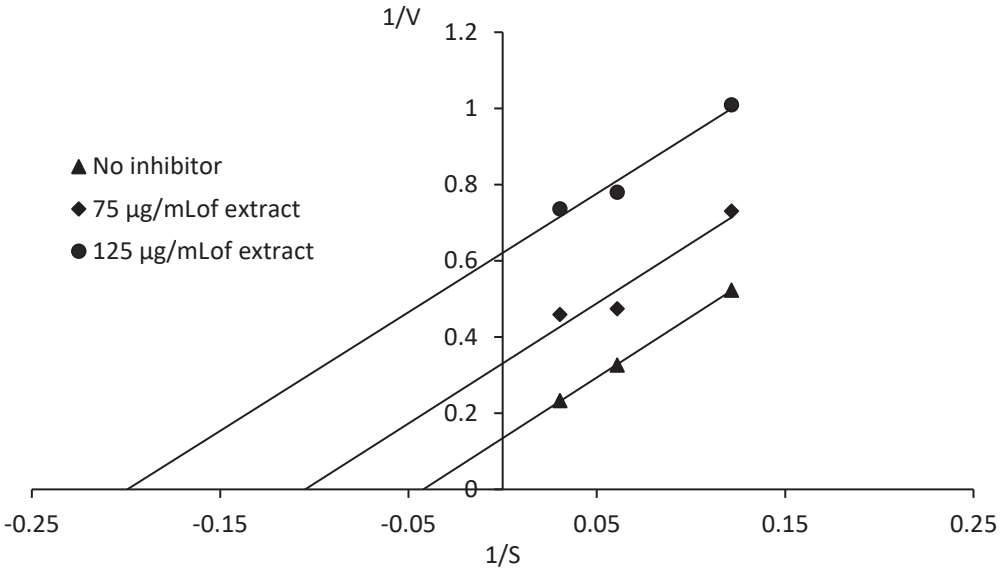

Figure 7. *Lineweaver-Burk plot analysis for HMG-CoA reductase in the presence of different concentrations of HMG-CoA (8, 16 and 32 mM) and two different concentrations of Sacha inchi nutshell extract (75 and 125 µg/mL). Data represents mean+SD of 3 independent experiments.*

Cholesterol esterase inhibitory activity

Sacha inchi extracts and Sacha inchi oil were evaluated for their cholesterol esterase inhibition activity. NaF used as a positive control markedly inhibited the cholesterol esterase about 26.0%. Among Sacha inchi extracts, only nutshell and baby nut extracts revealed cholesterol esterase inhibitory activity by 38.1 and 24.1%, respectively (Figure 8). Sacha inchi oil (750 µg/mL) had no effect on cholesterol esterase inhibition (data not shown).

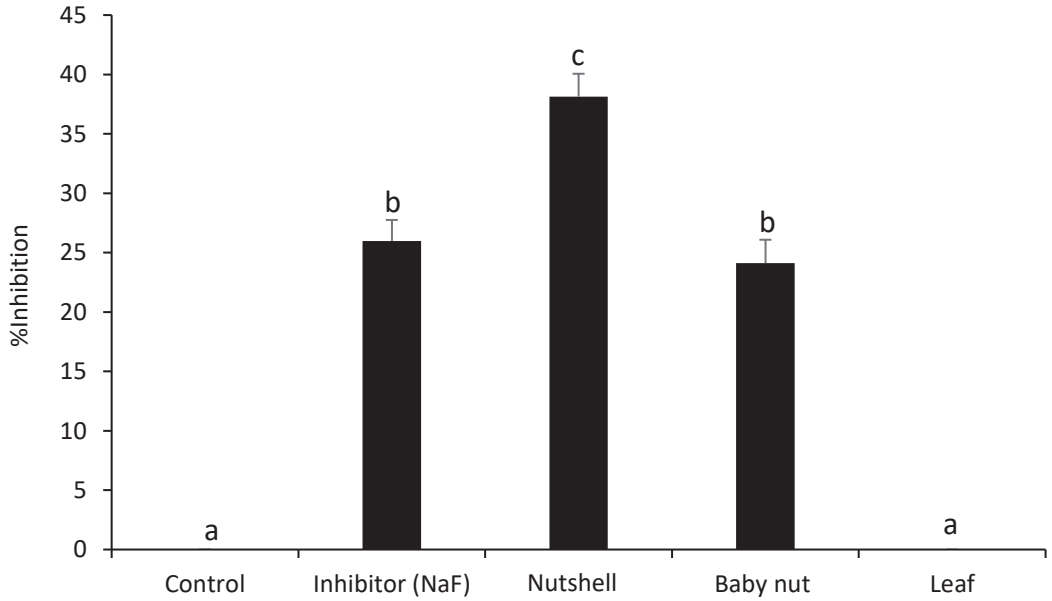

Figure 8. *Effect of Sacha inchi extracts (125 µg/mL) on cholesterol esterase activity. Percentage of enzyme inhibition were calculated. Control was represented the condition without inhibitor. Data represents mean+SD of 3 independent experiments. Values having different letters differ significantly (p<0.05).*

Discussion

Various parts of Sacha inchi are edible. Products of Sacha inchi in the market include roasted nut, cold press nut oil, and tea that made from leaves and nut shells.[11] Plants which contain antioxidant activity have been reported to also exert lipid lowering property.[21] As both oxidative stress and hyperlipidemia are involved in atherosclerosis, plant that exert both effects might be useful for CVDs prevention. Among various parts of Sacha inchi and sacha inchi oil, nutshell extract showed the highest antioxidant activity as well as total phenolic content. This data suggested that phenolic compound might be the active ingredient in Sacha inchi nutshell extract.

Sacha inchi products have been claimed to reduce cholesterol level. However, the scientific data and the exact mechanism are still limited. HMG-CoA reductase, a rate limiting step enzyme in cholesterol biosynthesis, is the target for treatment of hypercholesterolemia. Statins are synthetic drugs that competitively inhibit HMG-CoA reductase.[22] Statins are commonly used to treat hypercholesterolemia. However, the adverse side effects from long term use are in a great concern.[23] Thus, HMG-CoA reductase inhibitors from natural origin are in a great interest.[24]

This is the first report on anti-HMG-CoA reductase activity from Sacha inchi nutshell. Hot water extract of Sacha inchi nutshell inhibited HMG-CoA reductase activity ranging from 28-99% according to the concentrations of the extract (50-250 µg/mL). Previous studies on HMG-CoA reductase inhibitors from plant origin have been reported. HMG-CoA reductase inhibitory activity of crude extract of *Quercus infectoria*, *Basella alba, Rosa damascene*, *Myrtus communis*, and *Amaranthus viridis* leaf were 84%, 74%, 70%, 62% and 72%, respectively.[25-27] Fraction 18 of methanolic *Ficus virens* Ait extract showed a large number of HMG-CoA reductase inhibition about 98%.[28] Therefore, anti-HMG CoA reductase compounds from plant origin was very interesting because they can be used to lower blood cholesterol level. Cholesterol lowering effect of Sachi inchi via HMG-CoA reductase inhibition was firstly investigated in this study. The mechanism of enzyme inhibition of Sacha inchi nutshell extract was also studied. Unlike statin drug which competitively inhibit HMG-CoA reductase,[29] Sacha inchi nutshell extract showed uncompetitive inhibition pattern. This type of inhibitor is more specific and efficient compare to other types of inhibitors.[30] Uncompetitive inhibitors bind only to the enzyme-substrate complex, while competitive inhibitors have affinity for the enzyme and compete for substrate binding.

Besides inhibition of HMG-CoA reductase, inhibition of enzymes that control cholesterol absorption and transportation is important target for lowering blood cholesterol. Cholesterol esterase is one of those enzymes that plays an important role in the regulation of cholesterol metabolism by extending cholesterol intestinal absorption and transportation to enterocytes.[20] Sacha inchi nutshell extract showed the strongest anti-cholesterol esterase activity. Inhibition of cholesterol esterase could block cholesterol absorption and finally lower the blood cholesterol level. In this study Sacha inchi oil did not have either anti-HMG CoA reductase (data not shown) or strong antioxidant activity. However, previous studies have reported lipid lowering properties of Sacha inchi oil *in vivo*.[11,31] These data indicated that the mechanism of cholesterol lowering activity of Sacha inchi

oil did not cause by direct inhibition of HMG-CoA reductase.

All data suggested that Sacha inchi nutshell extract might be a potential lipid lowering agent which can be used as a supplement for treatment of hypercholesterolemia.

Conclusion

In conclusion, Sacha inchi nutshell extract showed the highest antioxidant activity and total phenolic content. It might be able to reduce blood cholesterol level through the uncompetitive HMG-CoA reductase inhibition, and cholesterol esterase inhibition. As oxidative stress and hyperlipidemia are contributed to CVDs development, consumption of Sacha inchi nutshell which contain high amount of antioxidant might prevent CVDs. However, more investigation in an *in vivo* model could further confirm the potential of Sacha inchi nutshell extract in treating hypercholesterolemia.

Conflict of interest

There are no conflicts of interest associated with this publication.

Acknowledgments

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