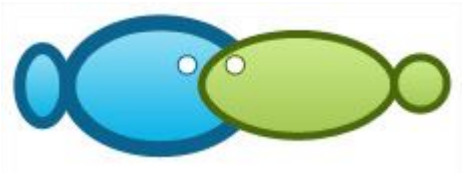

Influence of methanol-soaked treatment on the nutritional quality, antinutritional factors and nutrient digestibility of sacha inchi (*Plukenetia volubilis*) meal as protein ingredients in the diet of juvenile Nile tilapia (*Oreochromis niloticus*)

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Abstract. Sacha inchi meal (SIM), *Plukenetia volubilis*, is a by-product obtained from oil pressing process. It is rich in protein and omega-3 fatty acid. However, it contains antinutritional factors (ANFs) which cause low digestibility and growth of fish. This research was aimed to evaluate the nutritional quality, antinutritional factors and nutrient digestibility of SIM by methanol-soaked treatment (soaked-SIM), compared to SIM and soybean meal (SBM), as protein ingredients in the diet of juvenile Nile tilapia, *Oreochromis niloticus*. SIM was soaked in 80% methanol (1:10 w/v) for 48 h before drying. Crude protein of soaked-SIM (63.3%) was higher than those of SIM (56.5%) and SBM (45.5%). Crude fiber of soaked-SIM and SIM was lower than that of SBM. Soaked-SIM and SIM were rich in linoleic acid, linolenic acid, the ratio ω6/ω 3 was 0.92 and 1.01, respectively. The arginine and leucine content of soaked-SIM and SIM were higher than SBM while soaked-SIM had the lowest amounts of tannins, phenolic, saponins and trypsin inhibitor. The *in vitro* protein digestibility was conducted in juvenile Nile tilapia, result showed that soaked-SIM displayed significantly higher protein digestibility than any others ($p < 0.05$). Apparent digestibility coefficients of dry matter, protein, and lipid of soaked-SIM were significantly greater than of SBM ($p < 0.05$). Our finding suggested that methanol soaking method could improve the quality of SIM in terms of crude protein content, nutrient digestibility, and reduced amounts of ANFs. Besides, as nutritional value was improved in soaked-SIM, this suggests that soaked-SIM is a sustainable alternative protein source for fish feed.

Key Words: antinutritional factors, chemical composition, Nile tilapia, nutrient digestibility, sacha inchi.

Introduction. Fish meal is as a major source of protein in fish feeds due to an ideal resource to meet the essential amino acid requirement of the fish. The fish meal also contains high protein content and availability of micronutrients (Bandara 2018). However, fish meal prices have risen due to the decline in natural catches. The production cost of intensive aquaculture is commonly as high as 60-70% of total production that results from the feed cost (El-Sayed 2004). Therefore, there are several studies researching on reduction animal protein inclusion using alternative source of protein; especially plant protein ingredients, for reducing the production cost and increasing the aquaculture sustainability (Agbo et al 2021).

Incorporation of plant ingredients in the fish diet is a widely used practice driven by higher abundance and lower price compared to the fish meal. A substantial amount of research was already underway, testing potential protein sources that can replace fish meal in fish diets. The plant protein sources include cassava leaf meal (Ng & Wee 1989), rapeseed (Davies et al 1990), barley and alfalfa (Belal 1999), soybean meal (SBM) and cottonseed meal (Yue & Zhou 2008). A range of plant ingredients is used in aquaculture industry including grains (wheat and corn etc.), oilseeds (soybean, sunflower, rapeseeds, cottonseed etc.), and pulses (beans, lupins, and peas etc.) (Bandara 2018). Among those plant protein ingredients, SBM is the most common used in fish feeds due to its high protein content, high digestibility, relatively well-balanced amino acid profile, reasonable

price, and steady supply, SBM is widely used as a cost-effective feed ingredient for many aquaculture animals (Storebakken et al 2000; Nyirenda et al 2000; Koumi et al 2009); it is currently the most commonly used plant protein source in fish feeds (El-Sayed 1999). Banaszkiewicz (2011) report that SBM standardized on 44 and 49% of protein is on the feed market and the protein of soybean contains the considerable quantity of lysine 6.2 g per 16 g N), but value of protein is limited by methionine and cysteine content (2.9 g per 16 g N). Sacha inchi meal (SIM), *Plukenetia volubilis*, is a by-product obtained from oil pressing process. It is rich in protein (56.61%), amino acid (lysine, histidine and leucine) and omega-3 fatty acid (2656.13 mg $100g^{-1}$) (Rawdkuen et al 2016; Chirinos et al 2013; Kim & Joo 2019). In previous reports, Ortziz-Chura et al (2018) studied apparent digestibility coefficients of nutrients in SIM compared to other plant ingredients, such as kañiwa (*Chenopodium pallidicaule* Aellen), kiwicha (*Amaranthus caudatus* L), quinoa (*Chenopodium quinoa* Willd), beans (*Phaseolus vulgaris* L.), and jumbo squid (*Dosidicus gigas*) meal in rainbow trout (*Oncorhynchus mykiss*). Among tested ingredients, SIM showed the highest apparent digestibility coefficients of crude protein (98.0%) and digestible energy (4.15%) and the highest apparent digestibility coefficients of dry matter (73.5%). Araújo-Dairiki et al (2018) reported that 30% of dietary SIM did not cause negative impacts on feed intake, growth, and feed conversion ratio (FCR) of matrinxã (*Brycon amazonicus*). On the other hand, FCR of tambaqui (*Colossoma macropomum*) was significantly inferior when fed diet contained over 15% of dietary SIM. It was suggested that crude fibre is a reason for poor FCR in SIM fed fish. Recently, Khieokhajonkhet et al (2021) revealed that 11% of heated-SIM could be included in the diet for red hybrid tilapia (*Oreochromis niloticus* × *O. mossambicus*) with promoted growth and FCR. However, growth rate and FCR of fish fed over 11% of dietary SIM were negatively affected by ANFs.

It is known that solvent extraction method can remove ANFs and soybean peptides in SBM (Bureau et al 1998; Nguyen et al 2011). Dongmeza et al (2006) succeeded to remove some amounts of the saponins and tannins in moringa (*Moringa oleifera*) leaf by soaking the material in 80% methanol for 48h. This research was aimed to evaluate the phytochemical property and digestibility of SIM soaked in 80% methanol (soaked-SIM), compared to SBM, as a plant protein source for aquafeeds.

Material and Method. The experiment was conducted in March, 2020 at Department of Fisheries, Faculty of Agriculture, Khon Kaen University, Thailand.

Preparation of tested ingredients. Sacha inchi cake was donated by The Ultimate Bangkok Ltd. (UBM), Bangkok, Thailand. The processing of SIM and soaked-SIM is illustrated in Figure 1. Sacha inchi cake was finely ground and then sieved through a 30-mesh strainer to become sacha inchi meal (SIM). SIM was soaked with 80% methanol (1:10; w/v) for 48 h (Dongmeza et al 2006). Then soaked sacha inchi meal (soaked-SIM) was dried at 65°C for 12 h. Both SIM and soaked-SIM were stored at 4°C until further analysis.

In vitro protein digestibility. The juvenile Nile tilapia, *Oreochromis niloticus* (34.0±1.25 g) obtained from private farm (Khon Kaen province, Thailand). Fish were distributed to eight 150-L aquarium, each containing 15 fish in order to be acclimatized to the experimental condition for two weeks. The pooled sample of whole digestive tract of five tilapia in each aquarium was extracted for the crude digestive enzymes according to Rungruangsak-Torrissen (2007). In brief, the samples were homogenized in 50-mM Tris-HCl buffer pH 8 containing 200-mM NaCl (1:1; w/v). The homogenate was centrifuged at 4°C at 14000 rpm for 20 min and the supernatant was collected and kept at -20°C for the determination of the trypsin activity. The trypsin activity was assayed according to the method described by Rungruangsak-Torrissen et al (2002) and Benzoyl-L-arginine-p-nitroanilide (BAPNA) was used as specific substrate for trypsin. The total protein concentration of the crude enzyme extract was determined according to Lowry et al (1951). Trypsin activity was expressed as µmol p-nitroaniline produced h^{-1} mg $protein^{-1}$.

The *in vitro* protein digestibility was investigated in SIM and soaked-SIM compared with SBM by a modification by Rungruangsak-Torrissen et al (2002). Each sample with four replicates was suspended in 1:5 (w/v) of 50 mM phosphate buffer (pH 8.0). Then, each sample was added with 10 µL of 1% chloramphenicol. The crude enzyme extracts was added to each sample and incubated in shaking incubator at 25°C for 12 h, then reaction was stopped with 250 µL of 40% trichloroacetic acid and the samples were centrifuged at 4°C and 500 × g for 10 min. The supernatant was evaluated as liberated reactive amino groups of the peptides using the trinitrobenzene sulphonic acid (TNBS) method (modified from Ihekoronye 1986). The concentration of the reactive amino groups was calculated using DL-alanine as a standard. The unit was expressed as µMol DL-Alanine g^{-1} feed trypsin $activity^{-1}$.

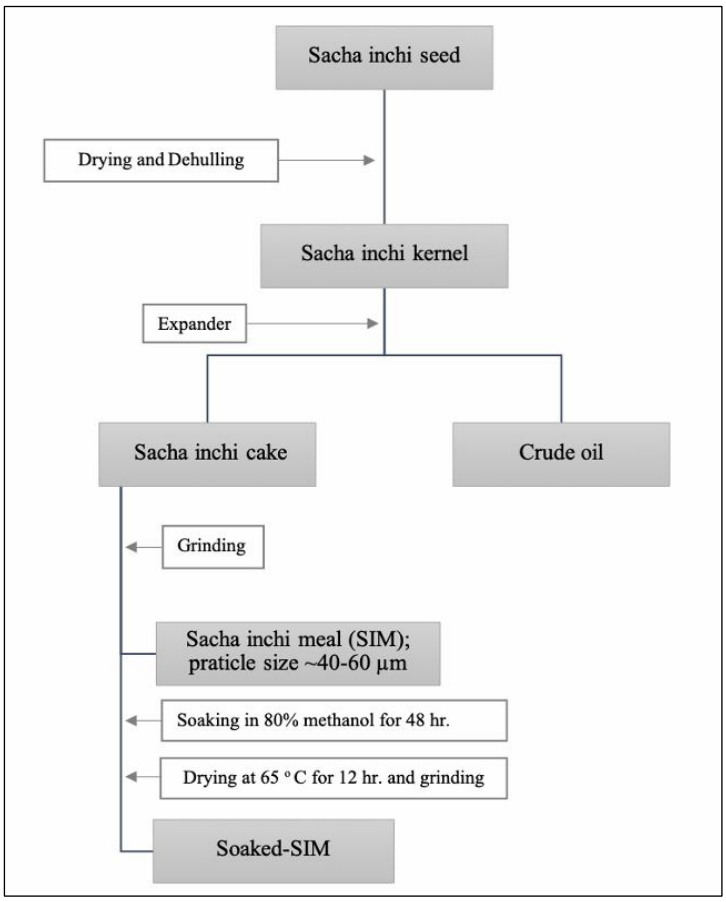

Figure 1. Flow chart of processed sacha inchi meal (SIM) and methanol soaked - sacha inchi meal (soaked-SIM) used in this study made from sacha inchi by-product.

Apparent digestibility coefficients. The apparent digestibility of dry matter, protein, and lipid in SBM, SIM, and soaked-SIM were investigated in Nile tilapia juvenile. The reference diet (RF) was formulated to satisfy the nutrient requirements of Nile tilapia according to NRC (2011). Test diets were made by combining 70% of the reference diet with 30% of each of the test ingredients (SBM, SIM and soaked-SIM). Chromic oxide (Cr_2O_3, 0.5%) was used as an inert marker and was incorporated into the reference and experimental diets (Table 1). Four replicates of each diet were followed and in each replicate 15 fish were stocked with an initial mean weight of 40.5±4.8 g.

After a week conditioning period, fish in each tank received the experimental diet prior to the collection of fecal samples. Fish faeces were collected using siphoning method at 2-3 hours after feeding. Triplicate groups of fish were fed the RF and experimental diets by hand to apparent satiation at 8:00 am. The fecal samples were collected daily following Mmanda et al (2020). Faeces were pooled and then stored at -20°C during the fecal collection period and dried at 60°C for 12 h for chemical analyses. All frozen fecal

samples were dried at 60°C for 12 h for chemical analyses. Chromic oxide in diets and faeces were assayed based on modified method of Divakaran et al (2002). The apparent digestibility of dry matter, protein, and lipid of the experimental diets were determined using the equation (1) proposed by (Forster 1999).

$$AD\ (\%) = 100\text{-}100 \times \left(\frac{MD}{MF}\right) \times \left(\frac{NF}{ND}\right) \quad (1)$$

where: AD = % the apparent digestibility; MD = % the marker in the diet; MF = % of the marker in the feces; NF = % of the nutrient in the feces; ND = % of the nutrient in the diet.

The digestibility of dry matter, protein and lipid of novel ingredients under study were estimated according to the equation (2) proposed by (Sugiura et al 1998):

$$ADi = ADCt + \left[\left(\frac{(1-s)Db}{s \times Dt}\right) \times (ADCt - ADCb)\right] \quad (2)$$

where: ADi = % of apparent digestibility of the ingredient under study; ADCt = % of apparent digestibility coefficient of the evaluated diet; ADCb = % of the apparent digestibility coefficient of the basal diet; Db = % of the nutrients of the basal diet; Dt = % of the nutrients of the test diet; s = the proportion of the ingredient evaluated in the diet; 1-s = the proportion of the basal diet in the test diet.

Table 1

Ingredients and chemical compositions of the reference diet and test diets

Ingredient (g 100 g^{-1})	*Reference diet*	*Soaked-SIM*	*SBM*
Fish meal	8	5.6	5.6
Poultry-by product meal	12	8.4	8.4
Soybean meal	30	21	21
Rice bran	12	8.4	8.4
Cassava meal	32.5	22.65	22.65
Premix[1]	1.5	1.0	1.0
Fish oil	1	0.7	0.7
Soybean oil	2	1.4	1.4
DL-methionine	0.5	0.35	0.35
Chromic oxide (%)	0.5	0.5	0.5
Reference diet[2] (%)	100	70	70
Soaked-SIM[3] (%)	-	30	-
SBM[4] (%)	-	-	30
Total	100	100	100
Proximate composition by analysis (% dry basis)			
Dry matter	94.3	94.1	93.3
Crude protein	30.4	41.0	33.4
Ether extract	5.8	6.0	2.9
Crude fiber	5.1	5.3	6.4
Ash	9.7	8.6	8.4
NFE[5] (%)	43.3	33.2	42.2
DE[6] (kcal 100 g^{-1})	286.5	291.8	281.9
DE P^{-1} ratio[7] (mg CP Kcal^{-1})	9.5	7.3	7.6

Note: [1] Composition of Vita pond vitamin 1 kg contains vitamin A 36,000 IU, vitamin D3 9,000 IU, vitamin E 187 mg, vitamin K 19 mg, vitamin B1 52 mg, vitamin B2 97 mg, vitamin B6 46 mg, vitamin C 69,800 mg activity, vitamin B12 60 mcg, Pantothenic acid 93 mg, Niacin 130 mg, Folic acid 10 mg, Inositol 225 mg, Biotin 450 mcg, Mn 105 mg, Cu 9 mg, Fe 90 mg, Zn 90 mg, I 1.8 mg, Co 450 mcg, Mg 1900 mg, Se 150 mg, Na 117 mg, K 3,600 mg, Ca 210 mg and K 3,600 mg; [2] % ratio of reference diet in test diet; [3] soaked-SIM: ratio of methanol soaked -sacha inchi meal in test diet; [4] SBM: ratio of soybean meal in test diet; [5] NFE: nitrogen free extracts calculated by 100% (crude protein + ether extract + crude fiber + ash + moisture); [6] DE: digestible energy calculated form digestible energy of ingredients as protein (4 kcal g^{-1}), lipid (8 kcal g^{-1}) and carbohydrate (2.5 kcal g^{-1}) in the diets; [7] DE P^{-1} ratio: ratio of digestible energy % protein^{-1}.

Analytical procedure

Chemical compositions. The chemical compositions in SBM, SIM, soaked-SIM, and experimental diets such as dry matter, crude protein, ether extract, crude fiber and ash

were analyzed in accordance to the AOAC method (AOAC 2000). The fatty acids of the oil samples were converted into methyl esters (FAME). The result was expressed as percentage of sample. Essential amino acids were determined using Automated Amino Acid Analyzer (Applied Biosystems 420A amino acid analyzer), and results were calculated as the percentage of dry matter of each sample. Fatty acid profiles in tested ingredients were measured in the crude lipid of each sample using gas chromatography (GC) according to AOAC (2012).

Antinutritional factors. The SBM, SIM, and soaked-SIM were dissolved in 30 mL of methanol and the solution sample was incubated in 25°C, shaking speed 100 rpm for 4 h. The solution of methanol-extracted sample was used for determination of phytic acid, total tannin and total phenolic content as following.

Total phytic acid content in tested ingredients was determined according to a modified method as described by Davies & Reid (1979) and Talamond et al (1998). The amount of 0.5 mL of filtered sample solution was added with 0.9 mL distilled water and 1 mL of $FeCl^{+3}$ and then the sample was heated in boiled water for 20 min before immediately cooled down. The cooled sample was added 7.5 mL of distilled water and 1 mL of NH_4SCN. The absorbance was measured at 465 nm. Phytic acid sodium salt hydrate (Sigma-Aldrich, Switzerland) was used as a standard solution.

Total tannin content was determined according to a modified method as described by Arabshahi et al (2007). The amount of 200 µL of solution sample was mixed with 2.3 mL of distilled water, 0.2 mL of 0.2 N Folin-Ciocalteu before incubated in the dark condition for 3 min. The sample solution was added with 0.3 mL of 1 N sodium carbonate and mixed well and then incubated in the dark condition for 1 h. The absorbance was measured at 760 nm. The tannin acid (Sigma, China) was used as a standard solution.

Total phenolic content was determined according to a modified method as described by Singleton et al (1999). The amount of 500 µL of sample solution was added with 2.5 mL of 0.2 N Folin-Ciocalteu reagent and mixed well before incubated in a room temperature for 5 min. The sample mixture was added with 2 mL of 7.5% sodium carbonate solution and then incubated in the dark condition for 2 h. The absorbance was measured at 760 nm. Gallic acid (Sigma-Alcridh, USA) was used as a standard solution.

The saponin content was determined by following the method of Jain & Shrivastava (2017). Briefly, 1 g of sample was soaked in 20 mL of 70% methanol for 48 h at a room temperature and then filtered through a paper filter (diameter 2.5 µm) (Tan et al 2014). The amount of 0.25 mL of sample extract was added with 0.25 mL of 8% vanillin reagent (4 g vanillin in 50 mL methanol) and then mixed well before added 2.5 mL of 72% sulfuric acid. The sample mixture was incubated in a water bath at 60°C for 10 min and then cooled down in cold water (approximately 1-2°C). The absorbance was measured at 544 nm. Diosgenin (Sigma-Alcridh, USA) was used as a standard solution.

Determination of trypsin inhibitor in tested ingredients was performed according to a modified method of Chen et al (2014) and Liu (2019). One gram of the sample was soaked in 50 mL of 10 mM NaOH and gently shaken using a shaker for 3 h at a room temperature. The sample solution was filtered through a paper filter (diameter 2.5 µm). Two mL of the sample extract was warmed at 37°C in a water bath. The sample extract was added with 5 mL of 0.04% (w/v) Na-Benzoyl-L-arginine 4-nitroanilide hydrochloride (BAPA) in Tris–HCl buffer (pH 8.2, 1% dimethylsulfoxide, v/v; 0.02 M $CaCl_2$) and mixed well. Afterward, 2 mL of 0.01% (w/v) trypsin in a 0.04 mM HCl solution was added to the sample mixture and incubated for 10 min before stopped the reaction by adding 1 mL of 30% acetic acid (v/v). The absorbance was measured at 410 nm. Trypsin inhibitor from *Glycine max* (soybean) was of analytical grade purchased from Sigma Aldrich (Sigma-Alcridh, USA) and it was used as a standard solution.

Statistical analysis. All data were expressed as mean value and standard deviation (SD). One-way ANOVA followed by Tukey's multiple comparisons test was performed using GraphPad Prism version 7.00 for Mac Os X (GraphPad Software; La Jolla, California, USA). Differences between treatments were considered significant at $p < 0.05$.

Results

Chemical compositions. The chemical compositions in tested ingredients consisting of SBM, SIM and soaked-SIM are shown in Table 2. The order of increasing content of crude protein in the tested ingredients was noticed to be soaked-SIM (63.3%), SIM (56.5%) and SBM (45.0%). Crude lipid content of SIM (9.8%) was higher than that of soaked-SIM (6.6%) and SBM (0.6%). Crude fiber content of SBM (9.4%) was higher than those of SIM (4.7%) and soaked-SIM (4.6%). Similarly, ash content of SBM (8.0%) was greater than those of SIM (5.8%) and soaked-SIM (5.8%). The highest content of nitrogen free extract was observed in SBM (25.7%) while SIM and soaked-SIM contained 15.0% and 13.3% of nitrogen free extract, respectively.

Fatty acid profile. The saturated fatty acids and unsaturated fatty acids found in the tested ingredients are shown in Table 2. The presence of palmitic, stearic, oleic, linoleic and linolenic FA were evidenced. SBM showed higher amount of palmitic acid (0.49%) than that found in SIM and soaked-SIM. The lowest amount of stearic acid and oleic acid were found in SBM (0.049 and 0.33% respectively) while SIM showed higher amount of stearic acid and oleic acid than that of soaked-SIM. SIM showed higher amount of linoleic acid (3.39%) and linolenic acid (3.69%). SIM presented values of polyunsaturated fatty acids (PUFA), monounsaturated (MUFA) and saturated fatty acids (SFA) like 7.08%, 0.76% and 0.70% respectively, while soaked-SIM presented values of PUFA, MUFA and SFA like 2.86%, 0.35% and 0.34%, respectively.

Phytochemicals. The phytochemical screening in tested ingredients revealed the presence of some active compounds such as phytic acid, tannin, phenolic, saponin and trypsin inhibitor as shown in Table 2. The highest content of phytic acid was found in SIM. Phytic acid content in soaked-SIM was higher than that of SBM. SIM and SBM showed similar tannin content that was greater than that of soaked-SIM. SIM had higher phenolic content than that of SBM. Soaked-SIM contained the lowest phenolic content. Soaked-SIM had lower saponin content than that of SIM and SBM. Both SIM and soaked-SIM showed lower levels of trypsin inhibitor than that of SBM.

Table 2

Chemical composition, fatty acids, and antinutritional factors of soybean meal (SBM), sacha inchi meal (SIM), and methanol soaked - Sacha inchi meal (soaked-SIM)

Parameters	*SBM*	*SIM*	*Soaked-SIM*
Chemical composition (%)			
Dry matter	88.6	91.1	93.3
Crude protein	45	56.5	63.3
Crude lipid	0.6	9.8	6.6
Crude fiber	9.4	4.7	4.6
Ash	8	5.8	5.8
Nitrogen free extract	25.7	15	13.3
Fatty acid (%)			
Palmitic acid (C16:0)	0.49	0.42	0.19
Stearic acid (C18:0)	0.05	0.28	0.15
Oleic (C18:1n9)	0.33	0.76	0.35
Linoleic (C18:2n6)	1.2	3.39	1.43
Linolenic (C18:3n3)	0.14	3.69	1.42
Antinutritional factors			
Phytic acid (mg Phytic acid g-1)	1.27	7.47	2.12
Tannin content (µg Tannic acid g-1)	4.83	5.23	2.04
Phenolic content (mg GAE g-1)	56.21	77.61	15.04
Saponin content (mg g-1)	2.43	4.74	1.18
Trypsin inhibitor activity (%)	0.18	0.05	0.04

Amino acid profile. The essential amino acids (EAA), non-essential amino acids (NEAA), total EAA, and total NEAA of the tested ingredients are presented in Table 3. For EAA, all tested ingredients were rich in arginine and leucine. Particularly, SIM and soaked-SIM contained larger amounts of arginine and leucine than those of SBM. Whereas the amount of phenylalanine in SIM and soaked-SIM was poorer than that of SBM. Both SIM and soaked-SIM contained higher amount of threonine, valine, and isoleucine than SBM. The lowest amount of EAA valuated in all ingredients was methionine. The amounts of serine, tyrosine, and cysteine in SIM and soaked-SIM were larger than SBM. Soaked-SIM displayed higher amounts of alanine and proline than those of SIM and SBM. The highest amounts of total EAA and total NEAA were found in soaked-SIM.

Table 3

Amino acid profile in soybean meal (SBM), sacha inchi meal (SIM), and methanol soaked - Sacha inchi meal (soaked-SIM)

Amino acid profile (%)	*SBM*	*SIM*	*Soaked-SIM*
Essential amino acid			
Arginine	3.1	5.2	6.3
Histidine	1.1	1.1	1.3
Isoleucine	2.0	2.3	2.8
Leucine	3.4	3.4	4.2
Lysine	2.6	2.2	2.8
Methionine	0.6	0.6	0.7
Phenylalanine	2.3	1.2	1.4
Threonine	1.8	2.3	2.9
Valine	2.0	2.6	3.2
Total EAA	18.9	20.9	25.6
Non-essential amino acid			
Alanine	1.9	1.8	2.2
Aspartic acid	5.0	5.9	7.6
Cysteine	0.6	1.3	1.6
Glutamic acid	7.2	6.3	7.4
Glycine	1.9	5.0	6.0
Proline	2.2	2.0	2.4
Serine	2.3	3.1	3.8
Tyrosine	1.4	2.3	2.8
Total NEAA	22.5	27.7	33.8

In vitro protein digestibility. The results of *in vitro* protein digestibility of tested ingredients are shown in Figure 2. The protein digestibility of soaked-SIM was not significant different between SIM and SBM ($p > 0.05$).

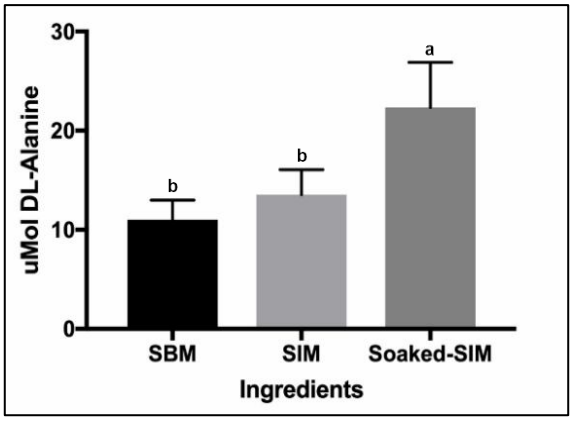

Figure 2. *In vitro* protein digestibility of soybean meal (SBM), sacha inchi meal (SIM), and methanol soaked - Sacha inchi meal (soaked-SIM). Values are means±SD (n = 5). The protein digestibility unit is expressed as µMol DL-Alanine g^{-1} feed trypsin $activity^{-1}$. Values with different superscript letters are significantly different ($p < 0.05$).

Apparent digestibility coefficients. The 30% inclusion of soaked-SIM and SBM did not result in reduced diet palatability. The ADCs for dry matter, protein, and lipid varied considerably (Figure 3) with significant differences ($p < 0.05$) detected among the fish fed diets containing soaked-SIM and SBM. The ADC for crude protein was higher in soaked-SIM (94.2%) than SBM (82.7%). Soaked-SIM diet had significantly greater apparent digestibility of lipid (98.3%) than that of SBM diet (90.6%).

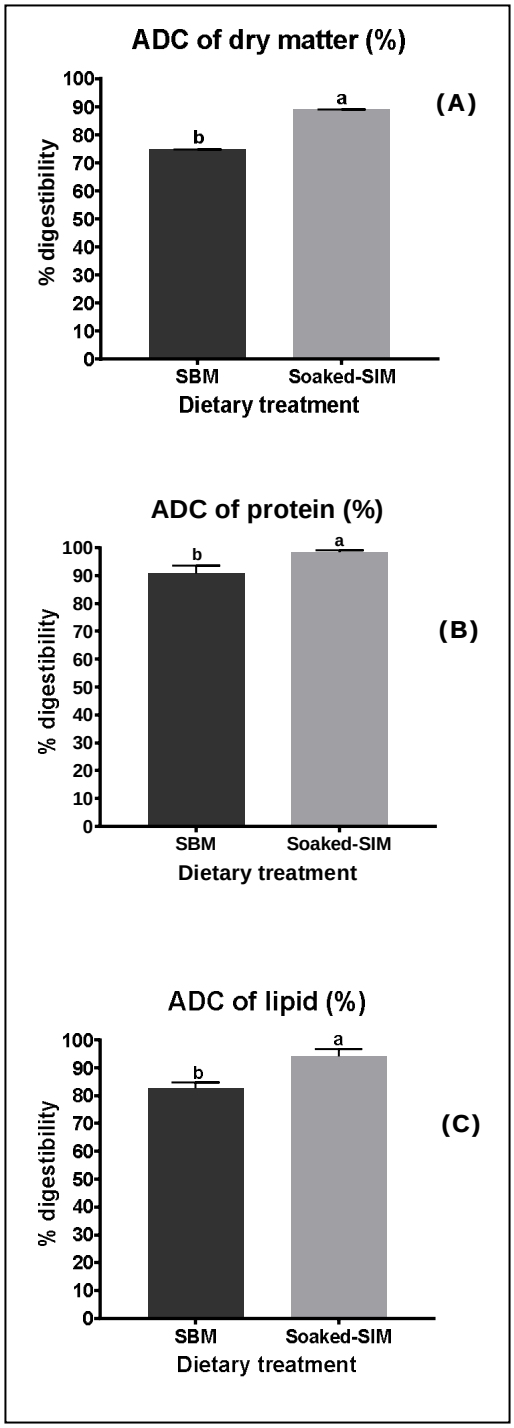

Figure 3. Apparent digestibility coefficients of dry matter (A), protein (B), and lipid (C) in juvenile Nile tilapia. Values are means±SD (n = 4). Values with different superscript letters are significantly different ($p < 0.05$). SBM: soybean meal, and soaked-SIM: methanol soaked - sacha inchi meal.

Discussion

Chemical compositions. Previous studies of Gutierrez et al (2011), Fanali et al (2011), and Bueno-Borges et al (2018) revealed that sacha inchi seeds (kernel) consisted of large amounts of crude lipid ranging from 35 to 60%, and crude protein ranging from 24 to 30%. The pressing process and the extraction of sacha inchi oil led the crude protein content in sacha inchi meal (pressed cake) to increase up to 53.9-56.6% (Khieokhajonkhet et al 2021; Rawdkuen et al 2016). In the present study, soaked-SIM contained 63.3% of crude protein which was 7% higher than that of SIM (56% of crude protein). These different contents of crude lipid in SIM depend on the source origins of SIM and the pressing process (Chirinos et al 2013; Rawdkuen et al 2016; Bueno-Borges et al 2018; Khieokhajonkhet et al 2021). The variation of crude lipid content in SIM has been observed in different studies; for example, crude lipid contents of SIM were 4.13% and 13.7% reported by Khieokhajonkhet et al (2021) and Rawdkuen et al (2016), respectively. In the present study, crude lipid content decreased in soaked-SIM (6.6%), which was lower than that of SIM (9.8%), indicating that the methanol (MeOH) soaking method could remove the crude lipid content of SIM, leading to enhance crude protein content in soaked-SIM.

One of the limitations of plant protein ingredients included in fish feeds is that they are high in crude fiber content which is generally indigested by fish (Sinha et al 2011). Consequently, fish has poor digestibility of nutrients, decreased growth performance, and increased organic waste (Storebakken et al 1999; Zhou et al 2004; Eusebio et al 2004). Eusebio et al (2004) suggested that dietary levels of crude fiber as low as 3.5% could be an advantage for fish growth; on the other hand, over 8% of dietary fiber might decrease dry matter digestibility of the diet and reduce the availability of other nutrients. In the present study, both SIM (5.8%) and soaked-SIM contained (5.8%) lower amounts of the crude fiber and nitrogen-free extract than those presented in SBM (8.0%), indicating that SIM has potential as a plant protein ingredient comparable to SBM.

Fatty acid profile. In general, saturated, MUFA and PUFA were observed in sacha inchi pressed cakes (Rawdkuen et al 2016). Khieokhajonkhet et al (2021) and Rawdkuen et al (2016) revealed that sacha inchi seed was rich in omega-3 fatty acids. Sacha inchi seed has a lower content of total saturated fatty acids than another oilseed such as canola seed, sunflower seed, flaxseed, corn, and cottonseed oils (Chirinos et al 2013; Maurer et al 2012). A surprising level of omega fatty acids (ω-) was found in sacha inchi seed. There were detectable levels of omega-3 (ω-3), omega-6 (ω-6). The highest level of ω-3 and ω-6 was found in the sacha inchi pressed-cake (Rawdkuen et al 2016), similarly the result of this study revealed that SIM showed higher amount of linoleic acid (3.39%) and linolenic acid (3.69%). Especially linolenic acid is considered as a source of essential fatty acid in fish feeds. Essential fatty acids, especially polyunsaturated fatty acids (18:3n-3) have roles in controlling and regulating cellular metabolism and fish physiology. Essential fatty acids are important for preventing fish pathology and optimal growth (Tocher et al 2010; Kanazawa et al 1980; Takeuchi et al 1983). In addition to linseed and rapeseed as an alternative source of omega-3 fatty acids (Yu et al 2019; Mu et al 2020), we suggest that SIM and soaked-SIM are also considered as good sources of essential fatty acids for fish feeds. In comparison to SBM, we found that only SFA (stearic acid) and MUFA (oleic acid) but also omega-3 (linolenic acid) and omega-6 (linoleic acid) were higher in SIM compared to that of SBM. Although a certain amount of the lipid was removed in soaked-SIM by the methanol soaking method, the amounts of linoleic acid, linolenic acid of soaked-SIM were still higher than those of SBM. The ratio ω-6/ω-3 found for sacha inchi seed was 0.81-1.12 reported by Gutiérrez et al (2011). Soaked-SIM and SIM were showed the ratio ω6/ω 3 was 0.92 and 1.01, this range is close to about one. Additionally, the ratio of ω-6/ω-3 was recommended as 1.0. As a result of our finding, we suggest that soaked-SIM could be not only as a source of protein but also the source of omega-3 in fish feeds.

Antinutritional factors. Rawdkuen et al (2016) and Bueno-Borges et al (2018) revealed that sacha inchi seeds contained antinutritional factors and phytochemicals, such as tannins, saponins, trypsin inhibitors, phytic acid, and phenolic content. Our finding is the first report showing the amounts of antinutritional factors and phytochemicals (data is showed in Table 2) in SIM as a by-product of sacha inchi seeds. The plant protein ingredients used as feedstuff commonly contain the antinutritional factors which cause detrimental effects on protein digestibility and retard the growth performance of fishes (Francis et al 2001). To improve the quality of the plant protein ingredients, the heating process and solvent extraction have been used to reduce the antinutritional factors in soybean, moringa leaf, *Basella alba* leaf, and sacha inchi seeds (Francis et al 2001; Venou et al 2006; Arndt et al 1999; Bueno-Borges et al 2018; Bureau et al 1998; Afuang et al 2003; Dongmeza et al 2006).

Arndt et al (1999) found that autoclaving at 121°C for 20 min has reduced 99.3% the trypsin inhibitor value of defatted-soybean meal compared with non-treatment group. Additionally, Bueno-Borges et al (2018) demonstrated that tannins, trypsin inhibitor, and phytic acid in sacha inchi seeds roasted at 120°C for 15 min were decreased 100%, 60.9%, and 34.2% of the antinutritional factor values presented in unroasted sacha inchi seeds, respectively. In terms of solvent extraction, Bureau et al (1998) reported that saponins in soybean meal were removed by alcohol extraction. Moreover, Dongmeza (2006) revealed that saponins and tannins in moringa leaf could be extracted with 80% methanol. Based on the values of antinutritional factors presented in soaked-SIM, the methanol soaking method used in the present study was succeeded to eliminate tannins, trypsin inhibitors, saponins, and phytic acid in SIM. Obviously, the amounts of tannins, trypsin inhibitor, and saponins in soaked-SIM, were even lower than those presented in SBM, except for phytic acid.

Amino acid profile. The overall content of amino acids in all tested ingredients in the present study showed that soaked-SIM contained a larger amount of both essential amino acids and non-essential amino acids than those presented in SIM and SBM, except for phenylalanine. For essential amino acids, soaked-SIM was rich in leucine and arginine. Further, soaked-SIM contained high amounts of valine, threonine, isoleucine, and lysine. Glutamic acid, aspartic acid, and glycine were the major non-essential amino acids in soaked-SIM. Similarly, Khieokhajonkhet et al (2021) reported that leucine, lysine, isoleucine, arginine, and valine were the major essential amino acids, while glutamic acid, aspartic acid, glycine, alanine, and serine were the major non-essential amino acids in the extruded SIM. Besides, Rawdkuen et al (2016) reported that SIM comprised of high amounts of lysine, histidine, and isoleucine as essential amino acids. Besides, SIM consisted of high amounts of tyrosine, glutamic acid, aspartic acid, and cystine as non-essential amino acids.

A comparison between SBM and soaked-SBM in the present study showed that both soaked-SIM and SBM were rich in leucine and arginine as essential amino acids; and aspartic acid and glutamic acid as non-essential amino acids. While the lowest amount of essential amino acid in both SBM and soaked-SIM was methionine. As a result of the amino acid profile in the present study, this indicates that the amino acid profile of soaked-SIM was comparable to that of SBM, we thus suggest that soaked-SIM is an appropriate alternative source of protein in fish feeds for aquaculture sustainability. However, owing to low methionine in both SBM and SIM, this should be therefore kept in mind when SIM is used as a fish meal replacer or SBM replacer in diets that methionine supplementation is vital for low fish meal plant-based diets (Arriaga-Hernández et al 2021).

In vitro protein digestibility. The *in vitro* protein digestibility was significantly improved in soaked-SIM diet due to high protein content and low ash content in soaked-SIM, compared to those of SBM and SIM (Rungruangsak-Torrissen et al 2002; Kopruc & Ozdemir 2005). The other possibility of retarded protein digestibility in SBM and SIM might be related to anti-nutritional factors, especially saponins and trypsin, of which amounts were high in SBM and SIM, compared to soaked-SIM. The major limitation with

plant-derived materials, such as legume seeds and SBM, is the presence of a wide range of anti-nutritional factors, such as trypsin inhibitors, non-digestible carbohydrates, lectins, saponins, phytates, and possibly allergenic storage proteins (Francis et al 2001). Francis et al (2002) reviewed that saponins possess detrimental effects on protein digestion, cholesterol metabolism, and function of the immune and nervous systems. However, the information of *in vitro* protein digestibility affected by saponins is limited in fish.

It was reported that tannins have negative impacts on protein digestion and utilization (Makkar et al 1987). Pinto et al (2001) found that feeding diets containing 0.69, 0.92, 1.37, and 1.82% of total tannin significantly caused a low growth rate in Piauçu (*Leporinus macrocephalus*). The authors suggested that tannins interfered with the metabolism and biological value of dietary nutrients resulting in the poor growth rate of fish. In the present study, we found that the amounts of saponins and tannins in soaked-SIM were lower than those presented in SBM. The improved value of the protein digestibility in soaked-SIM was probably attributed to the reductions of tannins and saponins removed by the methanol soaking method.

Apparent digestibility coefficients. The apparent digestibility coefficient of dry matter in the present study was higher than that reported by Ortiz-Chura et al (2018), whereas the apparent digestibility coefficient of protein (98.8%) in the present study was in accordance with the finding of Ortiz-Chura et al (2018). The values of apparent digestibility coefficients of dry matter, protein, and lipid were significantly higher in soaked-SIM diet compared to those of SBM diet. In the present study, the high apparent digestibility of dry matter in soaked-SIM diet could be explained by the low contents of fiber and nitrogen free extract in soaked-SIM diet. The fiber content in SBM diet was 9.7% which was higher than that in soaked-SIM diet (6.6%). Soaked-SIM diet consisted of 42.9% of nitrogen-free extract that was lower than that of SBM diet (46.2%). Suppression of apparent digestibility of dry matter in fishes was induced by feeding plant-based diets containing the high contents of crude fiber (Kopruc & Ozdemir 2005; Ortiz-Chura et al 2018; Sinha et al 2011). This phenomenon can be explained that fish as a monogastric animal lacks the enzymes, namely β-glucanase and β-xylanases, for non-starch polysaccharide digestion, are restricted (Kuz'mina 1996).

The improved apparent digestibility coefficients of protein in soaked-SIM diet was due to high content of crude protein in soaked-SIM diet, which the ADC for crude protein was 94.2% in soaked-SIM, higher than for SBM (82.7%). Murashita et al (2018) reported that feeding SBM diet caused acutely negative effects on digestive enzyme system of red seabream, *Pagrus major*. In the acute effect trial (< 48h), trypsin secretion in red seabream fed SBM diet was significantly suppressed. The authors suggested that some anti-nutritional factors are the key factors of suppressed secretion of trypsin in SBM fed fish. The similar effects of SBM diet have been reported in Atlantic salmon (*Salmo salar*) and yellowtail, *Seriola quinqueradiata* (Nguyen et al 2011; Romarheim et al 2006; Zhang et al 2018). In the present study, certain anti-nutritional factors, such as saponins (2.43 mg g^{-1}) and tannins (4.83 µg TA g^{-1}) were detected in SBM while lower amounts of saponins (1.18 mg g^{-1}) and tannins (2.04 µg TA g^{-1}) were detected in soaked-SIM, suggesting saponins and tannins are responsible for poor apparent digestibility of protein in SBM diet fed fish (Francis et al 2001; Francis et al 2002; Chikwati et al 2012; Makkar et al 1987; Pinto et al 2001). Improved value of apparent digestibility of protein in soaked-SIM also might be due to lower amounts of ANFs in soaked-SIM compared to SBM.

Plant protein ingredients contain high levels of non-starch polysaccharides (NSPs) which are indigestible for fish. In the present study, soaked-SIM had lower contents of the crude fiber and nitrogen-free extract than those presented in SBM. Sinha et al (2011) reported that the high intake of dietary NSPs could be inferior with emulsification of ingested lipid in the intestinal tract (Pasquier et al 1996; Ebiharam & Scheeman 1989). The apparent digestibility of lipid observed in SBM diet (79.7%) in the present study agreed with the reports of Sklan et al (2004) and Koprucu & Ozdemir (2005). Sklan et al (2004) reported that apparent digestibility of lipid in tilapia fed tested diets, containing

various protein ingredients, ranged from 72 to 90%. Similarly, Koprucu & Ozdemir (2005) found that apparent digestibility of lipid of SBM studied in tilapia was in the range from 72 to 97.5%. Worsened lipid digestion associated with feeding SBM-based diets was reported in several fishes, including yellowtail, red seabream, rainbow trout, and salmon (Nguyen et al 2011, 2017; Murashita et al 2018; Matsunari et al 2015; Krogdahl et al 2003; Yamamoto et al 2012). In the present study, lower levels of crude fiber in soaked-SIM also lead to greater apparent digestibility of lipid than that of SBM diet.

Conclusions. Methanol soaking method could improve the quality of SIM, in terms of increased values of crude protein content and *in vitro* protein digestibility and reduced the anti-nutritional factors in SIM. Soaked-SIM is a suitable candidate as SBM replacer due to it shows superior values of amino acid profile, omega-3 fatty acids, crude protein content, and apparent digestibility coefficients of dry matter, crude protein, and lipid, while it contains lower amounts of ANFs than SBM.

Conflict of interest. The authors declare that they have no conflict of interest.

Ethics statement. The study was approved by the Institutional Animal Care and Use Committee of Khon Kaen University (IACUC KKU); Reference No. 660201.2.11/381 (58).

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