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Properties of *Moringa oleifera* leaf protein from alkaline–acid extraction

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Abstract

Moringa oleifera (M. oleifera) is a perennial foliaged tree, widely cultivated due to it being an important source of essential nutrients. In this study, physical, functional, and biochemical properties of *M. oleifera* leaf protein were evaluated. The protein was obtained by alkaline–acid extraction at an amount of 77.44%. The leaf protein powder had 0.15 g/mL of bulk density and low water activity (0.35) and appeared with the value of *L** 57.83. Water holding capacities and oil holding capacities of leaf protein were adequate for use in food formulations such as 2.31 g water/g protein and 3.55 g oil/g protein, respectively. Protein solubility (PS) and emulsifying and foaming properties were significantly pH–dependent ($P<0.05$). The minimum solubility was obtained at pH 4 (9.28%), whereas the maximum PS was observed at pH 10 and pH 12 (58.01% and 58.30%, respectively). At pH 10, the foaming capacity was high, while it was low (up to 20 min) in terms of foaming stability (FS) since good FS was recorded at pH 3 and pH 6. The maximum emulsion capacity was greater at acidic pH (pH 3) and basic pH (pH 10). Contrarily, maximum emulsion stability (65.81%) was observed at pH 4 followed by pH 10 (62.26%). *M. oleifera* leaf protein powder showed an adequate digestibility of 75.54%. So, it illustrated on SDS–PAGE a complete digestion of all protein bands under reducing and non–reducing conditions after 24 h incubation with pepsin–pancreatin enzymes. Leucine (67.14 mg/g), tryptophan (12.10 mg/g), isoleucine (28.71 mg/g), and histidine (19.07 mg/g) were the most predominant amino acids that meet requirements of FAO/WHO (1991) for 2–5 years and 10–12 years old children. *M. oleifera* protein extract produced by alkaline–acid extraction can probably be used as an ingredient in food products.

Keywords: Biochemical properties, Functional properties, *Moringa oleifera* leaf, Leaf protein, Physical properties

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1. Introduction

Proteins are very important nutrients for the development of the human body and the maintenance of good health. People need an adequate amount of protein for normal maintenance of bodily functions and for growth, maturation, pregnancy, breastfeeding, and recovery from injuries and illnesses (Khan and Varshney, 2018). Global population growth combined with increasingly limited resources has led to the need for alternative protein sources to meet global protein requirements. However, increased production of animal-based protein is expected to have adverse impacts on environment and health (Gorissen *et al.,* 2018).

Thus, protein and calorie malnutrition are still taking place as the most widespread problems in developing countries; for example, the prevalence rate of stunting was 47% in Madagascar (USAID, 2017); 56% in Haryana, India (Kumari, 2018), and 45.5% in the Sidama zone, Southern Ethiopia (Rodamo *et al.,* 2018). There are two types of disastrous consequences that occur in children who have suffered from protein energy malnutrition such as marasmus and kwashiorkor. Since animal proteins are unavailable due to high price, plant proteins should be interestingly important in diet and are cheaper to produce than animal proteins. Conventional legumes have been playing a role as a food and feedstuff in most countries, but their production is not enough to meet the requirements of the increasing population and animal feed industries (Siddhuraju and Becker, 2003). The use of plant protein in food formulations has recently become attractive due to its greater sustainability and lower production costs (Gorissen *et al.,* 2018).

M. oleifera is a fast-growing perennial plant species native to India. Currently, it is cultivated in many areas worldwide where it is not native (Owusu *et al.,* 2008). This plant is considered one of the most useful plants in the world because almost all of its parts can be used as food, in traditional medicines, and for industrial purposes (Khalafalla and Abdellatef, 2010). Furthermore, people have used its seed and leaf flour in the formulation of infant food to increase protein content (Anwar *et al.,* 2007). In Brazil, there was an effort to spread the use and cultivation of *M. oleifera*, taking into account the high protein, vitamin, and mineral contents and the low toxicity of the seeds and leaves (Ferreira *et al.,* 2008).

Although its leaves are an important source of protein, the biological value of protein depends on the absolute and relative content of essential amino acids and its bioavailability after digestion. Protein digestibility can be affected by the low sulfur amino acid content, the compact structure, the presence of non-protein components (dietary fiber, tannins, and phytic acid), and anti-physiological proteins (protease inhibitors and lectins) (Teixera *et al.,* 2014).

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Alkaline Acid extraction has been used for conventional protein extraction. This method can affect the composition and physicochemical and functional characteristics of proteins. However, it can help to extract leaf proteins due to disruption of leaf tissue, increasing protein solubility and the degree of hydrolysis (Zhang *et al.,* 2015). For food application, extraction in alkaline solution and precipitation at isoelectric pH protein isolates or concentrates are prepared to extract proteins due to this extraction technique being relatively simple and possible to carry out at low cost (Mechmeche *et al.,* 2017). Proteins in various types of foods are elucidated as physical and chemical, which influences the protein behavior in food systems during processing, storage, and consumption as well as preparation, which can contribute to the quality and sensory attributes of food systems. Nevertheless, food properties can be influenced by protein, including water, fat holding capacity, emulsification, and foam formation (Feyzi *et al.,* 2017). For instance, proteins with high oil and water binding properties are desirable for use in meats, sausages, breads, and cakes, while proteins with high emulsifying and foaming capacities are desirable for salad dressings, sausages, soups, confectionery, frozen desserts, and cakes. However, water/oil bonding, emulsification, and foaming are affected by intrinsic protein factors, such as molecular structure and size, as well as by many environmental factors, including the protein separation, pH, ionic strength, and the presence of other components in the food system (Ulloa *et al.,* 2011).

Plant leaf protein concentrate (LPC) has been recognized as an additional source of protein since 1960s (Zhang *et al.,* 2015). A number of plant leaves (e.g., alfalfa, spinach, grass, cassava, cod, and tobacco) have been used to extract leaf proteins so far (Sun *et al.,* 2017). In addition, leaf proteins have become a major source of dietary protein in many developing countries and could potentially be a major source of protein for food applications and animal feed, as seen with, for instance, the use of LPC with common grains in the formulation of weaning foods (Meda *et al.,* 2017). Next, the use of LPC as a protein supplement in animal feed has also been demonstrated. It has also been mixed with a variety of inexpensive foods to make culturally acceptable dishes (Khan and Varshney, 2018). Other sources include leaves available as by-products of certain large-scale crops (e.g., cassava, barley, broccoli, and sugar beets) (Tenorio *et al.,* 2016).

Studies of *M. oleifera* have focused on the isolation of bioactive compounds, especially with antioxidant and hypotensive activities. However, there is little information on the usage of *M. oleifera* leaf protein as a food ingredient in human diet. This study aims to determine the physical, functional, and biochemical properties of *M. oleifera* leaf protein as an alternative source of protein.

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2. Materials and methods

2.1 Chemicals and materials

Casein from bovine milk, sodium dodecyl sulfate (SDS), and Coomassie Brilliant Blue R−250 were purchased from Fluka Chemica−Biochemika (Buchs, Switzerland). Pepsin and pancreatin were procured from Sigma Chemical Co. Ltd. (St, Louis, MO, USA). Trichloroacetic acid (TCA), sodium hydroxide, and other chemicals with analytical grade were obtained from Merck (Darmstadt, Germany). Distilled water was used for protein extraction and functionality experiments work. *M. oleifera* leaves were harvested at a maturity of three to four months, in April, by hand, from Tea Oil and Plant Oils Development Center located at 888 Moo 10, Tambon Wiang Phang Kham, Mae Sai, Chiang Rai, Thailand (20.4316^{o} N latitude and 99.8852^{o} E longitude). A dried *M. oleifera* leaf powder was used for protein extraction, and the protein extract was subjected to an analysis of the physical, functional, and biochemical properties. The study was conducted in triplicate.

2.2 Sample preparation

The fresh leaves were washed with tap water, directly dried at 40°C in a tray dryer for approximately 48 h, then grinded into fine powder and kept at −20°C until further use.

2.3 Alkaline−Acid (AA) extraction

AA extraction was carried out according to Mariod *et al.* (2010), with minor modification. The sample was added to distilled water at a ratio of 1:20 (w/v), and then it was mechanically stirred for 1 h by adjusting pH 9.0 with 1.0 M NaOH aqueous solution. The obtained filtrate (using white cheesecloth) was centrifuged at 10,000 rpm for 20 min at room temperature. The supernatant was transferred into a beaker and stirred again for 20 min, while the pH adjusted around 4.5 with 0.1 M HCl and left overnight thereafter to facilitate protein precipitation. The sediment protein slurry was centrifuged at 10,000 rpm for 10 min at room temperature, followed by dialysis overnight against water at 4°C. The protein precipitate was collected while adjusting pH to 7.0 and then freeze−dried (Delta−2−24/LSC plus, Germany). The protein powder was vacuum−packed and stored at −18°C for further experiment. Protein content was determined by the Kjeldahl method (%N x 6.25) according to Nielsen (2010).

2.4 Physical properties

2.4.1 Bulk density

Bulk density was measured according to Wang and Kinsella (1976), with some modification. The sample was poured into 25 mL graduated measuring cylinder. The cylinder was gently tapped ten times on the bench while the occupied volume was recorded. The bulk density was calculated as weight per unit volume (g/mL).

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2.4.2 Water activity

The water activity value of freeze-dried *M. oleifera* leaf protein powder was analyzed as a measure of storage stability using a dew point water activity meter (Aqualab, Decagon, USA), as described by Tunick *et al*. (2016).

2.4.3 Color

Color measurement was recorded according to the procedure of Ghribi *et al.* (2015). A colorimeter Hunter CIE lab system (ColorQuest XE, Hunter lab, Virginia, USA) was used to measure the protein color displayed as *L** (lightness), *a** (redness to greenness-positive to negative values, respectively), and *b** (yellowness to blueness-positive to negative values, respectively) values. The measurements were performed under standard illuminant D65. Before conducting the tests, the instrument was initially calibrated using a standard white plate (*L**= 94.64, *a**= −80 and *b**= 0.07).

2.5 Functional properties

2.5.1 Protein solubility

The solubility of the protein was analyzed according to the method described by Kumar *et al*. (2014), with some modifications. The sample (100 mg) was dispersed in 5 mL of distilled water. The pH of the mixture was adjusted to 2, 4, 6, 8, 10, and 12 using 0.1 M HCl or 1 M NaOH. The solution was stirred for one hour at room temperature and centrifuged at 4,000 x g for 20 minutes. The protein content of the supernatant was determined by the Kjeldahl method (Nielsen, 2010), and the percentage solubility of the proteins was calculated as follows:

$$\text{Solubility (\%)} = \frac{Amount\ of\ nitrogen\ in\ the\ supernatnat}{Amount\ of\ nitrogen\ in\ the\ protein\ extract} \text{ x } 100$$

2.5.2 Water holding capacity (WHC) and oil holding capacity (OHC)

WHC and OHC were determined by the technique of Mariod *et al*. (2010), with minor modification. The sample (0.5 g) was weighed into a 50 mL pre-weighed centrifuge tube and mixed with 10 g of distilled water for WHC determination, while 1.0 gram was mixed with 10 g of soybean oil for OHC determination. Then the obtained suspensions were vortexed for 2 min and left to stand for 30 min. The protein-water mixture was centrifuged at 3,000 g for 15 min. The supernatant was removed while the tube was re-weighed.

$$\text{WHC or OHC (g/g)} = \frac{W2-W1}{W1}$$

Where W_1 is the weight of the tube plus the dry sample and W_2 is the weight of tube plus sediment.

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2.5.3 Foaming capacity (FC) and stability (FS)

Foaming capacity was measured according to the method described by Lamsal *et al.* (2007) with some modifications. The sample (500 mg) was added into 50 mL of distilled water, and the pH was adjusted to 3, 4, 6, 8, 10 and 12 with either 0.1 M NaOH nor 0.1 M HCl. The solution was whipped using the maximum speed of a homogenizer (model RW 20 digital, IKA) for 5 min and transferred into a 100 mL graduated cylinder while the volume of foam (V_2) was immediately recorded at 0 min. The foam stability was determined by measuring the decrease in volume of foam as a function of time up to a period of 10, 20, 30 and 40 min.

$$\text{FC (\%)} = \frac{V2}{V1} \text{ x100}$$

Where V_2 is the volume after whipping and V_1 is the volume before whipping

$$\text{FS (\%)} = \frac{Volume\ after\ standing - Volume\ before\ whipping}{Volume\ before\ whipping} \text{ x100}$$

2.5.4 Emulsifying capacity (EC) and stability (ES)

EC was determined using the method described by Lamsal *et al.* (2007), with some modification. The sample (300 mg) was homogenized for 1 min in 20 mL distilled water, and the pH was adjusted to 3, 4, 6, 8, 10 and 12. The protein solution was mixed with 15 mL of soybean oil followed by homogenizing for 1 min. Then it was centrifuged at 5,000 rpm for 3 min. EC was calculated using the expression below.

$$\text{EC (\%)} = \frac{Height\ of\ emulsified\ layer}{Height\ of\ total\ content} \text{ x100}$$

ES was determined by heating the emulsion at 70°C for 30 min in a water bath; after which, it was centrifuged at 5,000 rpm for 3 min.

$$\text{ES (\%)} = \frac{Height\ of\ emulsified\ layer\ after\ heating}{Height\ of\ emulsified\ layer\ before\ heating} \text{ x100}$$

2.6 Biochemical properties

2.6.1 *In vitro* protein digestibility (IVPD)

IVPD was based on method described by Algadi and Yousif (2017). The aliquot protein (200 mg) was suspended in 15 mL of 0.1 N HCl containing 1.5 mg pepsin. The mixture was placed into a water bath at 37ºC for up to 3 h. In addition, the pepsin–digested hydrolysate was neutralized with 7.5 mL of 0.2 N NaOH, followed by the addition of 4 mg pancreatin in 7.5 mL of phosphate buffer (pH 8.0); then the samples were incubated (Incubator model

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MIR-154, Sanyo, Japan) for an additional 24 h at 37ºC. After incubation, the sample was treated with 10 mL of 10% trichloroacetic acid solution and centrifuged for 20 min at 5,000 g at room temperature to remove undigested protein and larger peptides while the collected supernatant was used to estimate the total nitrogen content using the Kjeldahl method (Nielsen, 2010). For comparative purpose, casein from bovine milk isolate was used as a standard reference. The values of the IVPD were calculated according to the equation:

$$\text{Protein digestibility (\%)} = \frac{Nitrogen\ in\ supernatant}{Nitrogen\ in\ sample} \text{ x } 100$$

The protein solutions after pepsin-pancreatin digestion was subjected to SDS-PAGE to test its digestibility by following the method described by Laemmli (1970) using 10% separating and 4% stacking gels. The protein solutions mixed with the sample buffer (reducing or non-reducing condition) contained 0.125 M Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, and 0.3 M beta-mercaptoethanol. There were 20 micrograms of protein loaded onto the electrophoresis gel and then subjected to separate at 15 mA/gel using Mini Protean Tetra Cell units (Bio-Rad Laboratories, Inc., Richmond, CA, USA). The protein was stained with Coomassie blue R-250 and distained by diffusion methanol-acetic acid solution.

2.6.2 Amino acid analysis

The amino acid profiles were analyzed by an in-house method based on the European Commission (1998), L257/16, using an amino acid analyzer. Ion exchange chromatography was used to separate amino acids and was determined by reaction with ninhydrin with photometric detection at 570 nm.

2.7 Statistical analysis

The experiments on protein extraction and its properties, with the exception of the amino acids profile, were performed in triplicate. The results were presented as a mean ± standard deviation. A two-way analysis of variance (ANOVA) with a post-hoc test was used to detect statistical difference, particularly in functional properties. Analysis was performed using SPSS version 16.0. Significance level was based on the confidence level of 95% ($P<0.05$).

3. Results and discussion

3.1 Protein extraction

The AA extraction technique is a conventional technique to extract protein. In this study, *M. oleifera* leaf had 77.44 ± 0.64% of extractable protein content. This finding is in the range of the result (71.5-79.3%) observed by Stone *et al.* (2015) about pea protein extracted

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by salt. Besides that, the protein obtained by AA extraction in this study was much higher than common leafy vegetables protein (35.1–54.9%), notably *Vernonia amygdalina, Solanum african, Amaranthus hybridus*, and *Telfaria occidentalis* reported by Aletor *et al.* (2002). The differences among alkaline–acid extraction efficiency results, due to the content of some other constituents besides proteins (non–protein) along with extracted protein, particularly carbohydrate or anti–nutritional components that affect protein recovery and yield (Lorenzo–Hernando *et al.,* 2019).

3.2 Physical and functional properties of *M. oleifera* leaf protein

3.2.1 Bulk density

Bulk density is an important property usually used to characterize powder products and is crucial for economical and functional reasons, for example, for reducing packaging costs. It is usually used to determine the packaging requirements, material handling, and volume of samples during storage and packaging (Jalgaonkar *et al.,* 2018). Bulk density of *M. oleifera* leaf protein extract was found to be 0.15 g/mL in Table 1. This value was closed to that of freeze–dried alfalfa leaf protein extracted by water (0.17g/mL) and NaOH (0.16 g/mL) reported by Wang and Kinsella (1976) and also to the protein isolate from cashew nut shells (0.14g/mL) (Yuliana *et al.,* 2014). In fact, low bulk density makes the protein isolates important in relation to packaging and would also improve the formulation of weaning foods (Adenekan *et al.,* 2018). Bulk density is certainly dependent on the combined effects of interrelated factors, like particle size, number of contact point, and intensity of attractive inter-particle forces (Peleg and Bagley, 1983).

3.2.2 Water activity (a_w)

Water activity was used to determine the potential of microbial growth or enzymatic reactions during storage of protein powder obtained after freeze–drying. Carvalho–Silva *et al*. (2013) mentioned that the value for water activity should be below 0.6 to retard alterations of powdered products. In this study, a_w was 0.35 (Table 1), where the chances of microbial contamination and presence of spores were not issues due to a_w lower value, which suggests that the generated powder would be stable during storage (Lafarga *et al.,* 2018). Nevertheless, the a_w values obtained herein were lower than the a_w of the freeze–dried protein powder (0.47 ± 0.01) of brown seaweed observed by Garcia-Vaquero et al. (2017), whereas it was reportedly higher than the a_w of Ganxet beans (0.180 ± 0.002) studied by Lafarga *et al.* (2018).

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Table 1 Physical properties and some functional properties of *M. oleifera* leaf protein

Properties	Value
Water activity	0.35 ± 0.00
Bulk density (g/ml)	0.15 ± 0.00
Color	
*L**	57.82 ± 0.66
*a**	1.83 ± 0.04
*b**	14.11 ± 0.32
WHC (g water/g protein)	2.31 ± 0.24
OHC (g oil /g protein)	3.55 ± 0.02

Note: values are given as mean ± S.D from triplicate determinations

3.2.3 Color

Color is one of the important parameters that might influence the overall acceptability of the product. The appearance of dried *M. oleifera* leaf powder before extraction is depicted in Fig 1(a). The colors of the leaf protein extract powder are presented in Table 1, which the colors had lightness of 57.82 ± 0.66, redness of 1.83 ± 0.04, and yellowness of 14.11 ± 0.32, as illustrated in Fig 1(b). This result showed that protein powder presented lower lightness (57.82 ± 0.66) compared to another source of protein isolate from alkaline extraction such as Seinat seeds (Siddeeg *et al.,* 2014) as well as safflower protein obtained by ultrafiltration, as reported by Ulloa et al. (2011). However, there was a similar color lightness (56.39 ± 0.29) of hemp seed observed by Hadnadev *et al.* (2018) from isoelectric precipitation, where the authors mentioned that the complex between phenolic compounds content in protein under alkaline extraction conditions result in the development of dark green to brown color. That might be the reason for the lesser lightness of protein powder since the *M. oleifera* leaf contains has an enormous number of phenolic compounds (Mohammed and Manan, 2015; Vongsak *et al.,* 2013).

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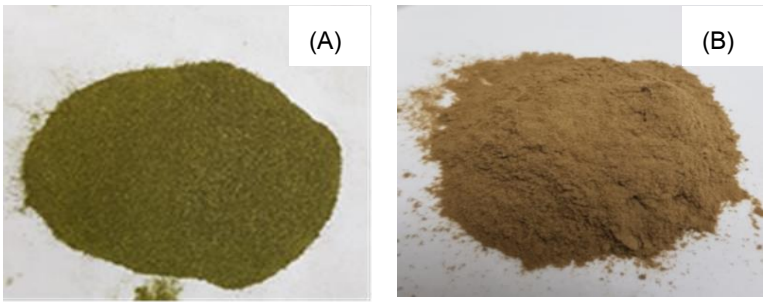

Fig 1 (A) *M. oleifera* leaf powder and (B) *M. oleifera* leaf protein

3.2.4 Water holding capacity

An extracted protein from alkaline–acid in this study had a WHC of 2.31 ± 0.24 g water/g protein (Table 1), which is included within the range of recommended WHC for four leafy vegetables reported by Aletor *et al.* (2002). As mentioned by Sun *et al.* (2017), the ability of protein to absorb water is considered a critical property in viscous foods such as soups, gravies, and baked products, especially when its values ranging from 1.49 to 4.71 g/g. Indeed, high water absorption of protein is important to reduce moisture loss for packaged bakery goods and also to maintain freshness and moist mouthfeel of baked foods (Kandasamy *et al.,* 2012). A dried leaf protein of *M. oleifera* may therefore be a good candidate for viscous foods or bakery products due to its WHC being within the required range of 1.49–4.72 g water/g protein.

3.2.5 Oil holding capacity

The OHC of *M. oleifera* leaf protein extract (3.55 ± 0.02 g oil/g protein) is shown in Table 1, higher than that of the *Moringa oleifera* seed protein isolate (1.9 g/g) reported by Jain *et al*. (2019). The study of Mune *et al.* (2016) also observed that the oil holding capacity of *M. oleifera* seed flour was significantly ($P<0.05$) lower than that of leaf flour. In addition, this result was much higher than seen with another source of protein concentrates such as edible green seaweed *Enteromorpha* species (1.08–1.34 g oil/g protein), found by Kandasamy *et al.* (2012). The OHC is a critical determinant of flavor retention. High OHC of protein is a good functional ingredient in meat based products, particularly sausages. The OHC is also required in ground meat formulation, doughnuts, and baked goods (Ulloa *et al.,* 2011). Thus, these results indicate that *M. oleifera* leaf protein extract possesses good oil absorption capacity.

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3.2.6 Protein solubility (PS)

As can be noticed from Fig 2, the solubility is pH–dependent. Once the pH close to the protein isoelectric point (around pH 4–5), there is a reduction of PS, as found by Mune *et al.* (2016), while above/below of the protein isoelectric point, the more protein solubility was observed. The minimum PS was observed at pH 4 (9.28 ± 0.64%), whereas the maximum PS occurred at pH 10 and pH 12 (58.01 ± 0.79% and 58.30 ± 1.46%, respectively), and there was no significant difference (P>0.05) between them. Seena and Sridhar (2005) mentioned that at highly acidic and alkaline pH, the protein acquires, respectively, a net positive and negative charge, which promotes the repulsion of molecules and thus increases the solubility of the protein. This result has been described in many food proteins according to the tendency of protein solubility as a function of pH, including microalgae proteins, mung bean proteins, and sunflower proteins, which have a minimum solubility around isoelectric point between a pH of 4–5 (Chen *et al.,* 2019; Du *et al.,* 2018; Ulloa *et al.,* 2011, respectively).

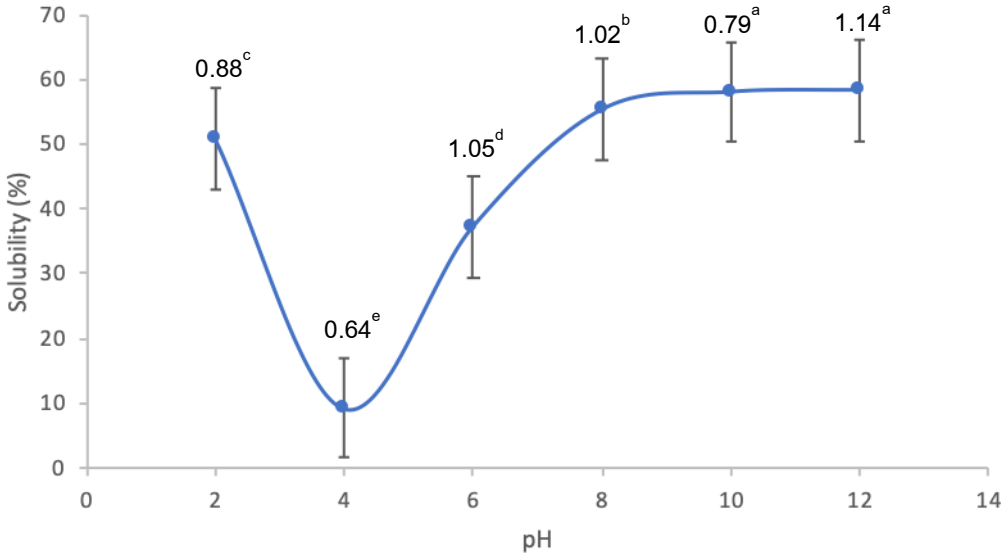

Fig 2 Effect of different pH on protein solubility. Data around bars at different pH represent as standard deviation (n=3).Bars with different letters among different pH values indicate statistically significant differences (P<0.05).

3.2.7 Foaming capacity (FC) and foaming stability (FS)

FC and FS are an important functional property for determining protein usage in different food systems–for example, toppings, baked foods, and ice–cream mixes (Shevkani *et al.,* 2015). The FC of *M. oleifera* protein was measured at time 0 min as the starting point. It was highly dependent on different level of pH; the lowest FC was recorded at pH 8 (7.55 ± 0.78%), whereas the highest FC was obtained at pH 10 (24.95 ± 2.30%) followed

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by pH 4 (19.52 ± 1.50%), as illustrated in Fig 3. The high FC at pH 10 might be due to the increases in the net charge of the protein molecules, which weakens hydrophobic interactions and also increases protein flexibility. In that case, it allows them to spread to the air–water interface quickly, thus encapsulating air particles and increasing foam formation (Lawal, 2004). This result was comparable to that found by Mune *et al*. (2016), who observed that pH 9 has the highest protein foaming capacity and found a lower FC at pH 7. Maximum duration of FS was obtained at pH 3 and pH 6, which can be reached in up to 40 min. On the other hand, pH 8 and pH 12 can only retain protein stability up to 10 min. Results were consistent with those obtained for other protein sources such as seaweed (Kumar *et al.,* 2014) and Ganxet beans (Lafarga *et al.,* 2018). Thus, protein stability was much higher in the neighborhood of the isoelectric pH than at other pHs. This observation is due to repulsive interaction at the isoelectric region minimized (for example, in the case of pH 3 and pH 6) and the formation of a viscous film at the interface, which led to the formation of stable molecular layers at the air–water interface that imparted stability to the foam (Lamsal *et al.,* 2007).

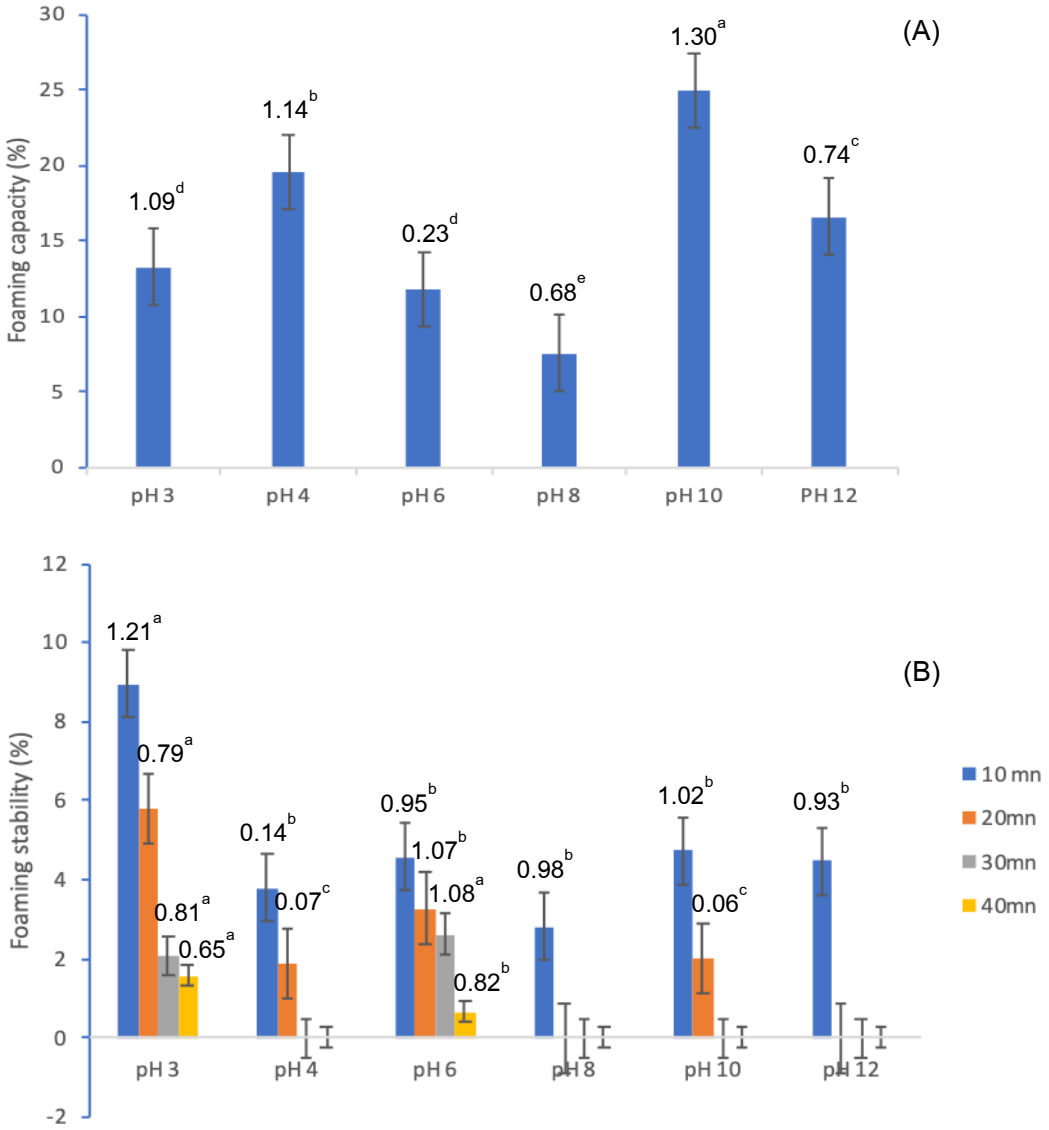

Fig 3 Effect of pH on the foaming capacity (A) and foaming stability (B).
Data around bars at different pH and time represent a standard deviation (n=3).
Different letters in the same color bar at different pH are significantly difference (P<0.05)

3.2.8 Emulsion capacity (EC) and emulsion stability (ES)

EC is used to measure the effectiveness of proteinaceous emulsifiers, in which protein has to absorb on the interface. EC is a pH−dependent property that influences the hydrophobic and hydrophilic balance of protein molecules Fig 4(A). In this result, at both acidic and alkaline pHs high in EC was observed, where the pH 10 had the highest EC value. Once the pH increased above 10, there was potential significant decreased of EC. Thus, a hydrophobic force decreased as a result of the increased net charge and the increased flexibility. So, this

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enables proteins to rapidly diffuse to the air-water interface, which results in poor EC (Yuliana *et al.,* 2014). This finding is similar to the result reported by Feyzi *et al.* (2015) about Fenugreek (*Trigonella foencem graecum*) seed protein isolate and *Lupinus luteus* (Burgos-Diaz *et al.,* 2016), where the highest EC was found in both extreme acidic and alkaline conditions. Furthermore, high ES was obtained at pH 4, pH 3, and pH 10, which was the result of good molecular rearrangement of the absorbed proteins at the oil-water interface, forming a thick layer and preventing coalescence. A huge protein ES at pH 4 is similar to the finding of Chen *et al.* (2019), who reported that the emulsifying properties are strong near the isoelectric point of proteins due to the repulsive forces between proteins being minimal while the protein adsorption and viscoelasticity at the oil-water interface are maximum. On the other hand, the low stability of the emulsion can be attributed to the increased interaction between emulsified droplets, resulting in facilitated protein aggregation and reduced emulsion stability (Chavan *et al.,* 2001).

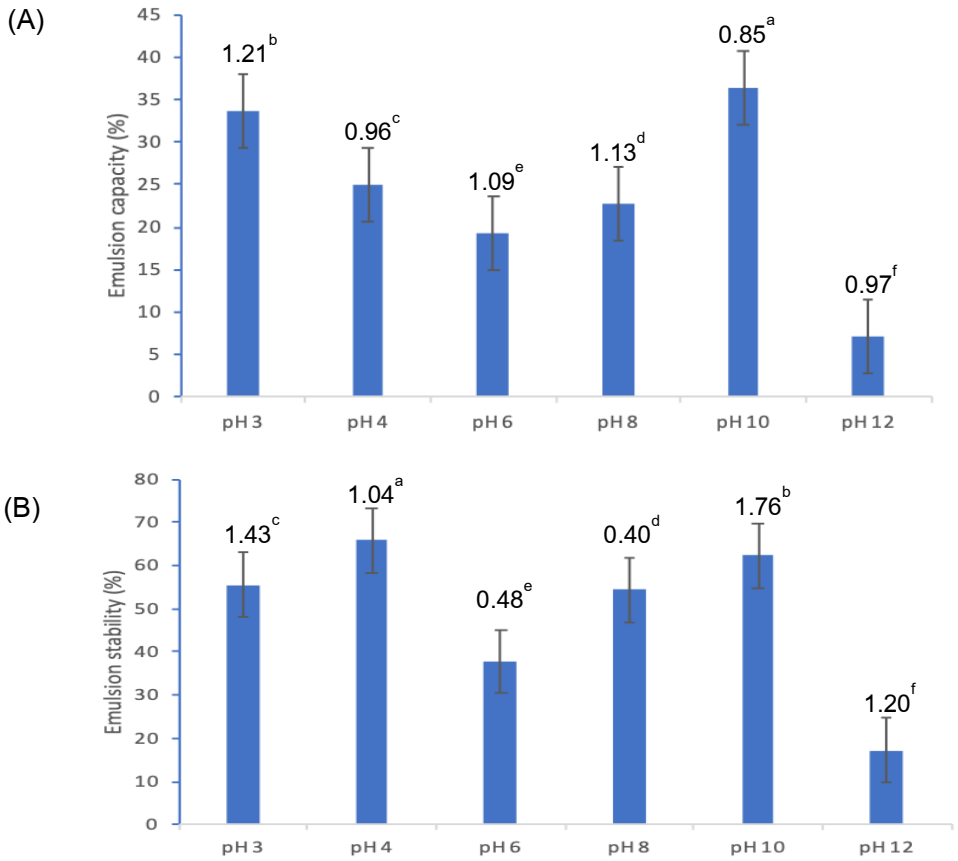

Fig 4 Effect of pH on the emulsion capacity (A) and emulsion stability (B). Data around bars at different pH represent a standard deviation (n=3). Bars with different letters among different pH values indicate statistically significant differences (P<0.05).

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3.4 Biochemical properties

3.4.1 *In vitro* protein digestibility

A digested protein of *M. oleifera* leaf was 75.53 ± 1.15%, which is lower than the casein standard (88.86 ± 0.61%) used (Table 2). This value was much higher compared to the protein digestibility of *Cassia obtusifolia* and Kawal leaves (49.43 ± 1.07% and 61.86 ± 1.05%, respectively) reported by Algadi and Yousif (2017). Digested protein of *M. oleifera* leaf protein was closer to that of soy protein isolate (71.04%) (Wang *et al.,* 2010). In comparison to the study reported by Almeida *et al.* (2015), *M. oleifera* leaf protein also had significantly lower digestibility than commercial whey and casein protein. Teixeira *et al.* (2014) obtained a lower value digested protein of *M. oleifera leaf* when applying sodium dodecyl sulfate and Mercaptoethanol treatment, but digestibility increases using heat treatment. Thermal conditioning could improve proteolysis that contributed to structures changes resulting in disruption of the tertiary and quaternary structures of proteins and favoring the enzymatic action (Teixeira *et al.,* 2014). Indeed, digestibility of plant protein is limited by the presence of the remaining anti-nutritional factors, which form a more complex protein structure and may decrease protein digestibility, such as trypsin inhibitors, phytates, and polyphenol (Aletor, 1993).

Table 2 *In vitro* protein digestibility

Protein	Digestibility (%)
Casein (Standard)	88.86[a] ± 0.61
M. oleifera extract	75.53[b] ± 1.15

Note: values are given as mean ± S.D from triplicate determinations.

SDS-PAGE of *M. oleifera* leaf protein extracted by alkaline-acid extraction, and its *in vitro* digestibility using pepsin-pancreatin enzymes is depicted in Fig 5. It was seen that there were four protein bands (64.78, 52.16, 49.85 and 45.99 kDa) under reducing conditions Fig 5(A)-1. In addition, one single high protein molecular weight band (183.40 kDa) was only observed under non-reducing conditions Fig 5(A)-2. After pepsin-pancreatin enzymes protein digestion for 24 h incubation Fig 5(B), there was no protein remaining on the gel, either under reducing or non-reducing conditions, which means that all protein bands were completely digested. This result was consistent with the study of Paula *et al.* (2017), where the authors mentioned that *M. oleifera* leaf protein extract was susceptible to pepsin and trypsin after incubation for 4 h, which caused complete digestion of all protein bands.

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3.4.2 Amino acids profile

The composition of amino acids (mg/g protein) of *M. oleifera* leaf protein extract is given in Table 3. Leucine (67.14 mg/g protein) was predominant among essential amino acid, whereas glutamic acid (75.06 mg/g protein) was the largest among non-essential amino acids. Thus, *M. oleifera* leaf protein was herein nearly higher compared to protein isolates from wild almond in terms of essential amino acids (Amirshaghaghi *et al.,* 2017). Nag and Matai (2000) also reported a large amount of glutamic acid, aspartic acid, leucine, and phenylalanine content in *M. oleifera* leaf protein concentrate. Studies on *Spirulina platensis* and *Sesamum indicum* protein isolates, by Bashir *et al.* (2016) and Amirshaghaghi *et al.* (2017), respectively, also observed a large proportion of leucine, glutamic acid, and aspartic acid. Witono *et al.* (2016) pointed out that the high glutamic acid content makes the samples a great alternative for use as flavoring/a flavor enhancer; then the presence of hydrophobic amino acids, such as leucine, methionine, proline, and alanine, also expressed an important performance in the functional properties of food proteins.

Certain values of amino acid were lower as compared to the FAO/WHO (1991) reference, but some of them met the requirements. Lysine and valine were limiting amino acids. Nevertheless, Lysine is particularly low in plant-based proteins (Gorissen *et al.,* 2018). Furthermore, Threonine reached the FAO/WHO (1991) requirements for 10-12-year-old children and was close to the 2-5-year-old children requirement. Based on essential amino acid requirement for infants, as infants have very critical nutritional requirements due to rapid growth and immaturity of gastrointestinal function (Behrman and Vaughan, 1983), essential amino acids in this study were not enough to fulfill the recommendation of the FAO/WHO (1991). However, all the essential amino acid contents were sufficient with regard to the recommendation of FAO/WHO (1991) for adults.

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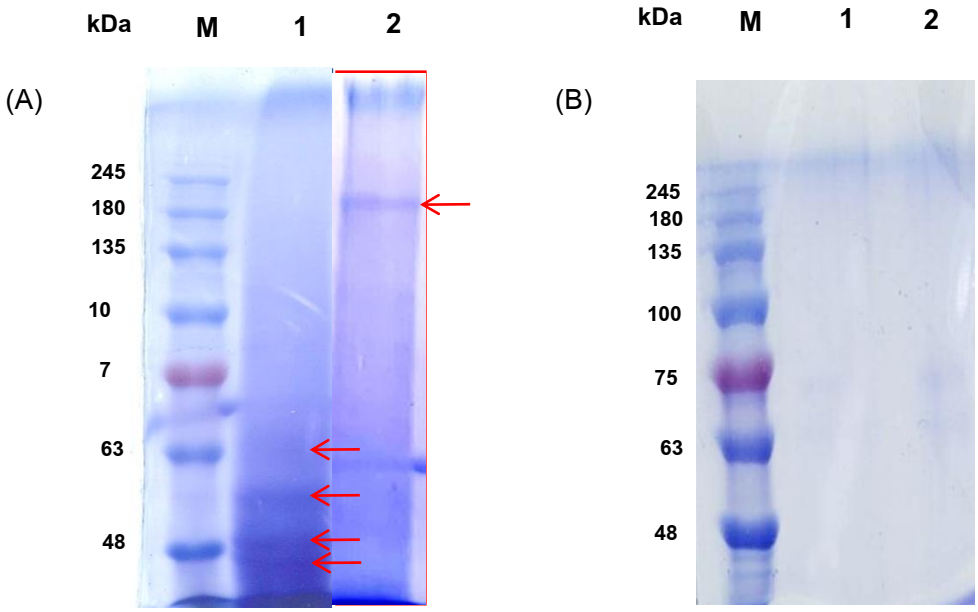

(A) (B)

Fig 5 (A) SDS–PAGE of *M. oleifera* leaf protein extracted by Alkaline–Acid extraction
(B) SDS–PAGE of *in vitro* protein digestibility of *M. oleifera* leaf
M: Standard Marker
1: Reducing condition
2: Non–reducing condition

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Table 3 Amino acids profile of *M. oleifera* leaf protein extract

Amino acids (AA)	Composition (mg/g protein)	2–5 years (mg/g protein) FAO/ WHO (1991)*	10–12 years mg/g protein FAO/WHO (1991)*
Essential AA			
Threonine	30.37	34	28
Methionine	13.18	-	-
Phenylalanine	35.92	-	-
Histidine	19.07	19	19
Lysine	37.91	58	44
Valine	18.60	35	25
Isoleucine	28.71	28	28
Tryptophan	12.10	11	9
Leucine	67.14	66	44
Essential AA–Non			
Serine	30.16	-	-
Glycine	33.20	-	-
Glutamic acid	75.06	-	-
Proline	28.20	-	-
Aspartic acid	67.88	-	-
Alanine	36.96	-	-
Tyrosine	31.16	-	-
Cystine	8.92	-	-
Arginine	41.64	-	-
Methionine + Cystine	22.1	25	22
Phenylalanine + Tyrosine	67.08	63	22

* Reproduced from FAO/WHO (1991) that focused only required amino acids

4. Conclusion

M. oleifera leaf possess a source of protein that can be used for a low-income population as a source of protein. Protein solubility and foaming, and emulsifying properties are greatly affected by pH. *M. oleifera* protein extract is included in the range of those other leaf and legume isolates obtained by isoelectric precipitation. Due to the pleasant solubility, WHC, and OHC and moderate foaming and emulsifying properties, *M. oleifera* leaf protein can play an important role in food systems, enhancing the quality or appearance of products such as salad dressing, ground meat formulation, and the production of beverages. In terms of digestibility, *M. oleifera* protein had lower digestibility than commercial casein protein but its digestibility was higher than other leaf proteins. By 24 h incubation, protein was completely digested. Accordingly, essential and non-essential amino acids were highly observed in *M. oleifera* leaf protein from alkaline-acid extraction. The protein isolates commonly used in the food industry are mainly derived from dairy, soy, or wheat; however, certain reports suggest that these could trigger allergic responses. Therefore, the *M. oleifera* leaf protein studied herein has demonstrated balanced functionality, digestibility, and essential amino acids, and can be used as an alternative source of protein.

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