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Extraction of Plant Based Protein from *Moringa oleifera* Leaves using Alkaline Extraction and Isoelectric Precipitation Method

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This study aimed to investigate the effect of solid-to-solvent ratio on the yield and properties of protein concentrate extracted from dried *M. oleifera* leaves powder by alkaline extraction and isoelectric precipitation method. Extraction of protein concentrate was carried out by manipulating the solid-to-solvent ratio (1:10, 1:20 and 1:30) that affects protein yield of MOLPC. The characterization of both chemical and physical functional properties was performed for dried *M. oleifera* Leaf Powder (MLP) and *M. Oleifera* Leaf Protein Concentrate (MOLPC). Fat absorption capacity (FAC) and water absorption capacity (WAC) of MOLPC were characterized at the different solid-to-solvent ratio and functional properties of MOLPC such as protein solubility, foaming and emulsifying capacity were characterized at different pH (2.0 – 11.0). The proximate analysis for the MLP had shown the moisture content (4.86 ± 1.18 %); ash (9.53 ± 0.07 %); crude protein (17.86 ± 0.23 %); crude fibres (12.54 ± 0.01 %) and fats (0.50 ± 0.38 %). Based on the findings, MOLPC tends to have higher protein content (26.58 ± 1.86 to 35.15 ± 3.4 %) compared to MLP. The FAC and WAC increased as the solid-to-solvent ratio increased (3.21 – 3.82 g/g and 5.19 – 6.41 g/g). For the functional properties of MOLPC; the protein solubility, foaming and emulsifying properties were all found to be pH-dependent.

1. Introduction

In the prevalent phenomenon of dietary protein deficiency, particularly afflicting the vulnerable pre-school children, pregnant or nursing mothers and elderly, it necessitates the production of protein concentrates from viable natural plant-based sources instead of expensive animal source. In the prospect of medicinal value, *M. oleifera* has been advocated as a traditional panacea since all the parts of the *Moringa* tree have long been used for the treatment of various diseases (Gopalakrishnan et al., 2016). The use of *moringa* leaves has been widely spread among medical experts and nutritionists to treat malnutrition and other illnesses due to its high quality of protein (Thurber and Fahey, 2005). Plant or vegetable proteins from various sources such as soybean (Preece et al., 2017), sunflower seed (Baurin et al., 2020), and chickpeas (Nguyen et al., 2021) have been studied for an alternative to animal protein in food application. Protein from *M. oleifera* can compete favourably with proteins from animal sources, especially for growth and enzymatic activity of human body (Bhargav et al., 2015).

It is important to have an appropriate extraction method that promote high yield of plant protein extract with high functional properties and nutrients for food supplement and nutraceutical production. The most common protein extraction protocol is by subjecting tissue to the exposure of distilled water or other weak buffers, which then causing rupture of cells with concomitant release of intracellular proteins as it responds to the hypotonic effect that gradually emerges (Maehre et al., 2018). In this context, animal cells in the absence of cell walls are suitable for this protocol, but it is not efficacious for plant cells with presence of cell wall. Due to their hydrophobic groups and disulfide connections between protein molecules, proteins in plant cells are rarely water soluble. For other alternatives, aqueous salt or alkaline extraction is one of the most often implemented technique for the isolation

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of plant-based proteins in laboratory scale because high alkalinity assists well in extracting leaf protein by breaking down the hydrogen bonds, disrupting the leaf tissue and enhancing protein solubility (Rawdkuen, 2020). Because the minimum protein solubility for M. oleifera leaf proteins is attained at the isoelectric point between pH 3.2 and 4.5, the acid precipitation method is an efficient method for isolating leaf proteins. At this isoelectric point, white cell sap protein of *M. oleifera* leaf concentrate is precipitated by acidification and it has the highest amino acids content and solubility as compared to concentrates extracted by heat coagulation or by addition of cationic or anionic flocculants (Santamaria-Fernández et al., 2019). Previous study by Ahmed (2016) has investigated the protein concentrate extraction from *M. oleifera* using alkaline extraction at fixed parameters. It has been reported that solid-to-solvent ratio is one of the significant factors that influence protein yield extracted from plant (Cui et al., 2017). Higher solid-to-solvent ratio coulld produce a higher yield of protein. The objective of this study was to investigate the effect of different solid-to-solvent ratio on the protein concentrate extracted from dried *M. oleifera* leaves powder (MLP) by using alkaline extraction and isoelectric precipitation method. The dried MLP was characterized for proximate analysis and *M. oleif*era leaves protein concentrate (MOLPC) was characterized for its functional properties such as fat and water absorption capacity and protein solubility, foaming and emulsifying capacity at different pH. The extraction of high yield protein concentrates from *M. oleifera* leaves can be incorporated into dietary supplements that has becoming a promising alternative for the food industry and treatment of malnutrition as well as has a great potential plant that is still underutilized in its application.

2. Methodology

2.1 Sample collection and pre-treatment of fresh leaves

Samples of fresh *M. oleifera* leaves (5 kg) were bought from local market in Skudai, Johor, Malaysia and further processed by tray-drying treatment at 60 ºC for 8 h. The dried leaves were collected, ground into fine MLP and kept in tightly sealed containers.

2.2 Extraction of *M. oleifera* leaves protein concentrate

Different parameters of solid-to-solvent ratio (1:10; 1:20; 1:30 w/v of MLP-water) were manipulated to extract the protein concentrate from MLP (Ahmed, 2016). The suspension was stirred for 1 h using a magnetic stirrer while adjusting the pH at 8.5 with sodium hydroxide solution (1.0 M). The mixture was then centrifuged at 3,200 rpm, 15 min at room temperature to obtain aliquots of supernatant (S1) and precipitate (P1). At the second stage of protein coagulation, the S1 was acidified to pH 4.5 by adding HCl (1.0 M), followed by second centrifugation at 10,000 rpm for 10 min to obtain supernatant (S2) and precipitate (P2). The P2 was rinsed with distilled water by three times, then subjected to third centrifugation at 10,000 rpm for 10 min to obtain supernatant (S3) and precipitate (P3). Supernatant (S3) was then discarded, and the slurry was adjusted to neutral pH of 7.0. The protein cake was freeze-dried to a constant weight until solid matter of MOLPC was achieved.

2.3 Characterization of MLP by proximate composition analysis

2.3.1 Determination of moisture content

Moisture content (MC) was determined following the standard method (AOAC, 1995). The final weight were being recorded and percentage of moisture (%) for each sample was calculated using Eq(1):

$$MC(\%) = \frac{w_1 - w_2}{w_1 - w_o} \times 100 \quad (1)$$

where w_0 is weight of porcelain crucible, w_1 is weight of crucible with fresh sample, w_2 is weight of crucible containing dried sample.

2.3.2 Determination of ash content

Ash content was determined according to standard method (AOAC, 1995) as shown in Eq(2):

$$Ash(\%) = \frac{w_2 - w_o}{w_1 - w_o} \times 100 \quad (2)$$

where w0 is weight of porcelain crucible, w1 is weight of crucible with fresh sample, w2 is weight of crucible containing dried sample.

2.3.3 Determination of crude protein content

The crude protein was conducted following Bradford assay (Kabbashi et al., 2018). A protein standard curve was performed by dissolving Bovine Serum Albumin (BSA) powder in 10 mL of water at room temperature. A

calibration standard curve of absorbance against BSA was plotted using Microsoft Excel and an equation of linear regression was generated. The unknown concentration of *M.oleifera* samples were determined by referral to the established protein standard calibration curve.

2.3.4 Determination of crude fibres

Determination of crude fibres was performed according to Offor et al. (2014). The crude fibre was determined using Eq(3).

$$\text{Crude fibre (\%)} = \frac{w_2 - w_1}{w} \times 100 \quad (3)$$

where w is weight of the original sample, w_1 is weight of the sample after pyrolysis, w_2 is weight of the dried sample.

2.3.5 Determination of crude fat content

Standard protocol of Soxhlet extraction with petroleum ether as solvent was performed to determine the fat content (Kabbashi et al., 2018). The crude fat content was calculated per 100 g of sample using Eq(4).

$$\text{Fat Content (\%)} = \frac{W_2 - W_1}{W} \text{ x } 100 \quad (4)$$

where w is weight of original sample, w_1 is weight of empty extraction flask, w_2 is weight of extraction flask with fat.

2.4 Characterization of the functional properties of *M. oleifera* leaves protein concentrate

2.4.1 Fat absorption capacity (FAC) of MOLPC

1.0 g of the MOLPC was mixed thoroughly with 10 mL of corn oil (Ahmed, 2016). The protein-oil mixture was subjected to centrifugation for 20 min. The supernatant was taken out, and the tube was reweighed. FAC was then determined by Eq(5):

$$\text{FAC (\%)} = \frac{W_2 - W_1}{W} \text{ x } 100 \quad (5)$$

where w is weight of dried sample, w_1 is weight of tube with sediment, w_2 is weight of tube with dried sample.

2.4.2 Water absorption capacity (WAC) of MOLPC

WAC was performed following method by Ahmed (2016) with slight modification. 1.0 g of the MOLPC sample was weighed into centrifuge tube. Distilled water (10 mL) was added in slowly to the tube and stirred continuously with glass rod. After 30 min, it was centrifuged for 20 min. Supernatant was taken out and the tube was reweighed. WAC was calculated using Eq(6) (Ahmed, 2016):

$$\text{WAC (\%)} = \frac{W_2 - W_1}{W} \text{ x } 100 \quad (6)$$

where w is weight of dried sample, w_1 is weight of tube with dried sample, w_2 is weight of tube with sediment.

2.4.3 Protein solubility of MOLPC

About 1g of the MOLPC was diffused in 100 mL distilled water and the mixture was adjusted to different pH (2 to 11) with 1.0 N sodium hydroxide and 1.0 N hydrochloric acid. The protein from sample and supernatant were weighed and the solubility was calculated as shown in Eq(7) (Ahmed, 2016):

$$\text{Protein solubility (\%)} = \frac{P_{supernatant} \times 50}{w \times \frac{P_{sample}}{100}} \text{ x } 100 \quad (7)$$

where w is weight of original sample, $P_{supernatant}$ is content of protein in supernatant (mg/mL) and P_{sample} is content of protein in sample (mg/mL).

2.4.4 Foaming capacity (FC) of MOLPC

0.5 g of the MOLPC was diffused in 50 mL distilled water (Ahmed, 2016). The protein solution was adjusted to different pH (2 to 11) with 1.0 M sodium hydroxide and 1.0 M of hydrochloric acid. The solution was whipped for 2 min by homogenizer in the graduated tube. FC was calculated as shown in Eq(8):

$$\text{FC (\%)} = \frac{V2 - V1}{V1} \text{ x } 100 \quad (8)$$

where v_1 is volume before whipping, v_2 is volume after whipping.

2.4.5 Emulsifying capacity (EC) of MOLPC

1.0 g of MOLPC was mixed in 50 mL of 0.1 N NaOH, followed by 50 mL of corn oil (Ahmed, 2016). The dispersion was magnetically stirred for 1 min at room temperature, then centrifuged at 1,100 rpm for 5 min. The emulsion was transferred to measuring cylinder. EC was determined as shown in Eq(9):

$$EC = \frac{V_A - V_R}{W} \quad (9)$$

where v_A is initial volume of oil, v_R is volume of oil released, w is weight of original sample.

3. Results and discussion

3.1 Analysis of chemical composition of MLP

The chemical composition of the MLP is presented in Table 1. The MC of dried MLP of less than 5 % was favourable for maximum nutrient and colour preservation of MLP (Senadeera et al., 2003). Similar range with previous studies was reported for ash content (8.0 to 9.8 %) (Kabbashi et al., 2018) and crude fibre content (3.4 to 19.4 %) (Yaméogo et al., 2011) in MLP. Crude fat values were lower as compared to previous study (2.3 to 17 %) reported by Yaméogo et al. (2011).

Table 1: Proximate analysis of chemical composition in MLP (dry weight basis)

Parameter(s)	Quantity (MLP), %
Moisture content	4.86 ± 1.18
Ash content	9.53 ± 0.07
Crude protein	17.86 ± 0.23
Crude fibre	12.54 ± 0.01
Crude fats	0.50 ± 0.02

3.2 Characterization and functional properties of MOLPC

3.2.1 Effect of solid-to-solvent ratio on protein content, FAC and WAC of MOLPC

Protein content of MOLPC increases with increasing solid-to-solvent ratio (Table 2). Similar trend was reported by Jain et al. (2019) showing an increase about 48.4 to 67.4 % of protein extractability with increasing solvent-to-flour ratio from 5:1 to 20:1. Increasing FAC was observed with increasing solid-to-solvent ratio (Table 2) due to high content of non-polar or hydrophobic amino acids in bulky protein concentrates of plant origin which is important for them to bind hydrocarbon chains. These findings were coherent within the range of 1.69 g/g to 3.87 g/g as supported by the previous studies (Ahmed, 2016).

Table 2: Protein content, fat absorption capacity and water absorption capacity of MOLPC

Parameters	Solid-to-solvent ratio (w/v)		
	1:10	1:20	1:30
Protein content (%)	26.58 ± 1.86	33.93 ± 0.00	35.15 ± 3.40
FAC (g/g)	3.21 ± 0.24	3.69 ± 0.13	3.82 ± 0.20
WAC (g/g)	5.19 ± 0.35	5.83 ± 0.45	6.41 ± 0.35

WAC increases with increasing solid-to-solvent ratio that aids in reducing moisture loss in MOLPC. WAC indicates the capacity of hydrophilic peptides in MOLPC binding to water molecules and high hydrogen bonding. High WAC property can be utilized in MOLPC products which require high water retention with low fat content to maintain its freshness in viscous form of servings. High WAC may possibly dehydrate other components of product formulation and moderate WAC in between 3.5 to 5.82 ± 0.47 g/g sample is much encouraged (Azubuike et al., 2018).

3.2.2 Effect of pH on protein solubility of MOLPC in water

Figure 1 shows the effect of protein solubility at different pH. A U-shaped curve was attained with a minimum solubility found at pH 3.5-4.0 and similar trends were indicated across three different solid-to-solvent ratios. At the isoelectric point (pI) which corresponds to minimum solubility at that particular pH, attractive forces are

significant and proteins have a net charge of zero, the protein becomes insoluble which resulted from association of the molecules (Ahmed, 2016). This indicates that interactions with water were minimal for protein molecules at pH values not far from pI. The net charge becomes negative for pH above pI and protein-water interaction is greatly enhanced at alkaline pH rather than acidic pH.

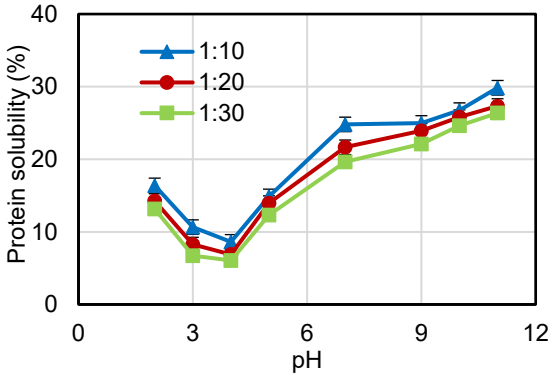

Figure 1: Protein Solubility of MOLPC in water against pH

3.2.3 Effect of pH on foaming capacity of MOLPC

Maximum foaming capacity was obtained at alkaline pH of 10-11 (Figure 2a) due to an increase of net charge of protein molecules which leads to repulsion and weakens the hydrophobic interactions. This enables the flexibility of protein molecules and allows a faster spreading of protein molecules to the air water interface and encapsulating air particles (Azubuike et al., 2018). There is an increase in foam formation through dispersions of gas bubbles with increasing solvent ratio from 1:10, 1:20 to 1:30. Protein isolates' foaming capacity is a key functional feature that determines their suitability for use in various food systems that require aeration (Shevkani et al., 2015).

3.2.4 Effect of pH on emulsifying capacity of MOLPC

The maximum emulsifying capacities were observed at pH 11 across three MOLPC-to-solvent ratios (Figure 2b). This could be due to larger contribution of protein in oil–water interfacial reactions by alkali-induced formation of more soluble protein through unfolding of polypeptide chains.

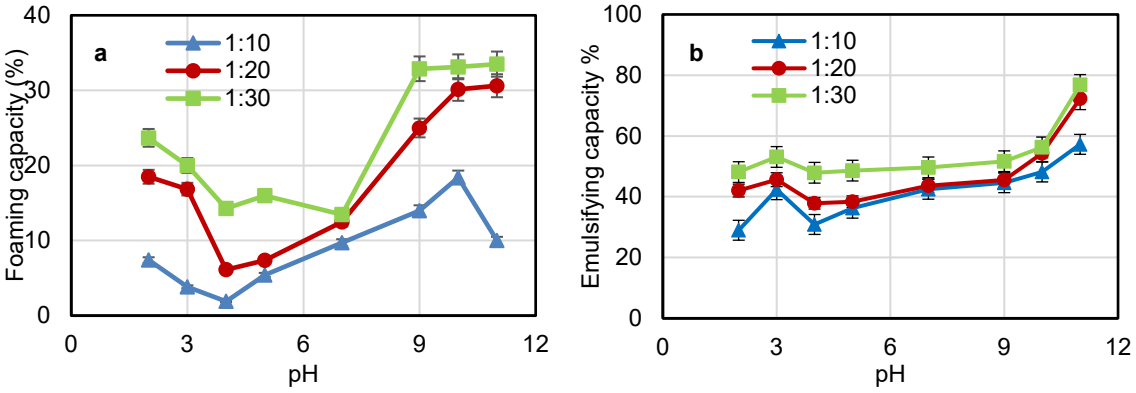

Figure 2: (a) Foaming capacity (b) Emulsifying capacity (EC) of MOLPC-to-solvent ratios against pH

If emulsifying properties exhibit a gradual decrease, it is due to the increase of protein in the aqueous phase that inadvertently increases the protein interaction at the protein and oil surface. This result indicates that emulsifying activity is pH dependent in which alkaline pH can improve the EC more than acidic pH (Okiki and Balogun, 2015).

4. Conclusion

In this study, protein concentrate was extracted from *M. oleifera* leaves using alkaline extraction method at pH 8.5, followed by isoelectric precipitation at pH 4.5. The yield of the protein concentrate increases with increasing

solid-to-solvent ratios. For functional properties, MOLPC has high WAC compared to common leafy vegetable protein concentrates. The solubility, foaming and emulsifying capacities were found to be dependent on pH. It was found that at alkaline pHs higher protein yield and improved functional properties of MOLPC was obtained compared to acidic pHs. Extracted protein from *M. oleifera* leaves protein concentrate (MOLPC) has great potential to be an alternative protein supplement in food formulation due to its high protein content than MLP, high productivity and better functional properties.

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