Extraction and Characterization of Protein from *Moringa oleifera* Leaf

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ABSTRACT

The aim of this study was to extract protein from *Moringa oleifera* leaf by using different extraction techniques. The protein obtained was subjected to test its biochemical and functional properties. Three different methods included alkaline-acid (AA) extraction, aqueous two-phase system (ATPS) and three phase partitioning (TPP) were used. Protein recovery and some physicochemical properties of each fraction were compared. The chemical composition of the fresh leaf contained moisture (75.18 ± 0.64%), crude protein (16.57 ± 0.51%), fat (3.04 ± 0.27%), ashes (10.16 ± 0.47%) and carbohydrate (17.55%). The AA extraction (231.73 ± 1.62 mg/g) showed an extreme method in terms of protein content followed by the inter-phase of TPP (201.75 ± 2.36 mg/g) while high recovery in top phase of ATPS (25.01 ± 0.41%). Hence, AA extraction showed high protein solubility (51.56 ± 1.13%) and water holding capacity (1.96 ± 0.22 g/g), whereas no significant difference appears on the protein solubility for the bottom phase (51.17 ± 2.94%) and water holding capacity in the inter-phase (1.696 ± 0.21 g/g) of TPP. The SDS-PAGE indicated a similar major protein molecular weight on AA and TPP's inter-phase under reducing condition, whilst a single protein band (52.16 kDa) in ATPS' top phase. Thus, AA extraction was a suitable technique that has a huge protein content and attributed functional properties, as well as a protein band appearance on SDS-PAGE. This study showed that these three extraction techniques can be employed for the extraction of protein from *Moringa oleifera* leaf.

Keywords: Extraction, Functional properties, Leaf protein

INTRODUCTION

The genus " *Moringa* " is estimated to include 13 species. Thus, according to their species, *Moringa oleifera (M. oleifera)* is the most cultivated tree (Bellostas et al., 2010). Historically, *M. oleifera* is a plant native to India, but is now grown both tropical and subtropical countries of the world because of its resilient adaptability characteristics, such as the ability to grow rapidly, survive under conditions drought and its longevity (Falowo et al., 2018). The consumption of this plant brings essential amino acids, functional ingredients which can contribute beneficial and health promoting phytochemicals in humans (Bamishaiye et al., 2011).

Recently, protein-energy malnutrition still plays a major role in half of all under-five deaths each year in developing countries and it represents a challenge for public health (Bernstein, 2017). So, in the developed countries, malnutrition in caloric proteins is one of the most serious problems, especially among children under 5 years old, who suffered from it in two serious forms: marasmus and kwashiorkor. Thus, the prevalence rate of stunting was 47% in Madagascar (USAID, 2017); 56% in Haryana, India (Kumari, 2017) and 45.5% in the Sidama zone, southern Ethiopia (Rodamo et al., 2016).

Literature review mostly focused on the isolation of its bioactive compounds content. However, there was a little information on the different techniques of protein extraction and its physicochemical characterization. Hence, this present study aimed to extract protein as new protein additional sources using different methods and to test its biochemical and some functional properties.

MATERIAL AND METHODS

Materials

M. oleifera leaves were harvested from trees in April by hand located in 888 Moo 10, Tambon Wiang Phang Kham, Amphoe Mae Sai, Chang Wat Chiang Rai, Thailand at maturity from 3 to 4 months of growing leaves. The preparation of sample was divided into two techniques. In purpose of proximate analysis determination, the fresh leaves were washed with tap water, drained until the water totally released out and then grinded. However, in the case of three protein extractions techniques; the washed sample was directly dried at 40°C in tray dryer approximately 48 h, then grinded into fine powder and kept at -20°C until further use as described by Teixeira et al. (2014) with minor modification.

Analysis of the chemical composition

The percentages of moisture, lipid and ash in the drained fresh leaves were determined by standard methods of the Association of Official Analytical Chemists (AOAC., 2000) while the percentage of protein was obtained using Kjeldahl method based on nitrogen percentage (%N); conversion factor was 6.25 (Nielson, 2010). Carbohydrates content was determined by difference.

Alkaline-acid (AA) extraction

AA extraction was determined according to Mariod et al. (2010) with some modification. The leaves powder was added distilled water at a ratio of 1:20 (w/v) and then mechanical stirring for 1h by adjusting pH 9.0 with 1.0 M NaOH aqueous solution. After that, the obtained filtrate (using white cloth) was centrifuged at 10,000 rpm for 20 min at room temperature. The supernatant was transferred into a beaker and stirred again for 20 minutes while the pH adjusted around 4.5 with 0.1M 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 which was 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 stored at -18°C for further analysis.

Crude extract preparation

The crude extract or clear supernatant was obtained using the method described by Bijina et al. (2011). Twenty grams of *M. oleifera* powder was extracted by adding 160 ml of phosphate buffer (0.1 M, pH-7) and made then homogenate on rotary shaker for 45 minutes at 150 rpm at room temperature. Furthermore, the homogenate mixture was followed by filtration using white cloth and centrifuge at 10,000 rpm at 4°C for 25 min. The clear supernatant (crude extract) was taken carefully to be used for ATPS and TPP.

Aqueous Two-Phase System (ATPS). ATPS was treated according to Srinivas et al. (1999) with some modification. ATPS (100 ml) comprising component referenced in the assumption PEG 6000 (8%, w:v), ammonium sulphate (15 %, w: v) and distilled water (20%, v:v) along with 30 ml of crude extract were prepared. The mixture was stirred for 15 min and allowed to stand for 10 min at room temperature. Furthermore, the mixture was centrifuged at 10,000 rpm for 10 min at room temperature. After centrifugation, it was obtained two phases such as top phase and bottom phase while both collected phases were separated and subjected to dialyze against water at 4°C overnight

and lyophilized thereafter. The sample after lyophilizing was stored at -18°C for further study.

Three Phase Partitioning (TPP). TPP was done according to Chaiwut et al. (2010) with modification. The clear supernatant obtained after crude extract preparation was added with ammonium sulfate to 30% saturation (w/v) and vortexed gently until salt totally dissolve, followed by the addition of *t*-butanol with the ratio of 1:1 (v/v). The mixture was left to stand for 1 hour at room temperature and then subjected to centrifuge at 10,000 rpm for 15 min. After centrifugation, three phases were shown such as the lower aqueous layer (bottom phase), the interfacial precipitate (inter-phase) layer and upper phase layer (top phase). Nevertheless, the bottom and middle phases were collected, where the middle phase was dissolved in H_20. Next, both phases were dialyzed against distilled water overnight at 4°C and lyophilized and then kept at -18°C for further experiment.

Protein isolates assay

Protein concentration was determined by the Biuret method by using BSA as a standard. The crude protein recovery and yield were calculated by using following formula as described by Sibt-e-Abbas et al. (2015).

$$\text{Recovery } (\%) = \frac{\text{weight of dry extract (g)}}{\text{weight of dry leaf powder (g)}} \times 100$$

$$\text{Yield}(\%) = \frac{\text{Isolate protein recovery x protein content of isolated protein}}{\text{Protein content of initial material}} \times 100$$

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE was carried out by the method of Laemmli using 10% separating and 4% stacking gels. The samples were mixed with sample buffer containing 0.125M Tris–HCl, pH 6.8, 20% glycerol, 4% SDS and 0.3M beta-mercaptoethanol in the sample with reducing buffer. Twenty micrograms of protein were loaded and then subjected to separate at 15 mA/ gel using Mini Protean Tetra Cell units (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After separation, the protein was stained with Coomassie blue R-250 and left overnight, then distained by diffusion methanol-acetic acid solution.

Functional properties

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 10 using 0.1 M HCl or 1M NaOH. The solution was stirred for 1 hour at room temperature and centrifuged at 4000 x g for 20 minutes. The protein content of the supernatant was determined by Kjeldahl method and the percentage solubility of the proteins was calculated as follows:

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

Water holding capacity (WHC). WHC was determined by technique of Mariod et al. (2010) with some modification. The sample (0.5 g) was weighed into 50 ml pre-weighed centrifuge tube and mixed with 10 g of distilled water. Then, the obtained suspensions were vortexed for 1 minute and left to stand for 30 minutes. The protein-water mixture was centrifuged at 2000 g for 15 minutes and the supernatant was removed while the tube re-weighed.

$$\text{WHC (g/g)} = \frac{W_2 - W_1}{W_1}$$

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

Statistical analysis

All experiments were conducted and analyzed in triplicate. Means and standard deviations were calculated and compared using the Duncan's test. Analysis was performed using SPSS version 16.0. Significant level was based on the confidence level of 95% ($p<0.05$).

RESULTS

Chemical composition

The results revealed that the fresh *M. oleifera* leaf contained of moisture (75.18 ± 0.64%), ashes (10.16 ± 0.47%), crude protein (16.57 ± 0.51%), lipid (3.04 ± 0.27%) and carbohydrate (17.55%) as shown in Table 1.

Table 1. Chemical composition of fresh *M. oleifera* leaves flour.

Nutrients	Mean composition (% ± SD)
Moisture	75.18 ± 0.64
Protein (DW)	16.57 ± 0.51
Lipid (DW)	3.04 ± 0.27
Ash (DW)	10.16 ± 0.47
Carbohydrate* (WW)	17.55

Note. Values are given as mean± standard deviation (SD), from triplicate determinations.
*Obtained by difference.
DW: dry weight
WW: wet weight; where, 4% protein, 0.75% fat, 2.52% Ash.

Protein isolates assay

The fraction of protein extracted from three different techniques was illustrated in Figure 1. Besides, the mean values for protein content, protein recovery and protein yield extracted are reported in Table 2. The protein content of the isolates ranged from 39.97 ± 1.11 mg/g (ATPS bottom phase) to 231.73 ± 1.62 mg/g (AA fraction) while the initial solubilized protein was 13.17 ± 0.97%. Overall, AA extracts and TPP inter-phase fractions were significantly ($p<0.05$) higher protein contents than other fractions. Moreover, the protein isolate recovery of these extractions observed that the ATPS top phase (25.01 ± 0.41%) followed by ATPS bottom phase (18.20 ± 1.83%) were greatly significant compared to the other methods ($p<0.05$). However, there was not significantly ($p>0.05$) difference between alkaline acid, TPP inter-phase on average isolate recovery; whereas they are significantly ($p<0.05$) lower protein recovery compared to TPP bottom phase (11.73 ± 0.85%). The protein yield is affected by the result obtained on protein recovery and protein content.

Here, TPP inter-phase takes a higher yield (9.46 ± 0.49%), which was significantly difference ($p<0.05$) as compared to the others. Nevertheless, there was no significant difference ($p>0.05$) found between ATPS top phase (8.57 ± 0.37) and AA fraction (8.16 ± 0.14%); yet they were significantly ($p<0.05$) higher protein yield compared to the bottom of ATPS and TPP fraction.

Table 2. Protein content, recovery and yield

Protein fractions	Protein recovery (%)	Protein content(mg/g)	Protein yield (%)
ISP		13.17 ± 0.97^{c}	
Alkaline-acid	4.64 ± 0.05^{d}	231.73 ± 1.62^{a}	8.16 ± 0.14^{b}
ATPS TP	25.01 ± 0.41^{a}	45.16 ± 2.10^{e}	8.57 ± 0.37^{b}
ATPS BP	18.20 ± 1.83^{b}	39.97 ± 1.11^{f}	5.53 ± 0.66^{c}
TPP IP	6.18 ± 0.25^{d}	201.75 ± 2.36^{b}	9.46 ± 0.49^{a}
TPP BP	11.73 ± 0.85^{c}	55.99 ± 1.39^{d}	5.59 ± 0.34^{c}

Note. ISP: initial solubilized protein; TP: top phase; BP: bottom phase; IP: inter-phase

Values are given as mean ± S.D from triplicate determinations

Means in the same column with different letters (a-f) are significantly ($p<0.05$) different.

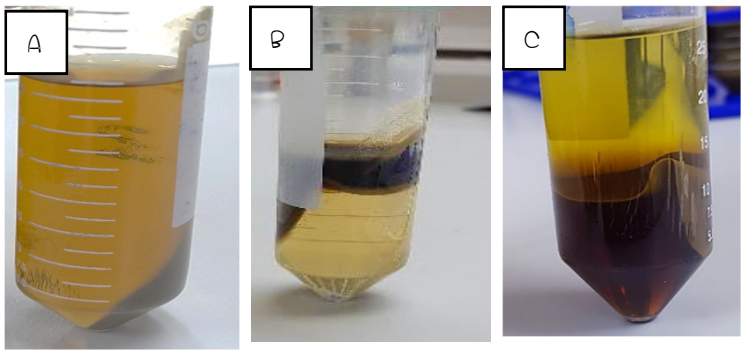

Figure 1. Three different methods of protein extractions from *M. oleifera* leaf (A) alkaline-acid extraction, (B) aqueous two-phase system and (C) three-phase partitioning.

SDS-PAGE

Protein patterns of the different protein fractions are shown in Figure 2. Under reducing conditions, the proteins in AA extraction (lane 2) and TPP inter-phase (lane 3) showed a similar protein bands pattern with the molecular weights (MW) of 64.78, 52.16, 43.85 and 41.99 kDa. However, the top phase of ATPS (lane 5) observed only one single major band with the MW of 52.16 kDa. Under non-reducing conditions, a single similar protein band was observed in the AA extract, TPP inter-phase and ATPS top phase fractions which had a high molecular weight (183.40 kDa). Both bottom phases of ATPS and TPP which are contained of ammonium sulfate did not observe clearly the protein band appearance, neither under reducing nor non-reducing conditions.

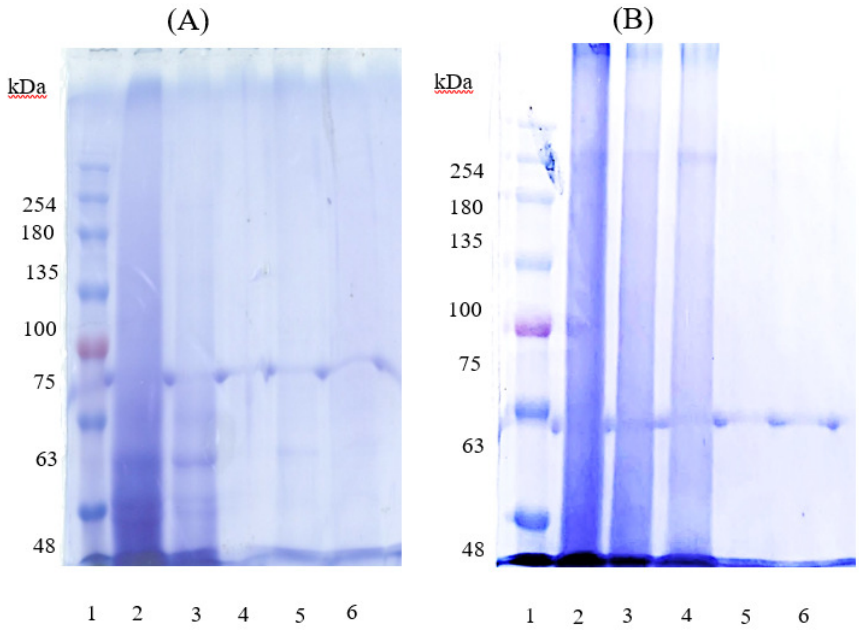

Figure 2. SDS-PAGE (A) under reducing 1-Standard marker, 2-Alkaline-acid extract, 3-TPP inter-phase, 4-TPP bottom phase, 5-ATPS top phase, 6-ATPS bottom phase. (B) under non-reducing 1-Standard marker, 2-Alkaline-acid extract, 3-TPP inter-phase, 4-ATPS top phase, 5-TPP bottom phase, 6-ATPS bottom phase.

Protein solubility

The variance of protein solubility was depended on the different protein fractions from the extraction methods. Here, Table 3 shows that the AA extract (51.56 ± 1.13%) and TPP bottom phase fractions (51.17 ± 2.94%) found a significant difference ($p < 0.05$) followed by TPP inter-phase (43.08 ± 2.89%) compared to the other fractions. Nevertheless, the ATPS extractions showed less protein solubility in which the phase that contains ammonium sulfate observed much higher protein solubility (20.65 ± 1.36%) than PEG phase (14.32 ± 2.78%).

Water holding capacity

There was no significantly difference ($p >0.05$) appeared between AA extract and inter-phase of TPP fractions (1.96 ± 0.22 g/g and 1.69 ± 0.21 g/g, respectively) as depicted in Table 3. However, less ability of protein to hold water found in ATPS top phase which was stated a negative result (-0.27 ± 0.02 g/g). The ATPS top phase was slightly high as compared to the bottom phase of ATPS and TPP in which they both bottom phases were apparently negative (-0.67 ± 0.20 g/g and -0.65 ± 0.14 g/g, respectively).

Table 3: Protein solubility and water holding capacity (WHC)

Fractions	Protein solubility (%)	WHC (g/g)
Alkaline-acid	51.56 ± 1.13[a]	1.96 ± 0.22[a]
ATPS TP	14.32 ± 2.78[d]	-0.27 ± 0.02[b]
ATPS BP	20.65 ± 1.36[c]	-0.67 ± 0.20[c]
TPP IP	43.08 ± 2.89[b]	1.69 ± 0.21[a]
TPP BP	51.17 ± 2.94[a]	-0.65 ± 0.14[c]

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

Means in the same column with different letters (a-d) are significantly (p<0.05) different.

TP: top phase; BP: bottom phase; IP: inter-phase

DISCUSSION

The chemical composition revealed that the moisture content (75.18 ± 0.64%), close to the result (75.90 ± 3.12%) reported by Foline et al. (2001). Furthermore, ash content was high over than the result obtained by Mgbemena and Obodo (2016), indicating that the leaves have a high mineral content, while it is lower than study reported by Leone et al. (2018). In addition, the average content of crude protein was within the range of Table (10.74 – 30.29%) reported by Falowo et al. (2018) and a similar observation on the study described by Ogbe and Affiku (2011). Then, it was higher than those found in many other leaves like in Carica papaya morphotypes (Nwofia et al., 2012) and Alchornea Cordifolia (Ngaha et al., 2016). A huge fat content was observed in this study compared to that reported by Bamishaiye et al (2011), whereas a lesser content than observed by Teixeira et al (2014). *M. oleifera* also contained an appreciable amount of carbohydrate. These all differences on proximate composition due to many factors such as growth environment, stage of harvest, soil type and method of processing (Falowo et al., 2018) and could be explained also due to the stage of maturity (Oduntan and Olaleye, 2012).

The results regarding protein isolate assay showed that the top phase containing PEG on ATPS was largely abundant in terms of protein isolate recovery due to the PEG itself is an inert, neutral, hydrophilic polymer and non- biodegradable; hence dialysis membranes can only reduce the concentration of smaller molecular weight of PEGylated proteins (Fee and Van Alstine, 2011). The protein content of AA extraction was 77.44 ± 0.64% (data not shown on kjeldahl method), which is closed to the result observed by Stone et al. (2015) about pea protein wherein the protein content was in the range of 71.5 – 79.3%. Besides, the extraction of AA obtained was much greater than the result reported by Aletor et al. (2002) of leaf protein concentrates. The differences can be explained due to the contain of some other constituents besides proteins (Chandi and Sogi, 2007). TPP showed that the inter-phase fraction contained high protein over the bottom phase. Indeed, the accumulation of protein in inter- phase depends on the nature of protein, initial protein concentration as well as the composition of the system (Kiss et al., 1998). For instance, Wati et al. (2009) mentioned that the total protein partitioned in the phases are different according the sample used. On the ATPS result, it was observed that the protein content in the top phase (PEG 6000) was higher than that of the bottom phase. Thus, less protein is recovered in the bottom phase which was similar to that seen on TPP. Nitsawang et al. (2006)

observed that the increase of ammonium sulfate concentration leads to the higher protein content in the top phase, that might be the reason of the higher protein in the top phase over bottom phase in this study. However, this result is unattributable to the findings of Lakshmi et al. (2012) where the authors remarked the protein contains in bottom phase is higher than found in top phase. Hence, the nature and size of the biocompound, molecular structure and chain size of the polymer, the initial composition of the system and the type of the salt also are the main factors that may be affected ATPS constituents (Carvalho et al., 2007).

The protein patterns appeared in AA extraction and TPP inter-phase were the same as the result illustrated by Paula et al. (2017) in which the authors reported that the apparent protein molecular weight from *M. oleifera* leaf over 29 kDa. In contrast, three protein bands appearance on AA extract and TPP inter-phase were disappeared in the top phase of ATPS (molecular weights, 64.78, 43.85 and 41.99 kDa) under-reducing condition as displayed in Figure 1 (A). From this study as the standard marker used from 48 kDa to 245 kDa, the *M. oleifera* leaf can be found high molecular weight (183.40 kDa) under non-reducing condition. Wati et al. (2009) mentioned that the tendency of the protein to concentrate in the fraction phases is depended upon the molecule inside. Thus, the inapparency of any protein in both ATPS and TPP bottom phases can be explained that the proteins did not have much enough to concentrate in the bottom phases.

As the pH 10 used in this study, the protein solubility of AA and TPP bottom phase, close to the results found by Mune et al. (2016) and figure reported by Kumar et al. (2014) at pH 10. In fact, there are many reasons that can be taken place which might affect the level of protein solubility to be lower or higher such as electrostatic interactions, hydrogen bonding as well as hydrophobic interactions (Al-Kahtani and Abou-Arab, 1993). WHC in AA was significantly ($p < 0.05$) higher than other seed like hemp seed meal protein (1.59 ± 0.05 g/g) reported by HadnaCev et al. (2018) and also shows a higher content as compare to *M. oleifera* leaf seed (Mune et al., 2016). The inter-phase of TPP is also contained an enormous value over than WHC (0.80 ± 0.03 g/g) observed by HadnaCev et al. (2018) in which they used a micellization extraction method. On the other hand, top and bottom phases of ATPS and bottom phase of TPP were coincidentally negatives result. These results might be due to the lesser protein in fractions. Indeed, polar amino acid residues of proteins and some composition of carbohydrate are the major factors affecting WHC (Sreerama et al., 2012).

CONCLUSION

M. oleifera leaves contained enormous protein. Each extraction technique was observed a different protein content and recovery. In the test of its biochemical and functional properties of the protein fraction in different extractions found that the AA extraction was the foremost suitable technique followed by TPP inter-phase concerning the attributed protein solubility and WHC as well as the protein band appearance on SDS-PAGE. Based on this research, *M. oleifera* leaves can be beneficial for the low-income population as a source of protein.

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REFERENCES

Aletor, O.L.U.W.A.T.O.Y.I.N., Oshodi, A.A. and Ipinmoroti, K. 2002. Chemical composition of common leafy vegetables and functional properties of their leaf protein concentrates. Food chemistry, 78(1): 63-68.

Al-Kahtani, H.A. and Abou-Arab, A.A. 1993. Comparison of physical, chemical, and functional properties of Moringa peregrina (Al-Yassar or Al-Ban) and soybean proteins. Cereal Chemistry, 70: 619-619.

AOAC. 2000. Official methods of the association of official analytical chemists. Association of Official Analytical Chemists, Washington D.C.

Bamishaiye, E.I., Olayemi, F.F., Awagu, E.F. and Bamshaiye, O.M. 2011. Proximate and phytochemical composition of Moringa oleifera leaves at three stages of maturation. Advance Journal of Food Science and Technology, 3(4): 233-237.

Bellostas, N., Christian Sørensen, J., Nikiema, A., Sørensen, H., Pasternak, D. and Kumar, S. 2010. Glucosinolates in leaves of Moringa species grown and disseminated in Niger. African Journal of Agricultural Research, 5(11): 1338-1340.

Bernstein, L.H. 2017. The Global Problem of Malnutrition. Food Nutrition Journal: FDNJ-159. DOI, 10: 2575-7091.

Bijina, B., Chellappan, S., Basheer, S.M., Elyas, K.K., Bahkali, A.H. and Chandrasekaran, M. 2011. Protease inhibitor from Moringa oleifera leaves: Isolation, purification, and characterization. Process Biochemistry, 46(12): 2291-2300.

Carvalho, C.P., Coimbra, J.S.R., Costa, I.A.F., Minim, L.A., Silva, L.H.M. and Maffia, M.C. 2007. Equilibrium data for PEG 4000+ salt+ water systems from (278. 15 to 318. 15) K. Journal of Chemical & Engineering Data, 52(2): 351-356.

Chaiwut, P., Pintathong, P. and Rawdkuen, S. 2010. Extraction and three-phase partitioning behavior of proteases from papaya peels. Process Biochemistry, 45(7): 1172-1175.

Chandi, G.K. and Sogi, D.S. 2007. Functional properties of rice bran protein concentrates. Journal of Food Engineering, 79(2): 592-597.

Falowo, A.B., Mukumbo, F.E., Idamokoro, E.M., Lorenzo, J.M., Afolayan, A.J. and Muchenje, V. 2018. Multi- functional application of Moringa oleifera Lam. in nutrition and animal food products: A review. Food research international, 106: 317-334.

Fee, C.J. and Van Alstine, J.M. 2011. Purification of pegylated proteins. protein purification: principles, high resolution methods, and applications, 149: 339.

Foline, O., Emenike, A.F. and Eunice, B.I. 2011. Comparative analysis of the nutritional composition of three different drying methods of Moringa oleifera leaves. International Journal of Applied Agricultural Research, 6: 131-138.

Gornall, A.G., Bardawill, C.J. and David, M.M. 1949. Determination of serum proteins by means of the biuret reaction. Journal of biological chemistry, 177(2): 751-766.

Hadnađev, M., Dapčević- Hadnađev, T., Lazaridou, A., Moschakis, T., Michaelidou, A.M., Popović, S. and Biliaderis, C.G. 2018. Hempseed meal protein isolates prepared by different isolation techniques. Part I. physicochemical properties. Food Hydrocolloids, 79: 526-533.

Joint, F.A.O. and World Health Organization. 1991. Protein quality evaluation: report of the Joint FAO.

Kiss, E., Szamos, J., Tamas, B. and Borbas, R. 1998. Interfacial behavior of proteins in three- phase partitioning using salt- containing water/ tert- butanol systems. Colloids and Surfaces A: Physicochemical and Engineering Aspects, 142(2-3): 295-302.

Kumar, K.S., Ganesan, K., Selvaraj, K. and Rao, P.S. 2014. Studies on the functional properties of protein concentrate of Kappaphycus alvarezii (Doty) Doty–An edible seaweed. Food chemistry, 153: 353-360.

Kumari, P. 2018. Prevalence of Protein Energy Malnutrition Among Under-Five Children Belonging to Rural Areas of Ambala, Haryana, India. Research & Reviews: Journal of Medicine, 7(3): 14-20.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. nature, 227(5259): 680.

Lakshmi, M.C., Madhusudhan, M.C. and Raghavarao, K.S.M.S. 2012. Extraction and purification of lipoxygenase from soybean using aqueous two-phase system. Food and bioprocess technology, 5(1): 193-199.

Mariod, A.A., Fathy, S.F. and Ismail, M. 2010. Preparation and characterisation of protein concentrates from defatted kenaf seed. Food Chemistry, 123(3): 747-752.

Mbailao, M., Mianpereum, T. and Albert, N. 2014. Proximal and elemental composition of Moringa oleifera (Lam) leaves from three regions of Chad. Journal of Food Resource Science, 3(1): 12-20.

Mgbemena, N.M. and Obodo, G.A. 2016. Comparative Analysis of Proximate and mineral composition of Moringa oleifera root, leave and seed obtained in Okigwe Imo State, Nigeria. J Mol Stud Med Res, 1(2): 57-62.

Mune Mune, M.A., Bakwo Bassogog, C.B., Nyobe, E.C. and René Minka, S.R. 2016. Physicochemical and functional properties of Moringa oleifera seed and leaf flour. Cogent Food & Agriculture, 2(1): 1220352.

Ngaha, N.M.I., Dahlan, I., Massoma, L.D., Mandengue, S., H.4 and Yusuf, A.A. 2016. Comparative proximate analysis of leaves and bark of alchornea cordifolia (Euphorbiaceae). Journal of Agriculture and Environmental sciences. 5(1): 84-90.

Nielsen, S.S. ed. 2010. Food analysis (pp. 139-141). New York: Springer.

Nitsawang, S., Hatti-Kaul, R. and Kanasawud, P. 2006. Purification of papain from Carica papaya latex: Aqueous two-phase extraction versus two-step salt precipitation. Enzyme and Microbial technology, 39(5): 1103-1107.

Nwofia, G.E., Ojimelukwe, P. and Eji, C. 2012. Chemical composition of leaves, fruit pulp and seeds in some Carica papaya (L) morphotypes. International Journal of Medicinal and Aromatic Plants, 2(1): 200-206.

Oduntan, A.O., Olalaye, O. and Akinwande, B.A. 2012. Effect of plant maturity on the proximate composition of Sesamum radiatum Schum leaves. Journal of Food Studies, 1(1): 69-76.

Ogbe, A.O. and Affiku, J.P. 2011. Proximate study, mineral and anti-nutrient composition of Moringa oleifera leaves harvested from Lafia, Nigeria: potential benefits in poultry nutrition and health. The Journal of Microbiology, Biotechnology and Food Sciences, 1(3): 296.

Paula, P., Sousa, D., Oliveira, J., Carvalho, A., Alves, B., Pereira, M., Farias, D., Viana, M., Santos, F., Morais, T. and Vasconcelos, I. 2017. A protein isolate from Moringa oleifera leaves has hypoglycemic and antioxidant effects in alloxan- induced diabetic mice. Molecules, 22(2): 271.

Rodamo, K.M., Fiche, Y.A., Geleto, F.B., Abebe, R.F. and Dangiso, D.D. 2018. Magnitude and Associated Factors of Protein Energy Malnutrition among Children Aged 6-59 Months in Wondogenet District, Sidama Zone, Southern Ethiopia. Journal of Gynecology and Obstetrics, 6(3): 47-55.

Sibt-e-Abbas, M., Butt, M.S., Sultan, M.T., Sharif, M.K., Ahmad, A.N. and Batool, R. 2015. Nutritional and functional properties of protein isolates extracted from defatted peanut flour. International Food Research Journal, 22(4): 1533.

Sreerama, Y.N., Sashikala, V.B., Pratape, V.M. and Singh, V. 2012. Nutrients and antinutrients in cowpea and horse gram flours in comparison to chickpea flour: Evaluation of their flour functionality. Food Chemistry, 131(2): 462-468.

Srinivas, N.D., Rashmi, K.R. and Raghavarao, K.S.M.S. 1999. Extraction and purification of a plant peroxidase by aqueous two-phase extraction coupled with gel filtration. Process Biochemistry, 35(1-2): 43-48.

Teixeira, E.M.B., Carvalho, M.R.B., Neves, V.A., Silva, M.A. and Arantes-Pereira, L. 2014. Chemical characteristics and fractionation of proteins from Moringa oleifera Lam. leaves. Food chemistry, 147: 51-54.

USAID. 2017. Madagascar: Nutrition Profile

Wati, R.K., Theppakorn, T., Benjakul, S. and Rawdkuen, S. 2009. Three-phase partitioning of trypsin inhibitor from legume seeds. Process Biochemistry, 44(12): 1307-1314.