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Isolation of the protein concentrate from the Leaves of *Moringa oleifera* and Study of its Functional properties

عزل مركز البروتين من أوراق شجرة المورينقا أوليفيرا ودراسة خواصه الوظيفية

A Dissertation Submitted in Partial Fulfillment of the Requirements of Master Degree in Chemistry

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Dedication

I dedicate this research work to my loving parents Mohieldin Osman and Faiza basher.

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First and foremost I would like to thank Allah for giving me strength and patience to complete this work.

Many thanks to my supervisor Dr. Essa Esmail Mohamed for his advice, comments, and guidance. I have acquired many things from him.

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Abstract

The purpose of this research work was to isolate protein concentrate from the leaves of *Moringa oleifera* and determine its functional properties. The results of the proximate composition have shown that the crude protein of the leaves was 19.94%. The protein concentrate was extracted by alkaline solution at pH 9, followed by isoelectric precipitation at pH 4.5. The yield of the protein concentrate was 38.02%. The functional properties of *Moringa oleifera* leaves protein concentrate (MOLPC) were investigated. The results have shown that the leaves protein concentrate had high water and oil absorption capacities (5.82±0.47 g/g and 3.87±0.04 g/g, respectively). The Solubility, foaming and emulsifying properties of the protein concentrate were all found to be pH-dependent. The protein concentrate showed minimum solubility at pH 2, 3, and 9. While foaming and emulsifying capacities were both increased at alkaline pHs. The maximum and minimum values of both emulsion and foaming capacities were obtained at pHs 11 and 4.5 respectively.

المستخلص

هدف هذا البحث لإستخلاص مركز البروتين من أوراق شجرة المورينقا أوليفيرا وتحديد خواصه الوظيفية. أوضحت نتائج التركيب الكيميائي أن الأوراق تحتوي علي ١٩.٩٤% من البروتين الخام. ومن ثم تم إستخلاص مركز البروتين بإستخدام محلول قلوي عند الأس الهيدروجيني٩ ثم رسب البروتين بالتعادل الكهربي عند الأس الهيدروجيني 4.5 . وقد بلغت نسبة مركز البروتين المستخلص 38.02%. تم تحديد الخواص الوظيفية لمركز البروتين لأوراق شجرة المورنيقا أوليفيرا. بينت النتائج أن مركز البروتين ذو قابلية كبيرة لإمتصاص الماء والزيت (5.82 g/g و 3.87g/g)على التوالي . كما وجد أن الذوبانية والقابلية لتكوين الرغوة والإستحلاب لمركز البروتين تعتمد على الأس الهيدروجيني بصورة وطيدة. حيث كانت أقل ذوبانية لمركز البروتين عند الأسس الهيدروجينية 2و3 و9. بينما زادت قابلية تكوين الرغوة وسعة الإستحلاب في الأوساط القاعدية. حيث لوحظ أن قيم الحد الأعلى والحد الأدني لكليهما عند الأسس الهيدروجينة ١١و٤,٥على التوالي.

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Introduction and literature review

1.1 Introduction

The problem of protein malnutrition in developing countries cannot be underestimated, as a large number of people in this part of the world still do not have access to cheap protein sources. Additionally, a number of factors, which include economical, environmental, and rapid increase in population in these countries, represent growing pressures for alternative-cheaper and renewable sources of proteins [1,2].

Based on source, food protein could be roughly grouped into animal proteins (e.g. milk and gelatin protein) and plant or vegetable proteins (e.g. peanut protein and wheat protein). The cost and risk factors associated with diseases from animal protein sources make nutritionists to consider alternative plant protein sources for human and feedstock preparation. Although plant proteins are lower in some essential amino acids as compared with animal proteins, however many plants (or vegetables) have attracted great deal of interest in recent years as a source of low cost protein to supplement human diets. In addition, plant proteins are important in food processing and food products development, because they are responsible for many functional properties that influence consumers acceptance of food products [3,4].

Many vegetables are used for the preparation of salads, including green leaves of lettuce, spinach, and Brassica spp. From a nutritional point of view, the consumption of leafy vegetables is recommended as source of functional components such as polyphenols, glucosinolates, dietary fiber and protein [5].

Moringa tree includes thirteen species and belongs to family Moringaceae. *Moringa Oleifera* is most widely cultivated species. It is present in west, east and South Africa, tropical Asia, Latin America, the Caribbean, Florida and the Pacific Islands. In Sudan, it is found in northern and central Sudan. All parts of this tree are edible [6-8].

The interest of utilization of *Moringa oleifera* leaves has widely increased, however few studies have been given to it is protein content. The leaves of this tree have been eaten in

many countries. In Philippines, boiled leaves are commonly used to feed babies [8]. The chemical composition of the leaves of *Moringa oleifera* was reported in previous studies. The results have demonstrated that the protein content is laid between 25% and 28.7% [9,10].

It could be concluded from previously that *Moringa oleifera* leaves are potential and promising source of protein.

1.1.1 Objective

The objective of the present study is to isolate protein concentrate from the leaves of *Moringa oleifera* originated from Sudan and study its functional properties.

1.2 Proteins

Proteins are complex macromolecules that contain a number of amino acid monomers liked by amide bonds. Amino acids are, therefore, the building blocks of polypeptides and proteins, which consist of a central carbon linked to an amine group, a carboxyl group, a hydrogen atom, and a side chain (R groups). R groups can be classified as nonpolar groups, uncharged polar groups or charged polar groups, in which their distribution along the protein backbone renders proteins with distinct characteristics [11]. Proteins are an abundant component in all cells, which vary in molecular mass, ranging from approximately 5000 to more than a million Daltons. They are composed of elements including hydrogen, carbon, nitrogen, oxygen, and sulfur. Twenty α-amino acids are the building blocks of proteins; the amino acid residues in a protein are linked by peptide bonds. Nitrogen is the most distinguishing element present in proteins; generally, proteins rich in basic amino acids contain more nitrogen [12]. Proteins have unique conformations that could be modified by physical, chemical, and enzymatic methods. Modification results in structural or conformational changes from the native structure without alteration of the amino acid sequence. Modifications that change the secondary, tertiary, or quaternary structure of a protein molecule are referred to as denaturation modifications (e.g., heat, acetylation, and Chemical hydrolysis) [11]. Proteins can be classified by their composition, structure, biological function, or solubility properties [12].

1.3 Source of proteins

1.3.1 Animal proteins

Proteins from animal sources contain essential amino acids needed for an adult's diet. The important examples of animal proteins are casein, egg albumin, and Sarcoplasmic. Animal protein is generally associated with high fat content and, because of this, when consumed in large a mounts, it leads to high risks of diseases, including high blood pressure and heart diseases. Despite of this it has a balanced combination of all amino acids; hence, it is called complete protein [13]. The cost and the risk factors associated with diseases from animal protein sources make nutritionists consider alternative plant protein sources for human and feedstock preparation [3,14].

1.3.2 Plant proteins

Plant proteins are amino acid polymers derived mainly from oilseeds (i.e., soybeans) and grains (i.e., wheat and corn), and are usually produced as byproducts of processing oils and starches [11]. Vegetables, legumes, and fruits are good sources of protein. Legume such as peas, beans and lentils have a higher content of protein than vegetables and fruits [13]. Plant

proteins are widely used as major ingredients for food, feed, pharmaceuticals, nutraceuticals, paper coating, textile sizing, and adhesives [11]. Although plant proteins sources are generally cheaper as compared with animal protein sources, these plant proteins supplements are lower in some essential amino acids [4,13].

1.4 Methods of proteins separation

Several separation techniques are used in sequence to purify a protein from a food. In general, the more the separation steps used, the higher the purity of the resulting preparation. The biochemical properties of a protein, such as molecular mass, isoelectric point (pI), solubility properties, and denaturation temperature are important to determine any unusual physical characteristics that will make separation easier [12,15,16].

1.4.1 Separation using Salts

Proteins can be separated into two groups depending on their behavior towards salts. For example, globulins are more or less globular proteins; their electrical charge is evenly distributed across their surface area. Globulins are soluble in distilled water, and can be precipitated by high salt concentrations; while Albumins have molecules with an elongated, rod-like shape with unsymmetrical distribution of electrically charged groups. As a result, albumins are insoluble in distilled water, because the molecules form head-to-tail aggregates held together by electrical forces [17]. Ammonium sulfate [$(NH_4)_2SO_4$] is commonly used, although other neutral salts such as NaCl or KCl may be used to salt out proteins [12]. A number of Studies have reported the use of ammonium sulfate and neutral salts to separate proteins [16,18]. One disadvantage of this method is that large quantities of salt contaminate the precipitated protein and must be remove by dialysis or gel filtration before the protein is re-solubilized in buffer [12].

1.4.2 Solvents fractionation

Proteins can be separated based on their solubility differences in organic solvent–water mixtures. The addition of water-miscible organic solvents, such as ethanol or acetone, decreases the dielectric constant of an aqueous solution and decreases the solubility of most proteins. Organic solvents decrease ionization of charged amino acids, resulting in protein aggregation and precipitation. The optimum quantity of organic solvent to precipitate a protein varies from 5 to 60%. Solvent fractionation is usually performed at 0˚C or below to prevent protein denaturation caused by temperature which results from mixing organic solvents with water [12,17]. A number of studies have reported the use of methanol and acetone in extraction of protein [4,19].

1.4.3 Isoelectric precipitation

The isoelectric point (pI) is defined as the pH at which a protein has no net charge in solution. Proteins aggregate and precipitate at their pI because there is no electrostatic repulsion between molecules. Proteins have different pIs; thus, they can be separated from each other by adjusting solution pH. When the pH of a solution is adjusted to the pI of a protein, the protein precipitates while proteins with different pIs remain in solution. The precipitated protein can be re-solubilized in another solution of different pH [12,17]. Zhong *et al.* [19] have examined the isoelectric and acetone precipitation methods to extract the protein isolates from Caragana korshinskii. The isoelectric precipitation exhibited good performance on functional properties than acetone precipitation. The effect of pI method on protein yield was studied by Stone *et al.* [15] to extract pea protein isolates. The results showed that isolate yield was greater for salt precipitation than isoelectric precipitation.

1.4.4 Heat (denaturation of protein)

Many proteins are denatured and precipitated from solution when heated above a certain temperature or by adjusting a solution to highly acid or basic pHs. Proteins that are stable at high temperatures or at extremes of pH are most easily separated by this technique because many contaminating proteins can be precipitated while the protein of interest remains in solution [12]. Heating between 80-90 C ^{o} was used to coagulate protein concentrates of common leafy vegetables [20].

1.5 Functional properties of vegetable proteins

Proteins in various types of food have unique food functional properties, which have been defined as the physical and chemical properties of protein molecules that affect their behavior in food products during processing, storage, and consumption. The most important protein functional properties in foods include solubility, emulsification, foaming**,** water absorption capacity, and oil absorption capacity. Other functional properties of proteins include gelation and dough formation that are closely related to viscosity [12,21].

1.5.1 Proteins Solubility

Solubility is one of the most popular tests of protein functionality. Many other important functional attributes of proteins are influenced by protein solubility, such as, foaming, emulsification, water binding, and gelation properties. Solubility is dependent on the balance of hydrophobic and hydrophilic amino acids that makeup the protein, especially those amino acids on the surface of the molecule. It is also dependent on the protein/solvent thermodynamic [12]. Protein solubility is influenced by solvent polarity, pH, ionic strength, ion composition, and interactions with other food components, such as lipids or

carbohydrates. Common food processing operations, such as heating, freezing, drying, and shearing, may all influence the solubility of proteins in a food system. The effect of pH on most vegetables protein solubility gave a u-shaped curve, where it is minimum solubility at isoelectric point, then increases above and below of isoelectric point (PI). In contrast, some proteins solubility was found to have low solubility at pH 10 and 8. [12,22-30]. A number of authors have studied the effect of ionic strength on protein solubility. Their results showed that, there was an increase in protein solubility as sodium chloride concentration increased up to 0.5 M, beyond this concentration the solubility decreased [16,31]. Contrariwise, the protein solubility of sweet and bitter lupin increased up to 1.0M sodium hydroxide [32]. Yu *et al.* [33] have examined the effect of processing methods such as heating or roasting on protein solubility of peanut, the result showed that heating or roasting of legumes and seeds reduces protein solubility, due to increase the surface hydrophobicity of the protein. Similarly, it was reported that the heat treatment significantly reduced the protein solubility of rice bran at pH 6, 8, and 10 [34].

1.5.2 Foaming capacity

Foams are coarse dispersions of gas bubbles in a liquid or semisolid continuous phase. It requires energy input during formation and are inherently unstable. Foams are formed through unfolding and absorption of the protein, at the air-water interface, as well as film formation around the air bubbles [12]. Whipping, shaking, and sparging (gas injection) are three common methods of foam formation. Foam volume and foam stability are two important parameters used to evaluate foams. Foam volume is dependent on the ability of a protein to lower surface tension between the aqueous phase and gas bubbles during foam formation. While Foam stability depends on the properties of the protein, film formed around the gas droplets. Foams are found in cakes, breads, marshmallows, whipped cream, meringues, ice cream, mousses, and beer. The extrinsic factors that affect the foaming properties are pH, extraction temperature, carbohydrates, sample concentration, and ionic strength [12,35]. The effect of pH on foaming capacity was investigated in some previous studies. The results indicated that the foaming capacity was increased in acid and alkaline regions, the highest foaming capacity of pigeon pea protein concentrates was found to be at pH 2, while the highest one of walnut protein concentrates and isolates was found to be at pH 11 [16,36,37]**.** In contrast, the decrease in foaming capacity at very acidic and alkaline pH was observed in cashew protein concentrates and isolates [4]. A number of authors have studied the effect of ionic strength on foaming capacity. The results showed that high concentration of ionic

strength decreases foaming capacity, while low concentration of ionic strength increases foaming capacity. This is because low ionic strength improves the solubility, which further affects the foaming capacity [36,38].

1.5.3 Emulsifying capacity

Emulsions are mixtures of two or more immiscible liquids, one of which is dispersed as droplets in the other. The droplets in an emulsion are collectively called the discontinuous or dispersed phase, whereas the liquid surrounding the droplets is the continuous phase. Proteins migrate to the surface of a droplet during emulsion formation to form a protective layer or membrane on the surface, thus reducing interactions between the two immiscible phases. The quality of an emulsion is dictated by many factors including droplet size, droplet size distribution, density differences between the two phases, viscosity of the two phases, electrostatic and steric interactions between molecules at the interface, and thickness, and viscosity of the adsorbed protein layer [12,35]. Emulsifying properties can be evaluated by the protein's emulsion stability (ES) and emulsion activity (EA). The ES is a measure of the stability of the emulsion over a certain time span and EA is a measurement of how much an oil can be emulsified per unit protein. Food emulsions include margarine, butter, milk, cream, infant formulas, mayonnaise, processed cheese, salad dressings, ice cream, and some highly comminuted meat products, such as bologna. Generally the emulsifying capacity of protein are affected by their molar mass, hydrophobicity, conformation stability, charge and physic-chemical factors such as pH, ionic strength, and temperature [35,39]. The effect of pH on emulsifying activity has been studied by a number of authors. The results showed that emulsifying activity is pH dependent, alkaline pHs improve the emulsifying capacity more than acidic pH. This could be due to greater participation of protein in oil–water interfacial reactions brought by alkali- induced formation of more soluble protein through unfolding of polypeptide chains [18,25,37]**.** In contrary, it was reported that the emulsifying capacity of sweet lupin protein concentrates is the same and tend to decreased, as the pH is increased [30]. The effects of temperature and ionic strength on the emulsifying properties of pea and rice bran proteins were reported. The results have shown that as the temperature increases the emulsifying properties decreases. On the other hand, addition of ionic strength (NaCl) increased the emulsifying capacity of pea protein and rice bran protein [34,35].

1.5.4 Proteins Gelation

Protein gels are made by treating a protein solution with heat, enzymes, or divalent cations under appropriate conditions. While most food protein gels are made by heating protein solutions, some can be made by limited enzymatic proteolysis (e.g., chymosin action on casein micelle to form cheese curd), and some are made by addition of the divalent cations Ca^{2+} or Mg^{2+}. Proteins are transformed from the "soluble" state to a cross-linked "gel-like" state in protein gelation [12]. Protein gels are thermally reversible, while some protein gels are thermally irreversible. The stability of a protein gel is affected by a variety of factors, such as the nature and concentration of the protein, temperature, rates of heating and cooling, pH, ionic strength, and the presence of other food constituents. Studies of gelation properties of soy and pea protein showed that strong gel form at low temperature and high protein concentration [12,35].The least gelation concentration (LGE) which is defined as the lowest protein concentration at which gel remained in inverted tube was used as an index of gelation capacity. The lower the LGE is the better the gelating ability of the protein ingredient [21,36,38].

1.5.5 Water and oil absorption capacities

Oil and water absorption capacities are defined as the ability of protein to bind water and oil [34].The interactions of water and oil with protein are very important in food system because of their effects on the flavor and texture of food [33]. Intrinsic factors affecting water-binding capacity of food protein include size, shape, steric factor, conformational characteristic, hydrophilic-hydrophobic balance of amino acid in protein molecules as well as lipids, carbohydrates and tannins associated with protein [34,37]. Food processing methods such as roasting and fermentation have important impacts on the protein conformation and hydrophobicity. The roasting of peanut protein reduced both water and oil capacities of peanut flour. This could be due to irreversible denaturtion caused by roasting which might destroy both hydrophilic and hydrophobic group of peanut protein. On the other hand, fermentation increased water absorption capacity and oil absorption capacity of raw, and roasted peanut flours [33].

1.6 Uses of plant proteins

Vegetables and legume proteins have been used widely in replacing parts of wheat flour in bread and cookies. Vegetable proteins have also been used as an alternative protein source for animal and fish. Abobaker *et al.* [40] used the leaf protein concentrates from some Egyptian groups as dietary supplement to improve the quality. The results indicated that the addition of protein concentrate of tomato leaves to the dehydrated vegetable soup in proportion higher

than 7.5% was unacceptable, while that containing 7.5 was acceptable and the sample containing 5% was accepted as well as the standard (the sample without addition of protein). In addition, the meat of sausage was replaced by broad bean and leaf protein. The result of taste tasting that was carried out on fired product indicated that the leaf protein containing more than 15% was unacceptable, while those containing lower level were acceptable.

Soy proteins are used as processing aids to bind and emulsify water and fat in making processed meats, as meat replacement in texturized form, and the protein solutions pumped or absorbed into meat tissue. The same technology was applied to poultry and fish. Up to 12% of soy flour, soy protein concentrates or soy protein isolates were used spaghetti with meatballs or Salisbury steak, and up to 30% hydrated soy protein was permitted in commercially made hamburger. Soy protein was used in bakery products and cakes. The results showed that the use of soy flour improved the crumb body and resiliency, crust color, and toasting characteristics of breads, while usage of defatted soy flour in cake at (3-6%) produces smoother batter with more distribution of air cells [41]. Rapeseed protein concentrates and isolates were incorporated into wheat dough to obtain loaf volume and crust color. The results indicated that the bread containing 5% rapeseed protein concentrates and isolates decreased the loaf volume by 10% to 15% and 20% respectively. Crust color of isolates and concentrates containing bread was slightly darker than that of control [42]. Moringa leaves were used as alternative protein source for Nile tilapia. The results showed that the fish fed with higher levels of Moringa (20% and 30% of protein base) was inferior to those fed with the control diet and the diet containing 10% Moringa leaf meal [9].

1.7 *Moringa Oleifera*

1.7.1 Taxonomy

Kingdom: *Plantae.*
Class: *Magnoliopsida.*
Family: *Moringaceae.*
Order: *Brassicales.*
Genus: *Moringa Adans.*
Species: *Moringa Oleifera Lam.* [43]

There are about 13 species of Moringa trees in the family *Moringaceae*. *Moringa Oleifera* is most widely cultivated species. The plant is known by many of names such as, mother's best

friend, malunggay, benzolive tree, horseradish tree, clarifier tree, ben oil tree, and drumstick tree [8,44].

Moringa tree is small, fast- growing, deciduous tree that grows up to 7m high, with light and smooth bark, large lenticels, and soft wood. Leaves imparipinnate, rachis 12-25 cm, pubescent, with 2-6 pairs of pinnules, 6-3 cm long, with 3-5 pairs of leaflets, 1-2 cm long and slightly larger terminal leaflet. The basal pair of the leaflets may be tri-pinnate. Leaflets obviate, pale green. Flowering during the dry season, in panicles, sweet-scented, cream colored, 5 petals unequal and slightly larger than sepals. Fruits in pods of triangular cross section, approximately 30-50 cm long or more, containing round, black (3-winged), oily seeds [6,8].

1.7.2 Distributions

The tree is native to India and Arabia. It is an important crop in Ethiopia, Philippines, and Sudan. In addition, it is being grown in West, East and South Africa, tropical Asia, Latin America, the Caribbean, Florida and the Pacific Islands. In Sudan, it is found in northern and central Sudan [6,7].

1.7.3 Propagation

Moringa Oleifera can be originating from seeds or cuttings. Cuttings should be taken from woody parts of the branches with more than 1 m length, and then planted in field. Direct seeding, grows rapidly. The life span is about 20 years [6].

1.8 Chemical composition of *Moringa oleifera* leaves

Different parts of this plant contain profile of important minerals and are good source of protein, vitamins, ß-carotene, amino acid, and various phenolics. *Moringa* leaves have been reported to be a rich source of β-carotene, protein, vitamin C, and minerals [45,46].The ash, moisture, fiber, and protein of leaves have been analyzed in previous studies. The ash was found to be high ranged from 7 to 11% which indicates that the leaves are rich source of minerals. The moisture content was less than 10%. The leaves contained high yield of crude protein 25% and 28.7% [9,10,46].

1.9 Uses of *Moringa oleifera* leaves

Moringa is one of the very valuable multipurpose trees for semiarid areas. It provides wind protection, shade, and support for climbing garden plants. All the parts of Moringa tree which includes bark, pods, leaves, nuts, seeds, roots, and flowers are edible [6,44]. Seeds with high oil content up to (38%) produce sweet, non-sticking oil used in preparation of food [6]. High percentage of oleic acid in the seeds oil was reported to be desirable in terms of nutrition and high stability cooking and frying oil [47].The cake from seed is used for purify drinking

water. In Sudan, seed crude extract is used instead of alum by rural women to treat the highly turbid Nile water. In addition, seeds have specific protein fractions for skin and hair care [45]. The seedpods are picked and eaten like green beans, taste bitter and are only used as condiment. The flowers provide good honey [6] and sometimes it is steeped in boiling water to yield fragrant tea [48].

The roots have flavor of horseradish and are eaten as vegetables in East Africa. It is also used in water purification. The bark has coarse fiber and exudes a reddish gum (ben gum). In addition, it is used for tanning hides, and when beaten, produce a fiber for ropes [6,49].

Leaves are edible and used fresh or dried and then ground into powder [6]. People in many countries boil the tiny leaflets and eat them like spinach. In Philippines, boiled leaves are commonly used to feed babies [8,47]. Leaves are also good source of natural antioxidant due to presence of various types of antioxidant compounds such as ascorbic acid, flavonoids, phenolics, and carotenoids [45]. In addition, it has medicinal use in maintaining a healthy liver and gall bladder function as well as for other medicinal applications due to chlorogenic acid [46]. Moringa leaves have been also used as protein supplement for fish such as Nile tilapia [9].

Chapter Two

Materials and methods

2.1 Collection and pretreatments of the sample

Moringa oleifera leaves were collected from Alsalha in Omdurman (Khartoum state-Sudan), cleaned thoroughly by washing with tap water and shade dried. The dried leaves were ground to powder by using a grinder.

2.2 Chemicals

Hydrochloric acid (Assay = 35-38%, density = 1.18 g cm^{-3} at 20 ˚C, purchased from Loba Chemie, India). Sodium hydroxide (Assay = 97.0%, purchased from Central Drug House, India). Kjeldahl catalysis tablets copper (purchased from Chemicals ltd Poole, England). Bromo-cresol green (purchased from Fisher Scientific Company, U.S.A). Methyl red (purchased from Hopkin and Williams L.T.D). Sulfuric acid (Assay = 90.0%, purchased from Loba Chemie). Acetic acid (Assay = 99.7%, density = 1.048-1.050 g cm^{-3}, purchased from Oxford Lab Chem, India). Tri-chloro acetic acid (Assay = 99.0%, purchased from SD Fine–Chem. Limited, Mumbai). Nitric acid (Assay = 69-72 %, density = (1.41- 1.42) g cm^{-3}, purchased from Trust Chemical Laboratories, India).

2.3 Proximate composition

2.3.1 Determination of moisture content

2.0g of the sample was weighed in a pre-weighed porcelain crucible and placed into an oven which was set at 105˚C for 18 hours. The crucible was taken to a desiccator, cooled, and reweighed. The moisture content was calculated using the following equation:

$$\text{Moisture \%} = \frac{w2-w1}{w2} X100 \quad \ldots\ldots (2.2)$$

Where w_1 is the weight of sample and crucible after drying and w_2 is the original weight of sample.

2.3.2 Determination of ash content

2.0g of the sample were weighed into a pre-weighed porcelain crucible. The crucible was then placed in a temperature controlled furnace and heated up to 550 ˚C for 3 hours The crucible was removed from the furnace, transferred to a desiccator, left to cool, and reweighed again. This process was repeated several times until constant weight was attained. The ash content was calculated using the following equation:

Ash % = $\frac{w2-w1}{w2}$ X100 …….. (2.1)

Where w_1 is the weight of the sample and the crucible after combustion and w_2 is the original weight of sample.

2.3.3 Determination of fiber content

2.0g of the sample were weighed into a conical flask and 100 mL of acid fiber solution (acetic acid, nitric acid, tri-chloro acetic acid, distilled water) was added. The mixture was refluxed for 40 min at boiling temperature. The solution was filtered and the filtrate was transferred to a porcelain crucible and heated in an oven for 18 hour at 105˚C. The crucible was cooled, reweighed, and placed into a furnace at 550°C for 3 hours. Finally, the crucible was cooled to room temperature and reweighed. The fiber content was calculated using the following equation:

Fiber % = $\frac{w2-w1}{w}$ X100 …… (2.3)

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

2.3.4 Determination of crude protein

1.0g of the sample was weighed and placed into the digestion flask. 5.0g of Kjeldahl catalysis tablets copper was added to the flask which followed by careful addition of 20 mL of sulfuric acid. The flask was placed on a digestion rack, heated for 3 hours, and cooled. 200 mL of distilled water were added continuously to the content of the flask. 25 mL of boric acid were placed into a distillation flask and 3-4 drops of methyl red/bromo-cresol green were added. The flask was connected to the distillation apparatus and the content of the conical flask were distilled until 100 mL of the distillate were collected. Finally, the distillate was titrated against 1.0 N hydrochloric acid. The percent of nitrogen content (N) was calculated using the following equation:

N % = $\frac{\text{titration volume X diluton factor X normality of std acid X 14 X 100}}{\text{1000 X weight of the sample}}$ ……. (2.4)

Crude protein % = N x 6.25 (nitrogen conversion factor…….. (2.5)

Where (14) is the atomic weight of nitrogen.

2.4 Extraction of protein concentrate

90g of the powdered leaves were suspended in a180mL-distilled water (1:20 w/v). The suspension was stirred for 1 hour using a magnetic stirrer while adjusting the PH at 9.0 with sodium hydroxide solution (1.0M). The mixture was centrifuged for 15 min at room temperature. The supernatant was transferred into a beaker and stirred for 30 min, and the pH

was adjusted to 4.5. The sediment protein slurry was washed three times with distilled water by centrifuging for 10 min, and finally the pH of the slurry was adjusted to 7.0. The slurry was kept for overnight in a freezer and dried using a freeze drier [37].

2.5 Determination of the functional properties of protein concentrate

2.5.1 Water solubility

200mg of the sample were dispersed in 20mLdistilled water and the pH of the mixture was adjusted to 2, 3, 4, 5, 7, 9, 10, 11 with 1.0 M sodium hydroxide and 1.0M hydrochloric acid. The mixture was stirred at room temperature for 30 min and centrifuged for 20 min. The sediment of protein was dried and weighed. The weight of soluble protein was determined by weight difference between the original sample and residual protein. Protein solubility was calculated by the following equation:

$$\text{solubility } \% = \frac{\text{the weight of souble protein}}{\text{the weihgt of sample}} \text{X100} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (2.6)$$

2.5.2 Fat absorption capacity (FAC)

1.0g of the sample was weighed into 50mL pre-weighed centrifuge tube and thoroughly mixed with 10mL of groundnut oil. The sample was allowed to stand for 30min. The protein-oil mixture was centrifuged for 20 minutes and the supernatant was carefully removed, and the tube was reweighed. Fat absorption capacity (FAC) (gram of oil per gram of protein) was determined by:

$$\text{FAC} = \frac{w2 - w1}{w} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (2.7)$$

Where w is the weight of the dry sample, w_1 is the weight of the tube plus the dry sample and w_2 is the weight of tube plus the sediment[37].

2.5.3 Water absorption capacity (WAC)

1.0g of the sample was weighed into 50 mL pre-weighed centrifuge tube and 10 ml of distilled water were added in small increments to the tube under continuous stirring with glass rod. The sample was allowed to stand for 30 min, and then was centrifuged for 20 min. The supernatant was carefully removed, and tube was reweighed. Water absorption capacity (WAC) expressed as gram of water per gram of protein, was calculated by:

$$\text{WAC} = \frac{W2 - W1}{W} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (2.8)$$

Where W is the weight of the dry sample, W_1 is the weight of the tube plus the dry sample and W_2 is the weight of tube plus sediment[37].

2.5.4 Foaming capacity (FC)

500 mg of the sample were dispersed in 50 mL distilled water. The pH of the protein solution was adjusted to 2, 3, 4, 5, 7, 9, 10, 11 with1.0M sodium hydroxide and 1.0M of hydrochloric acid. The solution was whipped for 2 min in graduated tube. The volume was recorded before and after homogenization. Foaming capacity (FC) (the volume (%) increased due to stirring) was calculated by:

$$FC\ \% = \frac{V_2 - V_1}{V_2} X100 \quad \text{..... (2.9)}$$

Where V_2 is the volume after whipping and V_1 is the volume before whipping [37].

2.5.5 Emulsifying capacity (EC)

1.0g of the sample was homogenized for 1min at room temperature in 25 mL distilled water. The pH of the solution was adjusted to2, 3, 4, 5, 7, 9, 10, 11 with 1.0M of sodium hydroxide and 1.0M of hydrochloric acid. The protein solution was mixed with 25 mL of groundnut oil followed by homogenization for 1min. Finally, the emulsion was centrifuged for 5min, and emulsifying activity was determined by:

$$EC\ \% = \frac{\text{Hieght of emlisified layer}}{\text{Hieght of the content of the tube}} X100 \quad \text{..... (2.10)}$$

Chapter three

Results and discussion

3.1 Proximate composition

The chemical composition of the leaves of *Moringa oleifera* is shown in table (3.1). As can be observed from the table, the ash content is 9.30%, which indicates that the leaves have a high mineral content. Previous study by Coppin has shown that *Moringa oleifera* leaves from Sub-Saharan Africa is a rich source of minerals. Calcium was found to be 161 mg/100g, potassium 52 mg/100g, magnesium 60mg/100g, iron 40.65mg/100 g, manganese 14.60 mg/100 g and copper 0.95 mg/100 g [46]. Furthermore, the crude protein of the leaves reaches 19.94 %, which is lower than the reported values by Estelamar *et al.* [46] and Richter *et al*. [9], which are 28.65% and 25 % respectively. These differences could be attributed to the differences in locations and climatic conditions. The moisture content was 5.93%, which lies in the range of the previous studies of chemical composition of *Moringa oleifera* leaves (less than 10%) [9,10,46].

Table 3.1: Proximate composition and functional properties of *Moringa oleifera* leaves.

Parameter	Quantity
Ash%	9.30±0.12
Fiber%	13.94±0.01
Moisture%	5.92±.03
Crude protein%	19.94±0.16
Protein concentrate content %	38.02±3.9
Water absorption capacity(g/g)	5.82±0.47
Oil absorption capacity(g/g)	3.87±0.04

3.2 Functional properties of protein concentrate of *Moringa oleifera* leaves (MOLPC)

3.2.1 The water and oil absorption capacity (WAC and OAC)

The water and oil absorption capacities of the protein concentrate of the leaves are presented in table (3.1). As can be seen from the table, the water absorption capacity is 5.82 g water/g PC. This value is higher than the reported values for common leafy vegetables protein concentrates (PC). Literature survey have shown that the water absorption capacities of

protein concentrates of *S. Africana*, *A. hybridus*, and *T. occidentalis* were 1.491, 2.71, and 1.733 g water/g PC respectively. However, the above value is closely similar to the one reported for protein concentrate of *V. amygdalina*; 4.715 g water/g PC [20]. High water absorption capacity of protein concentrate of *Moringa oleifera* leaves may be attributed to many factors that enhance WAC such as amino acids composition, surface hydrophobicity/polarity, and carbohydrates content. High water absorption capacity helps to reduce moisture loss in package bakery goods and maintain freshness and moist mouth feel of baked foods [16,33]. On the other hand, fat absorption capacity of protein concentrate of *Moringa oleifera* leaves is 3.87 g oil/g PC. This is rather higher than the values reported for PCs of the leaves of S. Africana, A. hybridus, V. amygdalina, and T. occidentalis which are laid in the range 0.19 to 0.47 g oil/g PC [20]. However, this value is noticeably lower than the reported values for rice bran PC, 9.18 ± 0.74g oil/g PC, and Basmati Rice386, 8.14 ± 0.72 g oil/g PC, and HBC19, 8.14 ± 0.72 g oil\g PC [50]. It has been reported that the fat absorption capacity of protein is influenced by the chemical composition of amino acids. Xiaoying et al. [37] reported that hydrophobicity improves fat absorption capacity. Fat absorption capacity is a critical factor in flavor retention [21,33].

3.2.2 Effect of pH on Protein Solubility of MOLPC

The solubility of protein concentrate of *Moringa oleifera* leaves was studied at different pHs and are shown in figure (3.1).

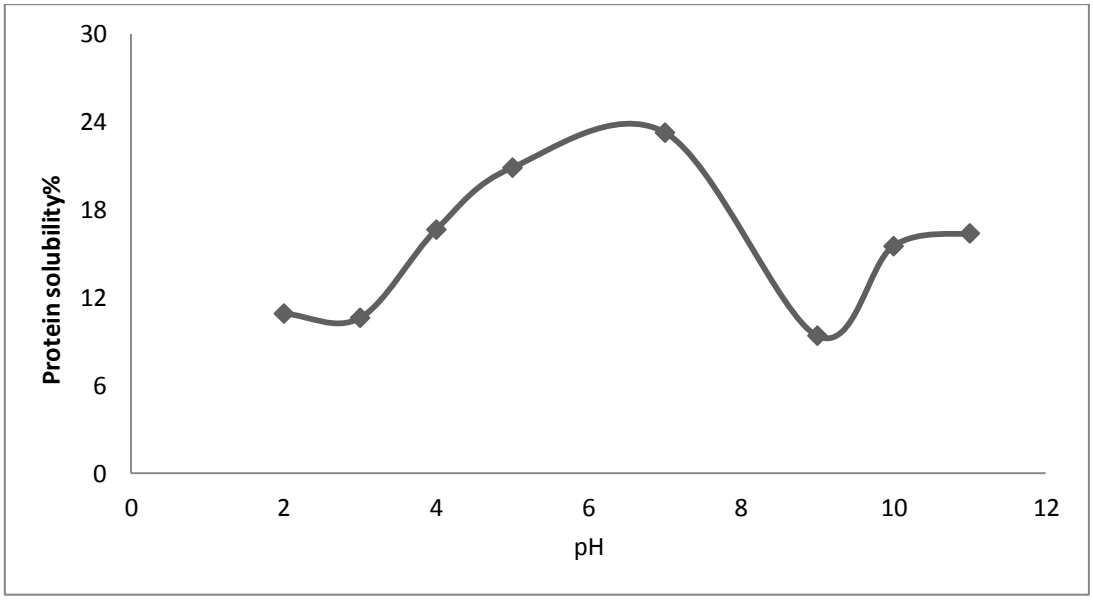

Figure 3.1: Variation of protein solubility of MOLPC with pH

As can be noticed from the figure that the solubility is pH dependent. The minimum solubility occurred at pH 9, 2, and 3 whereas the maximum protein solubility was observed at pH 7 and 5. Similar results were reported by Aletor *et al.* on protein concentrates of common leafy vegetables [20]. In another study, Akintayo *et al*. [28] have studied the effect of pH on protein solubility of akee pulp and seed flours. The minimum solubility was found to be at pH 3 and 7 for seeds and for pulp at 4 and 10. This indicates that two different isolates might be possible, at pH 4.0 and 10.0, for the pulp extraction, while, for the seeds, one would be extractable at pH 3.0 and another at pH 7.0.

3.2.3 Effect of pH on Foaming and emulsifying capacities of MOLPC

The effect of pH on foaming and emulsifying capacities of MOLPC were studied and the results are displayed in figures (3.2) and (3.3) respectively. As it appears from the figures, the foaming and the emulsifying capacities are both pH dependent. The alkaline pHs were found to improve the emulsifying and foaming capacities more than the acidic pHs. The maximum foaming and emulsifying capacities were observed at pH11. Similar results were obtained for other protein concentrates for the emulsifying capacity. This could be due to greater participation of protein in oil–water interfacial reactions brought on by alkali-induced formation of more soluble protein through unfolding of polypeptide chains [18,39]. High foaming capacity of protein could be attributed to flexibility of protein. In previous studies, maximum foaming capacity was obtained at alkaline pH and explained by an increase of net charge leads to repulsion, which facilitates the flexibility of protein molecules and thus increased foaming capacity [37,38].

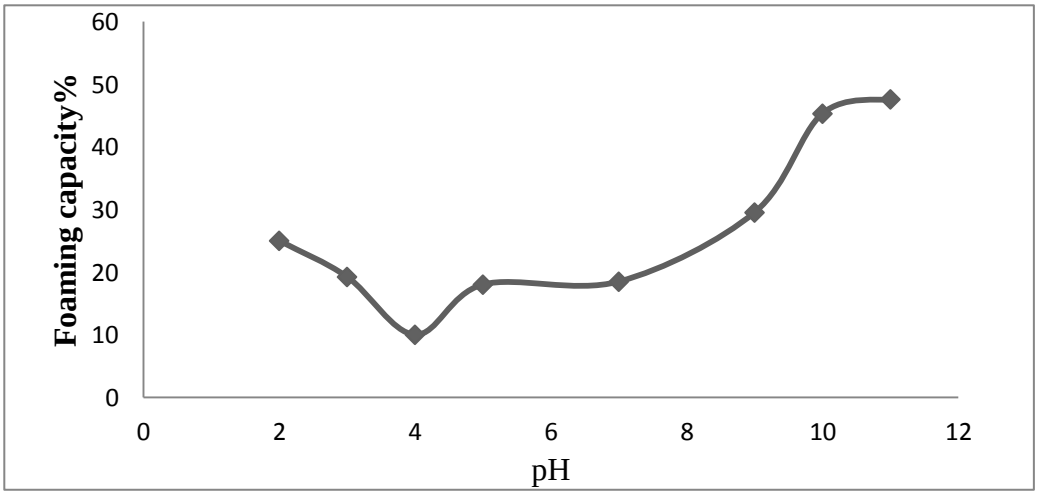

Figure 3.2: Variation of foaming capacity of MOLPC with pH

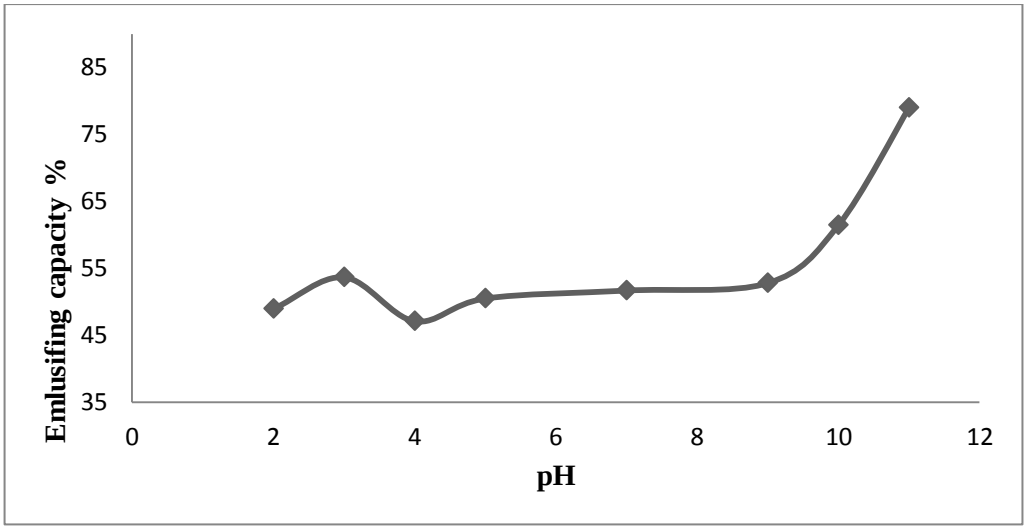

Figure 3.3: Variation of emulsifying capacity of MOLPC with pH

Conclusion

In the present study, protein concentrate was isolated from the leaves of *Moringa oleifera* using alkaline extraction method at pH 9, followed by isoelectric precipitation at pH 4.5. The yield of the protein concentrate reaches 38.02%. The MOLPC has high water absorption capacity compared to common leafy vegetable protein concentrates. The solubility, foaming and emulsifying capacities were found to be pH dependent. Moreover, alkaline pHs were found to improve foaming and emulsifying capacities more than acidic ones.

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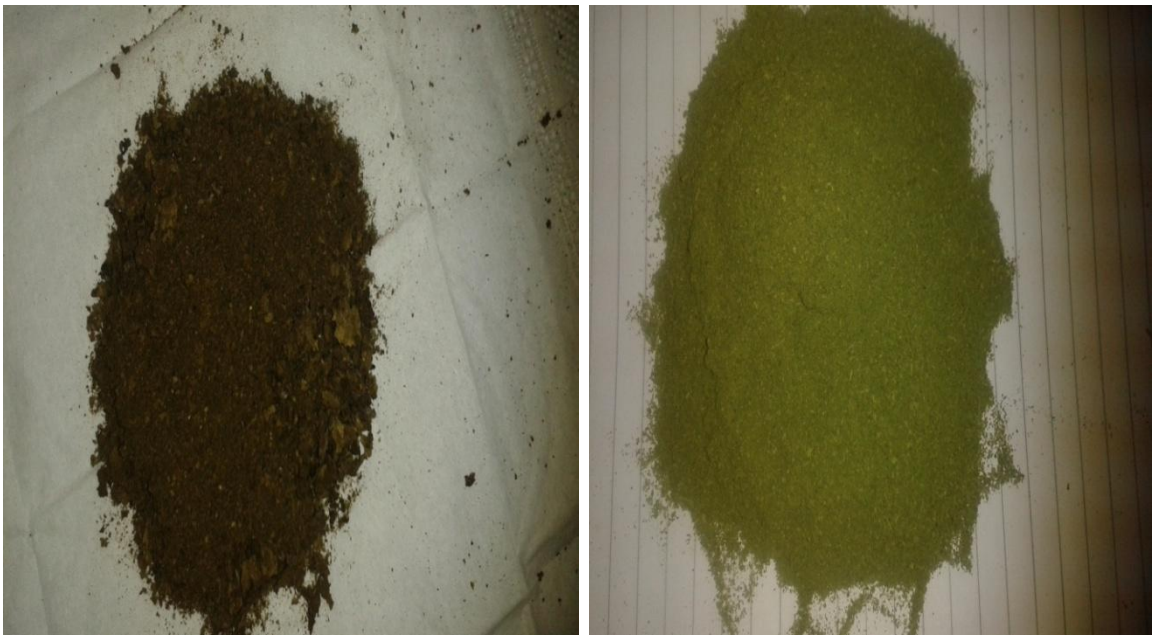
Appendix

Figure A.1: *Moringa oleifera* tree (The photo was taken from Omdurman- Wadnobawi)

Figure A.2: *Moringa oleifera* leaves

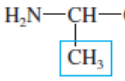
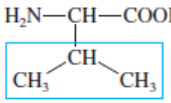
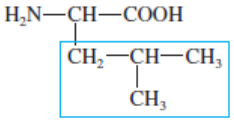
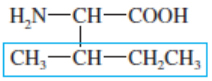
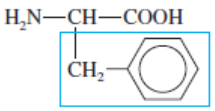
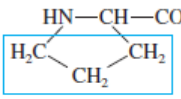
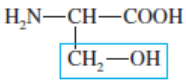
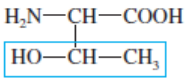


X **Y**

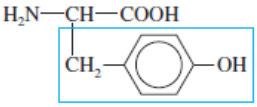
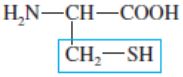
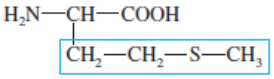
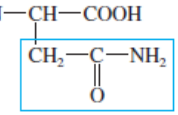
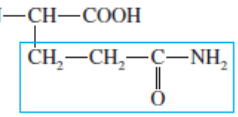
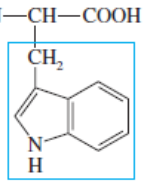
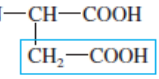
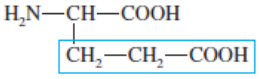
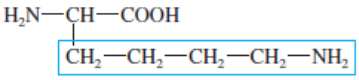
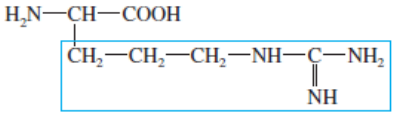
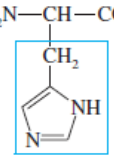
Figure A.3: (Left) Protein concentrate of *Moringa oleifera* leaves and (right) powder of *Moringa oleifera* leaves

Amino Acids Structures

The Standard Amino Acids

Name	Symbol	Abbreviation	Structure	Functional Group in Side Chain	Isoelectric Point
side chain is nonpolar, H or alkyl					
glycine	G	Gly		none	6.0
alanine	A	Ala		alkyl group	6.0
*valine	V	Val		alkyl group	6.0
*leucine	L	Leu		alkyl group	6.0
*isoleucine	I	Ile		alkyl group	6.0
*phenylalanine	F	Phe		aromatic group	5.5
proline	P	Pro		rigid cyclic structure	6.3
side chain contains an —OH					
serine	S	Ser		hydroxyl group	5.7
*threonine	T	Thr		hydroxyl group	5.6

The Standard Amino Acids (*continued*)

Name	Symbol	Abbreviation	Structure	Functional Group in Side Chain	Isoelectric Point
tyrosine	Y	Tyr		phenolic —OH group	5.7
side chain contains sulfur					
cysteine	C	Cys		thiol	5.0
*methionine	M	Met		sulfide	5.7
side chain contains nonbasic nitrogen					
asparagine	N	Asn		amide	5.4
glutamine	Q	Gln		amide	5.7
*tryptophan	W	Trp		indole	5.9
side chain is acidic					
aspartic acid	D	Asp		carboxylic acid	2.8
glutamic acid	E	Glu		carboxylic acid	3.2
side chain is basic					
*lysine	K	Lys		amino group	9.7
*arginine	R	Arg		guanidino group	10.8
*histidine	H	His		imidazole ring	7.6