The Effect of Fermentation on Nutritional Quality of 
*Cassia obtusifolia* Leaves (kawal)

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DEDICATION

To my parents

For their everlasting love, care, hope and dedication

For their patience and perseverance

Assuring my every success.

To my loyal teachers,

Past, present and future.

To all my friends.

With love and respect

Mutasim
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LIST OF CONTENTS

Page
ACKNOWLEDGEMENT......................................................... i
LIST OF CONTENTS........................................................... ii
LIST OF TABLES............................................................. vii
ABSTRACT................................................................. viii
ARABIC ABSTRACT........................................................ x

CHAPTER ONE

INTRODUCTION............................................................ 1

CHAPTER TWO: LITERATURE REVIEW

2.1. General characteristics of fermented foods.................................4
   2.1.1 Types of fermentation............................................. 5
   2.1.2 Fermented food in nutrition..................................... 5
   2.1.3 Use of fermentation as a food preservation method.................6

2.2. Indigenous fermented foods................................................ 7
   2.2.1 Shelf life of indigenous fermented foods...........................9
   2.2.2 Toxicity and safety of indigenous fermented foods............... 10

2.3 Cassia obtusifolia......................................................... 11
   2.3.1 kawal preparation method....................................... 11
   2.3.2 Utilization of sicklepod......................................... 13
2.3.3 Medical uses of kawal…………………………………………………13
2.3.4 Nutritive value of Cassia obtusifolia…………………………………14

2.3.5 Kawal as meat substitute……………………………………………15

2.4 The chemical composition of Cassia obtusifolia leaves and kawal…………………………………………………………………..15
2.4.1 Fiber content…………………………………………………………….15
2.4.2 Fat content…………………………………………………………….16
2.4.3 Protein content…………………………………………………………17
2.4.4 The ash content ……………………………………………………..18

2.5 Protein fractionation………………………………………………………19

2.6 In vitro protein digestibility……………………………………………….20

2.7 Amino acid composition…………………………………………………..20

2.8 The mineral content……………………………………………………21
2.8.1 Potassium and sodium content………………………………………23
2.8.2 Calcium, phosphorus and magnesium content…………………..23
2.8.3 Iron content……………………………………………………………..24

2.9 Anti-nutritional factors…………………………………………………..24
2.9.1 Phytic acid ………………………………………………………………..25
2.9.2 Tannin content…………………………………………………………….26
2.9.3 Total polyphenols………………………………………………………..27

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CHAPTER THREE: MATERIALS AND METHODS
3.1 Materials................................................................................................. 29
3.3 Methods................................................................................................. 29
3.4 Proximate composition ................................................................. 29
  3.4.1 Crude fiber ................................................................................. 29
  3.4.2 Fat content ................................................................................ 30
  3.4.3 Protein content ........................................................................ 30
  3.4.4 Ash content.............................................................................. 31
3.5 Protein fractionation due to solubility......................... 32
  3.5.1 Determination of water soluble proteins (Albumins) ............... 32
  3.5.2 Determination of salt soluble proteins (Globulins) ............... 33
  3.5.3 Determination of alcohol soluble proteins (Prolamins) ....... 33
  3.5.4 Determination of alkali-soluble proteins (Glutelins) ........... 34
  3.5.5 Protein content of insoluble fraction...................................... 34
3.6 In vitro protein digestibility...................................................... 34
3.7 Amino acid analysis................................................................. 35
3.8 Determination of mineral content ........................................ 36
3.9 determination of Anti-nutritional factors.......................... 36
  3.9.1 Determination of phytic acid content................................. 36
  3.9.2 Determination of tannin content ........................................... 38
  3.9.3 Total polyphenol (TP) determination...................................... 39
  3.9.4 Standard curve preparation................................................... 39
3.10 statistical analyses................................................................. 40
CHAPTER FOUR: RESULTS AND DISCUSSION

4.1. Chemical composition of green leaves of *Cassia obtusifolia* and kawal………………………………………………………………………………41
  
  4.1.1 Fiber content…………………………………………………………41
  4.1.2 Fat content…………………………………………………………41
  4.1.3 Protein content……………………………………………………42
  4.1.4 Ash content………………………………………………………43

4.2 Protein fractionation of green leaves of *Cassia obtusifolia* and kawal…………………………………………………………………………43
  
  4.2.1 Albumin content………………………………………………43
  4.2.2 Globulin content………………………………………………45
  4.2.3 Prolamin content………………………………………………45
  4.2.4 Glutelin content………………………………………………45
  4.2.5 Insoluble protein content……………………………………46

4.3 *In vitro* protein digestibility of green leaves of *Cassia obtusifolia* and kawal……………………………………………………………………46

4.4 Amino acid content……………………………………………………49

4.5 Minerals content of green leaves of *Cassia obtusifolia* and kawal………………………………………………………………………………49
  
  4.5.1 Sodium content………………………………………………….49
  4.5.2 Calcium and phosphorous content…………………………….51
  4.5.3 Magnesium and manganese content…………………………51
  4.5.4 Iron and zinc content…………………………………………52

4.6 Anti-nutritional factors of green leaves of *Cassia obtusifolia* and kawal…………………………………………………………………………52
CHAPTER FIVE: CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusion ........................................................................................................56
5.2 Recommendations ..........................................................................................57
REFERENCES ........................................................................................................58
LIST OF TABLES

1. Chemical composition of green leaves of *Cassia obtusifolia* and kawal (on dry matter basis) ............................................................ 44
2. Protein fractionation of green leaves of *Cassia obtusifolia* and kawal (on dry matter basis) ............................................................ 47
3. *In vitro* protein digestibility of green leaves of *Cassia obtusifolia* and kawal ........................................................................ 48
4. Amino acid contents of green leaves of *Cassia obtusifolia* and kawal (on dry matter basis) ............................................................ 50
5. Mineral content of green leaves of *Cassia obtusifolia* and kawal (no dry matter basis) ................................................................. 53
6. Anti-nutritional factors of green leaves of *Cassia obtusifolia* and kawal (on dry matter basis) ............................................................. 55
ABSTRACT

In this study *Cassia obtusifolia* leaves and Kawal were obtained from Nyala, west Sudan. The objective of this study was to evaluate the effect of fermentation on chemical composition, protein fractions, *in vitro* protein digestibility, amino acid contents, mineral content, phytic acid, tannin, and total polyphenol of *Cassia obtusifolia* leaves. Fermentation was found to cause significant changes in *Cassia obtusifolia* leaves major nutrients; fat content increased from 3.50 to 4.50 %, protein content from 24.81 to 35.13 %, ash content from 13.67 to 18.00 %, and fiber content decreased from 13.04 to 12.90 %.

The effect of fermentation on protein fractions showed an increase in globulin from 58.52 to 63.38 %, albumin 12.59-14.43 %, prolamin 8.69-13.83 %, glutelin 5.03-8.32 %, and a decrease in insoluble protein from 17.81 to 5.41 %. Globulin and albumin are major fraction of kawal protein.

The *in vitro* protein digestibility significantly increased from 49.43 to 61.87 %. Fermentation was found to cause significant changes in amino acid composition; valine increased from 946.6 to 1086.9, methionine 127.188 - 147.588, isoleusine 737.388-850.813, leucine 1322.275-1474.613, and threaonine decreased from 664.113 to 574.788, phenylalanine 768.225-96.313, lysine 878.725-766.61, histidine 525.7-407.025, mg/100g.

Also, the fermentation caused significant change in mineral content, it showed an increase in Ca from 3.87 to 4.17, P 0.27-0.29 g/kg, Mn 75.33-112.33
mg/kg, and decrease in Na from 1.42 to 0.88, Mg 0.37-0.46 g/kg, Fe 533.67-84.33, Zn 536.00-84.67 mg/kg. Fermentation was found to cause decrease in anti-nutritional factors. Tannin content decreased from 2.39 to 2.24 %, phytic acid 649.13-340.92 mg/100g and total polyphenol 4.77-3.80 %.
الملخص الدراسة

اجريت هذه الدراسة على عينة من اوراق نبات الكول، وعينة من الكول، تم الحصول على العينات من نبات غرب السودان، وكان الغرض من الدراسة معرفة تأثير التخمير على المكونات الكيميائية، تجزئة البروتين، نسبة البروتين المهمضون، الاحماض الأمينية، المعادن، حمض الفايتيك، التانين، واليثالي فينول لأوراق نبات الكول.

وجد أن التخمير قد أظهر تغيرات واضحة للمعالج في المكونات الكيميائية الرئيسية لأوراق نبات الكول حيث ارتفعت معنوي نسبة الدهون من 3.50 الى 4.50 %، نسبة البروتين من 24.81 الى 35.13 %، نسبة الربوتن من 13.67 إلى 18.00 %، والمقابل قلت نسبة الألياف من 13.04 إلى 12.90 %.

تأثر التخمير على نسبة تجزئة البروتين دلت على زيادة معنوية في نسبة القلوبيولين من 58.52 الى 63.38 %، الأليبيومين 12.59-14.43 %، البرولامين 8.69-13.83 %، القلوتيلين 5.03-8.32 %، ونقصان نسبة البروتين غير الذائب من 17.81 الى 5.41 %.

الإجزاء الأكثر وجوها في بروتينات الكول.

اظهرت النتائج أن نسبة البروتين المهمضوم ازدادت معنوي بعد التخمير من 43.43 الى 61.87 %.

وجد أن التخمير قد أظهر تغيرات واضحة في الاحماض الأمينية حيث ارتفعت نسبة الفالين من 6.56 الى 946.63، الميثونين 127.183-147.588، الأليبيومين 373.888-850.813100g /mg، البرولامين 1322.275-1474.610، الشيربين 664.113-768.225، هيستيدين 257.700-525.025، الهيدروجين 127.183-147.588، الأليبيومين 373.888-850.813100g /mg، البرولامين 1322.275-1474.610.

وجد أن التخمير قد أظهر تغيرات واضحة في المعادن فقد زاد كل من الكالسيوم 3.87 الى 4.17، الفسفور 0.29-0.37، والمنغنيز 75.33-75.33، وقل كل من الصوديوم 1.42-0.88، الماغنيزيوم 84.67-533.67، الحديد 0.46-0.37.
وقد اثبتت التجارب أن التخمير يودي إلى تقليل مضادات الأغذية فقد قل الثانين بنحو 2.39 إلى 2.24 %، أما البوتلي فيتول فقل من 649.13-340.92 mg\100gm. حمض الفايتك 4.77 إلى 3.80 %.
CHAPTER ONE
INTRODUCTION

The word "fermentation" had many shades of meaning in the past; in
the broad sense it is a process in which chemical changes are brought about in
an organic substrate through the action of enzymes elaborated by
microorganisms (Jay, 1986).

Furthermore, "fermented foods" have been defined as those foods which
have been subjected to the action of microorganisms or enzymes so that
desirable biochemical changes cause significant modification of the food
(Campbell-Platt, 1987). When microbes produce unpleasant aromas, flavors or
toxins in food, the food is spoiled and man has had to learn to avoid it. In
other cases, microbes produce attractive aromas, flavors and texture, and man
has learnt to appreciate and desire such fermented foods (El Faki, 1991). Food
fermentations are responsible for many enrichment diverse flavors and aromas
that enrich the human diet (Steinkraus, 1989).

The fermentation process that an African woman employed was behind the
dramatic improvement in the protein value of the food (Dirar, 1992). The
fermentation of meals of cereals and legumes are known to increases the
protein content (El Tinay et al., 1979).

The international community of food scientists has, in the past decades,
shown a deep interest in three areas of food science and technology. The first
of these is the area of indigenous fermented foods, where a preponderance of
literature has revealed interesting facts including a substantial enhancement of the food as a result of microbial growth in it (Steinkraus, 1978).

The second field of interest is the areas of solid substrate fermentation in which the substance to be fermented albeit wet, is not fluid (Stanton, 1978).

The third area is that of leaf protein (Pirie, 1978). Scientists, in their relentless quest for new protein sources to help feed an ever-increasing world population, found that the plant leaf can be a truly commendable candidate. *Cassia obtusifolia* (Family Leguminosae) is a wild African plant found in wastelands in the rainy season. Its leaves can be fermented (named kawal) and is used by people from the eastern part of Chad and the western part of Sudan as meat replacer or meat extender (Dirar, 1993).

The role of kawal and the like is in providing the sauces which make these staples palatable. During famine years, kawal, a protein source, probably protected many children against kwashiorkor. Until a few years ago, kawal was little known to most Sudanese, for it was a product confined to the western provinces of the country, away from populated areas and centers of influence. Then as today, kawal was shunned by the elite who consider it unfit for modern social life because of its repugnant, fetid odor that lingers on the fingers for hours.

**Objectives:**

The objectives of this study were to assess:

- The effect of fermentation on the chemical composition of *Cassia obtusifolia*.
- The effect of fermentation on protein fractionation.
- The effect of fermentation on *in vitro* protein digestibility.
- The effect of fermentation on amino acid composition.
- The effect of fermentation on mineral content.
- The effect of fermentation on anti-nutritional factors (phytic acid, tannins, and total polyphenols).
CHAPTER TWO
LITERATURE REVIEW

2.1. General characteristics of fermented foods

Fermentation is one of the oldest and most economical methods of producing and preserving foods. In addition to preservation, fermented foods can also have improved nutritional value. During fermentation, it is the unique properties of the bacteria and fungi present that increase the level of proteins, vitamins, essential amino acids and fatty acids in the food. Some microorganisms produce flavor compounds, complex polysaccharides or organic acids (Khalid, 2003).

Fermented food industry may very well be one of the largest world wide. Well known fermented products range from alcoholic beverages, to cheeses, soured milk products, various types of breads, yeast products and antibiotics. In technologically developed regions, these food products have evolved into the large-scale industrial production of fermented consumer goods. Almost unknown in the west are the fermentation of vegetables, such as legumes, nuts and oil seeds, practiced in Asia and Africa (Desphande et al., 2000).

Fermented foods constitute a diverse range of products for many regions of the world. Campbell-Platt (1987) listed some 250 categories of fermented foods, with over 3500 individual products. The range of fermented products is extremely diverse; there are over 900 known types of cheeses, over 200 varieties of fermented meats and over 200 types of bread.
Odunfa (1985) reported that Africa has over 300 different fermented foods, and that one country such as Nigeria has over 20 (Odunfa, 1981). The Sudan alone, however, has over 80 fermented foods and beverages that are distinctively different from each other (Dirar, 1993). Kuboye (1985) noticed that most of the traditional Nigerian staple foods and soup condiments are fermented products.

### 2.1.1 Types of fermentation:

Depending on the type of organism involved, there are two major types of fermentation:

A- Alcoholic fermentation.

B- Lactic acid fermentation.

For many indigenous fermentations the microbial interaction are complex and mixed; fungal -bacteria, fungal –yeast, and yeast bacteria combination (Rogers, 1989). These interactions play important role in the nutritional, safety and sensory characteristics of the end product (Hall, 1989).

### 2.1.2 Fermented food in nutrition:

The reasons for fermenting foods are various: preservation, improvement of digestibility and enrichment of substrates with essential vitamins, proteins, and amino acids. Additionally, fermentation may transform vegetable proteins into meat-like flavors and textures (Steinkraus, 1989).
The advantages of food fermentation have been dealt with El Faki (1991). These advantages may be summarized as follows:

a- Fermentation is a food preservation method.

b- Fermentation may destroy undesirable factors in the raw product.

c- Fermented food may have a better flavor than the raw products.

d- Fermented food may be safer.

e- Fermentation improves the texture of the food.

f- The methods used are inexpensive.

g- The process involves little waste.

2.1.3 Use of fermentation as a food preservation method:

Fermentation is generally less expensive than other forms of preservation, particularly in the developing countries where canning, refrigeration and freezing facilities are limited (Yagoub, 2003).

According to Steinkraus (1996), fermentation plays at least five important roles in the diets of the developing countries. These include:

- Dietary enrichment through development of diversity of flavors, aromas and textures in food substrates.

- Preservation through lactic acid, alcohol and alkaline fermentations.

- Biological enrichment of food substrates with protein, essential amino acids, essential fatty acids and vitamins.

- Detoxification.

- Decreasing cooking times and fuels requirements.
2.2. Indigenous fermented foods

Traditional food fermentations are characterized by their simplicity, rapidity and lack of requirement for high levels of expensive technology (Fardiaz, 1980). Also fermented foods require much shorter cooking times or lower cooking temperature than unfermented foods (El Faki, 1991). Indeed proteins are indispensable, integral fundamental food components both nutritionally and functionally, i.e., they are not only source of energy and amino acid essential for growth and maintain, but also because of the unique properties they provide to foods (Yagoub, 1998).

The animal proteins are relatively expensive if compared with plant proteins and there is no possibility that they can be produced in quantities adequate for supplementing cereal proteins (Desrosier, 1961). In a comprehensive study Bressani and Elias (1968). Indicated that the combat against protein malnutrition must be based on the use of plentiful and cheaper protein foods.

With an over-increasing world population, nutritionists and food technologists look for new protein sources. Plant proteins available in variety of forms (oil seeds, legumes, pulses …etc) could efficiently be used, especially for those for whom the availability of protein is a constraint. Accordingly, the international community of food scientists has shown a deep interest in the areas of indigenous fermented foods (Steinkraus, 1978), and solid substrate fermentation (Stanton, 1978), and in leaf protein as well (Pirie, 1978).
Raymond (1961) concluded that from the point of view of the human diet, traditional fermentation serves several important functions such as:

1- Enhancement or creation of unique flavors, examples of which are kawal in western Sudan and Nigerian fermented Iru (fermented locust beans).

2- Improvement in their nutritional quality, edibility and digestibility, such as Nigerian fermented Iru (fermented locust beans).

3- Changes in the textural properties.

4- Removal of some objectionable odor.

5- Removal of toxic substances: the use of toxic plants as food after fermentation is well known in Africa. The fermentation of castor oil seeds (*Ricinus communis* L) and melon seeds (*Citrullus vulgaris* L) are only some examples. Furthermore, the traditional fermentation is found to provide beneficial multi-micro-flora responsible for successful mixed–culture fermentation. Hesseltine (1992) established that mixed cultures are unquestionably the rule in nature and have many advantages over single-culture fermentation. These are concluded in the following points:

a- The product yield may be higher.

b- High growth rate.

c- Mixed-cultures are able to bring bought multi-steps transformations that would be impossible for single microorganism.

d- Compounds made by a mixture of microorganisms often complement each other and work to exclude undesired microorganisms.
e- Permit better utilization of the substrate.
f- Assure protection against contamination.
g- Mixed-culture fermentations enable the utilization of cheap but impure substances.
h- Provide necessary nutrients for optimum performance. Despite these mixed cultures advantages; this king of fermentation has some drawbacks namely:
a- Scientific study of mixed-culture is difficult.
b- One of the worst problems of mixed-culture fermentation is the control of the optimum balance among the microorganisms involved.

The development of the indigenous fermented foods of plant origin implies that though their raw materials are unpalatable in their native state they are successfully converted edible if subjected to natural fermentation process.

The major and most serious food shortages common in rural Sudan are those related to cereal-stable foods and the major ingredients of the stew (mulah), i.e., meat and milk consequently, the villagers have developed milk and meat substitutes primarily from plant material, which they manipulate by fermentation to give flavor more or less simulating those of proteolitic meat and sour milk. The traditional Sudanese fermented food ‘kawal’ prepared from leaves of *Cassia obtusifolia* is considered to be one of these popular fermented foods of plant origin (Yagoub, 1998).
2.2.1 Shelf life of indigenous fermented foods:

Fermentation is one of the oldest and most economical methods of producing and preserving food (Reddy et al., 1986). The fermentation process is widely thought to stretch the shelf life of cooked cereals and legumes (Hesseltine and Wang, 1980).

This fact has been consistently ascertained by the storage of sun dried fermented products of western Sudan, such as Kawal, Sigda and Furundu indefinitely without deterioration (Harper and Collins, 1992).

2.2.2 Toxicity and safety of indigenous fermented foods:

Many plant substrates of fermented foods contain significant levels of anti-nutritional and toxic components. The use of toxic plants as food after fermentation is a well known practice in Africa. The traditional fermentation processing of cassava (*Manihot esculenta*) and castor oil seed (*Ricinus communis*) that were reported to contain toxic compounds (Odunfa, 1985) are only some examples.

Processing steps such as soaking plant materials, boiling, steaming, roasting and fermentation contribute to a significant reduction in toxic and anti-nutritional components in many foods. These processing were reported to detoxify legumes and oil seeds for human consumption (Yagoub, 1998). Many oriental and African fermented food-processing operations, for instance, include a soaking or cooking step prior to fermentation. Discarding the soak or cook water prior to further processing can result in the removal of significant amount of harmful compounds from the foods. Further elimination of toxic
compounds may also occur during the actual natural fermentation process (Yagoub, 2003).

2.3 Cassia obtusifolia

This plant is commonly named as sicklepod, and locally as kawal or sorib. Botanically, sicklepod is leguminous plant and was named Cassia obtusifolia (Dirar, 1993).

Cassia obtusifolia compound of pinnate leaves with three leaflets which are obovate and the stem is slender which grows up to 2 m high; flowers are yellow to orange. The pot is slender and the plant has many avoid seeds (Andrews, 1952).

Cassia obtusifolia is distributed widely in Africa and America. In the Sudan Cassia obtusifolia grows as a wild plant during rainy season on the central clay plains and southern reason (Dirar, 1993).

2.3.1 Kawal preparation method:

kawal fermentation method was carried according to Dirar (1993), the green leaves are first freed of all extraneous matter, such as leaves of other plants, pods and flowers of the kawal plant itself, caterpillars and insect-damaged leaves. This process of sorting out the kawal leaves is strictly observed and in fact this part of the preparation procedure is the most tedious step as it takes hours of painstaking work. Green flower buds and delicate young pods may, processed with the green leaves.

The unwashed, healthy green leaves, now clean from all adulteration are
beaten in a mortar-and-pestle to give a green paste. Pounding is done in such a way that the leaves are crushed without releasing their juice. In the final paste can be seen partially crushed leaves, twigs, mid-ribs and petioles.

Meanwhile, a pit is dug in the ground in a shaded cool place. An earthenware pot (Burma) is fitted into the pit, leaving only the neck of the container above ground. The green paste is now packed into the pot by hand.

Next, green sorghum leaves are folded onto the surface of the leaf paste in the Burma so that it is completely covered. Washed, dry stones are then placed on top of the sorghum leaves to weight them down. The mouth of the pot is then covered with some metal tray or dish and the whole sealed off with mud to prevent insect from entering.

Every 3-4 days the jar is opened, the now yellow and dry sorghum leaves removed and the Burma thoroughly hand-mixed and repacked, this time a little loose. Fresh sorghum leaves are folded on the surface of the paste and weighted down as before. The Burma covered and sealed off again. The paste is next molded into small, irregular balls or flattish cakes which are then sun dried for 3-4 days.

The duration of the fermentation is about 15 days for the supply of an average family.

2.3.2 Utilization of sicklepod:

The important part of the plant is the leaves which are fermented to produce kawal which is considered a source of dietary protein for a large number of people in Kordofan and Darfur states (the west of Sudan).
Dirar (1993) Stated that the Fur have been subjected repeatedly to famine in the past decades. In 1983-1985 where starvation, drought and desertification had been setting hard on the region, the poor Fur had survived famine better than might have been expected by using protein–rich food from kawal plant (Arthur, 1986).

2.3.3 Medical uses of kawal:

From medical point of view kawal plant seems to contain chemical ingredient of pharmacological importance. For example, the fresh leaves are used as laxative and the roots used as diuretic and for treatment of bites; roots and leaves are both used to cure skin diseases, for example, ring-worm and itches (Anon, 1982). In Sudan Zaghawa tribe of northern Darfur used seed for making a kind of tea that can be drunk for headache, stomach pain, fatigue or unidentified illness (Tubiana and Tubiana, 1977).

In addition to its role as a food, kawal plant is used in tribal tradition for other respects such as hut building materials, making rough mats or as fire wood. Another major use for the plants is connected to an interesting practice; the kawal plants with their tall, woody stems, act as natural wind break for sorghum and millet plants against wind, especially in noisome wind in the Nuba Mountains (Corkill, 1939).
2.3.4 Nutritive value of *Cassia obtusifolia*:

*Cassia obtusifolia* is not the only leguminous herb found widely in the Sudan. But in particular, it is the only one suitable for making kawal food due to the high nutritive value of the plant (Dirar, 1984).

Regarding the nutritional value it has been reported that the plant is rich in calcium and iron, excellent source of vitamin B and in addition, it contains high levels of phosphorous, ash, riboflavin and ascorbic acid. Kawal plant contains high level of protein. The value of kawal leaf protein seems to lie in the quality rather than in the quantity, i.e., it contains high values of cysteine and methionine (Taweel, 2003).

*Cassia obtusifolia* leaves are certainly among the richest plant materials with respect to various nutritional components of the human diet. Duke (1981) tabulated the chemical composition of the beans, leaves fruits, inflorescences, shoots and sprouts of over 30 leguminous plants. It can be seen from his tables, that the leaves of *Cassia obtusifolia* have the highest calcium and riboflavin of all the legumes listed. Moreover, the leaves of this plant rank second in iron and beta-carotene, and third with respect to ash and ascorbic acid. Further, if only the leaves are taken into consideration, those of *Cassia obtusifolia* have the highest phosphorous, calcium, riboflavin and ascorbic acid levels out of some 16 legumes.
2.3.5 Kawal as meat substitute:

The traditional Sudanese fermented food ‘kawal’, defined as a meat substitute derived by fermentation of leaves of *Cassia obtusifolia* falls in the family of West African strong smelling flavor foods (Dirar, 1993).

The major role of kawal in the diets is as a meat substitute both in its capacity as source of high-quality protein and as a source of meaty flavor (Dirar *et al*., 1985).

2.4 The chemical composition of *Cassia obtusifolia* leaves and kawal

Proximate composition of plant material consists of determining the major classes of chemical components, which include moisture, crude fiber, fat content (ether extract), crude protein, ash and carbohydrates (by difference).

Proximate composition provides good initial impression of relative nutritive value and utility of an agricultural product and allows basis of comparison between different species, plant parts and cultivation conditions (Abdel-Rahim, 2004).

2.4.1 Fiber content:

There are two terms of fiber known in the feed and food composition; dietary fiber and crude fiber. Crude fiber is the insoluble organic residue that remains after boiling defatted sample successfully with dilute sulphuric acid followed by dilute sodium hydroxide and ignition. It consists of cellulose and
hemicelluloses. The dietary fiber is the non-starch polysaccharides and lignin that are not digested or absorbed in the human small intestine (Asp, 1987).

In human nutrition, however, a high fiber diet exerts beneficial effects by aiding water retention during passage of food along the gut and they’re by producing larger softer faeces. In addition, the more, insoluble fibers such as cellulose and lignin are beneficial with the clinic functions, while the more soluble fiber e.g. gums and pectin’s lower blood cholesterol, possibly by binding bile acids and cholesterol. In contrast, the crude fiber, i.e., cellulose and hemicelluloses, are found to influence adversely the rate of digestibility, in such away that the mobility of the gastrointestinal tract is increased by the presence of these fibers, thus shortening the time for enzymes action (Abdel-Rahim, 2004).

Yagi (1997) reported that the fiber content of Cassia obtusifolia leaves 13.5 % as dry matter. Dirar et al., (1985) calculated the fiber content of Cassia obtusifolia leaves and kawal were 13.5 and 12.1 % on dry matter basis respectively. Moreover, Ousman et al., (2005) mentioned that the fiber content of Cassia obtusifolia leaves was 13.6 % on dry matter basis.

2.4.2 Fat content:

Lipids (fats) are heterogeneous compounds, which are classified according to their solubility in organic solvents such as chloroform, ethyl ether, petroleum ether or benzene. This solubility differentiates them from other constituents such as protein, carbohydrates and nucleic acids in seeds.
Lipids include free fatty acids, mono-glycerides, di-glycerides, tri-glycerides, phospholipids, steroesters glycerols (Abdel-Rahim, 2004).

The fat content of *Cassia obtusifolia* leaves is low and has no commercial value. Yagi (1997) reported that the fat content of *Cassia obtusifolia* leaves 2.5 % as dry matter. Dirar *et al.*, (1985) calculated the fat content of *Cassia obtusifolia* leaves and kawal were 2.5 and 3.8 % on dry matter basis respectively, while Ousman *et al.*, (2005) found that the fat content of *Cassia obtusifolia* leaves was 4.6 % of dry matter.

**2.4.3 Protein content:**

Protein is the most important component in the organic matter. Since protein is the principle constituent of the organic and soft tissues of the human body, its continues supply is needed thought the human life. Proteins are high molecular weight polymers of amino acids. The main function of proteins are the provision of amino acids for building and maintenance of body and synthesis of nitrogen containing substances which are important for body function, such as enzymes, antibodies, hormones, ..etc. Besides the building function, proteins are sometimes used for energy supply. Protein content of food stuffs can easily be estimated by determining the nitrogen content and multiplying that by 6.25 (since each 100 grams protein contain 16 grams nitrogen) (Abdel-Rahim, 2004).

Protein play a central role in biological systems in addition to functioning as enzymes, proteins also function as structural components of cells in complex organisms. They are highly complex polymers, colloidal in nature, and made up of 20 amino acids. Different proteins vary in molecular weight
and amino acids sequence. At the elemental level proteins containing 50-55 % C, 6-7% H, 20-23% O, 12-19% N and 0.2-3.0 %S (Damodaran, 1996). Proteins are either homoprotein or hetroprotein conjugated with non-protein components. Structurally, proteins also can be classified into globular and fibrous (Yagoub, 2003).

Concerning crude protein content, plant leaves generally contain between 20 and 30 % on dry basis (Pirie, 1983). Dirar (1984) reported that the leaves of *Cassia obtusifolia* have been shown to contain 22 %, while Duke (1981) found 27.6 %. Dirar *et al.* (1985) reported that the protein content of *Cassia obtusifolia* leaves and kawal were 24.3 and 26.2 % of dry matter, respectively. Moreover Abdella (1989) calculated the protein content of kawal as 28 %.

### 2.4.4 The ash content:

The ash, generally known as the residue that remains after ignition of the organic matter, is expressed as percentage from the total weight. In addition, the ash may be used as a starting point for the determination of the elemental composition of the food material (Yagoub, 1998).

According to Dirar (1993) *Cassia obtusifolia* has very high ash content compared to other plants, the fresh leaves contain between 12.6 and 19.6 % ash. Dirar *et al.* (1985) reported that the ash content of *Cassia obtusifolia* leaves and kawal were 12.8 and 19.6 % of dry matter, respectively. Also Abdella (1989) found that the ash content of kawal was 19.5 % as dry matter. Moreover Ousman *et al.*, (2005) calculated that, the ash content of *Cassia obtusifolia* leaves was 10.4 % as dry matter.
2.5 Protein fractionation

One of the oldest criteria for protein classification is the selective solubility of proteins. Proteins are divided into five major groups: albumins which are soluble in water; globulins which are salt soluble; prolamins 70-80% ethanol soluble; glutelins which are sodium hydroxide soluble; and scleroproteins which are insoluble in aqueous solvents (Fruton and Simmonds, 1959).

The respective protein fractions found in wheat are also applicable to other cereals and generally known as albumins, globulins, prolamins and glutelins (FAO, 1999). Among the Osborn fractions in cereals, the prolamin fractions have been the most studied. This fraction is called gliadin in wheat, kafarin in sorghum, scalin in rye, hordein in barely, avenin in oat and zein in maize (Eliasson and Larsson, 1993).

Fruton and Simmonds (1959) reported that albumin in sunflower comprises a very small fraction (0.5%) of the total protein, while Gheyasuddin (1970) reported albumin as 22%. The result reported by Gheyasuddin was not necessarily in conflict with their report since water-soluble fractions contain soluble globulin in addition to albumin. Besides, salt present in the meal would probably dissolve some salt-soluble globulins.

With the growth of knowledge about proteins, the lines of demarcation established in these classifications have proved to be extremely fluid. A sharp distinction between albumins and globulins on the basis of solubility cannot be made (Gheyasuddin, 1970). The prolamin fraction is negligible. This is characteristic feature of all oil seeds. Glutelin comprises about one fifth of the total nitrogen.
Klimenko et al. (1964) consider the glutelin fraction as globulins firmly bonded to the components of the insoluble residue. Their conclusion is based on the observation that an increase in concentration of neutral salts leads to a greater extraction of proteins.

2.6 In vitro protein digestibility

The nutritive value of protein, referred to as protein quality, depends on its content of amino acids and in vitro digestibility (Hahn et al., 1984). The percentage of an essential amino acid that is in greatest deficiency as compared to the amount in a standard or reference protein is known as the limiting amino acid or chemical score of protein. However, today it is more common for score to be calculated as a percentage of adequacy rather than a percentage deficiency.

One of the main factors affecting the nutritive value of legume protein is their limited susceptibility to hydrolysis by digestive enzymes. This photolytic resistance has been attributed to the structural characteristic as well as to the presence of anti-nutritional seed compounds such as trypsin inhibitors polyphenols and phytic acids (Clemente et al., 2000). Babiker et al. (1998) mentioned that the in vitro protein digestibility of Cassia obtusifolia leaves was 67.9 %.

2.7 Amino acid composition

The unfermented leaves contain much higher values of the essential amino acids than the FAO reference protein. The combined values of the
sulphur acids, cysteine and methionine, is just equal to that of the reference protein, i.e. 3.5 g per 16 g nitrogen (Dirar et al., 1985). Cassia leaves are generally considered to be rich in methionine, with value ranging from 1.6g N for *C. emarginata* to 1.9 g N per 16 g N for *C. occidentalis* (Duke, 1981). Leaves of *C. obtusifolia* from the Sudan were found to be exceptionally rich in this sulphur-containing amino acid, with value of 2.1 g N per 16g N (Dirar, et al., 1985).

Fermentation, however, brings about some changes in the amino acid composition of kawal protein. The essential, branched-chain amino acids, valine, leucine and isoleucine, are not degraded to the same extent as several non-essential amino acids such as glutamic and aspartic acids. Similar behavior has been observed in the pattern of amino acid changes in some silages (Oshima, et al., 1979). Some losses in both cystine and methionine occur during fermentation. Nevertheless, depletion was not rendering these sulphur amino acids limiting in the kawal protein (Dirar, et al., 1985).

### 2.8 The mineral content

The mineral matter comprises large number of inorganic elements present in foodstuff with varying amounts. Parts of these are recognized to perform essential functions in human nutrition; therefore, they must be consensually supplied by soft stuffs. Accordingly, 14 different mineral elements are essentially required by human body for good health and growth (Wilson, 1967); Ca, P, K, Mg, Na, S, and Cl are needed in appreciable
amounts, i.e., macro-elements. Others such as Fe, Cu, Mn, I, Co …etc are required in traces, i.e., microelements.

Functionally, the essential elements are considered to play two main roles in animal body: as building constituents and as regulating substances. As structure constituents they serve through 3 general routes:
1- As building units in the hard tissue of the body, the bones and teeth giving strength and rigidity to their structures (e.g.: Ca, P and Mg).
2- Components in soft tissue: such as muscle protein, which contains P and S, nervous tissues contain P.
3- As components essential for the functioning of the body, for instance, I₂ in thyroxin, Fe in hemoglobin, Zn in insulin, and as co-factors in enzyme systems, e.g., Mg as component in body fluids.

Some anti-nutritional components such as phytate if present in the food-stuff may impair the absorption of the essential elements, by binding with them, this rendering these elements non-available (Erdaan, 1997). Moreover, an antagonistic action of certain elements may hamper the absorption of specified elements. For instance, a large intake of Fe and Mg interferes with phosphorous absorption through binding, to form insoluble phosphates.

The mineral constituents have shown important regulatory functions:
1- They contribute to the osmotic pressure of body fluids and maintain base balance e.g. K and Na.
2- They make possible normal rhythm in heart beat.
3- They are indispensable in the formation of blood clots.
2.8.1 Potassium and sodium content:

The human body contains approximately 1.0 % K, 0.4% Na. Potassium exists primarily as cellular constituent. Contrarily, much sodium is concentrated in extra cellular fluids (Maynard and Loosli, 1962).

Yagi (1997) reported that the *Cassia obtusifolia* leaves contain 1.24 % as dry mater sodium. Dirar *et al.*, (1985) mentioned that the *Cassia obtusifolia* leaves and kawal contain 1.42 and 0.87 % as dry matter sodium, respectively.

2.8. Calcium, phosphorus and magnesium content:

Calcium and phosphorus, quantitatively, are believed to be the major constituents of the elementary composition of the human body (4 % Ca and 2.5% P). They are closely associated with each other in most parts and therefore inadequate supply of either in the diet limits the nutritive value of both. Accordingly, calcium and phosphorus levels in the blood are important indicators of the state of nutrition. Moreover, it should be emphasized that levels represent a balance between several apposing factors: absorption that half or more of the phosphorus of most seed and their products is so combined to phytin, excretion, disposition and mobilization (Maynard and Loosli, 1962). Magnesium though present in the body in much smaller amounts (about 0.1 %), is closely associated functionally with calcium and phosphorus. From another angle, an informative study of the calcium composition of *Cassia obtusifolia* leaves gave 2.9 % (Duke, 1981); while Dirar *et al.*, (1985) obtained
a higher value of 3.85 % Ca, 0.26 % P, 0.3 % Mg and kawal contained 4.13 % Ca, 0.28 % P, and 0.42 % Mg as dry matter. Yagi (1997) found that *Cassia obtusifolia* leaves contained 2.82 Ca, 0.93 P, 0.50 % dry matter.

### 2.8.3 Iron content:

Although the human body contains only about 0.01 % iron, this vital element plays a significant role in the life process as a constituent of the respiratory pigment (hemoglobin). Iron is essential for the functions of energy and tissue in the body, known as heme, not only in the hemoglobin but also in protein that is a component of cytochrome peroxides (Yagoub, 1998).

Yagi (1997) reported that the *Cassia obtusifolia* leaves contained 534 mg/kg as dry matter iron. Dirar *et al.*, (1985) found that the iron contents of *Cassia obtusifolia* leaves and kawal were 534 and 82 mg/kg as dry matter, respectively. Ousman *et al.*, (2005) mentioned that iron content of *Cassia obtusifolia* leaves was 28.9 mg/100g dry matter.

### 2.9 Antinutritional factors

*Cassia obtusifolia*, like other legumes, has the capacity to synthesize various chemical compounds, which when consumed by animals or humans may result in a reduction in the nutritive value as compared to that predicted from amino acid data. The presence of biologically active components, such as phytates and phenolic compounds, are found to have adverse effects on interstice properties of protein. Consequently, the *in vitro* measures for the changes in the activity of these components, as might be effected by
processing, could be an indication to changes in nutritional as well as physicochemical behavior of proteins (Yagoub, 2003)

2.9.1 Phytic acid:

Phytates or salts of phytic acid, myo-inositol hexaphosphate or more properly myoinositol 1, 2, 3, 4, 5, and 6 hexakis (dihydrogen phosphate), a naturally occurring organic component, have long been recognized as constituents of certain seeds and plant tissue, especially cereal grains and oil seeds. Phytates which are believed to be the principal sources of phosphorous in most seeds which are not digested by human beings. Recent information indicated that if phytate comprises substantial part of the diet they can interfere with mineral element absorption and utilization, thus lowering their bioavailability as necessary cofactors (Harland and Oberleas, 1986). Although less attention has been focused on phytate protein interactions, it has lately been reported that phytic acid can indeed inhibit pepsin, trypsin, and alpha amylase (Sinhg and Krikorian, 1982).

Most of the investigations (Odell, 1969; Oberleas, 1973) suggested that the formation of insoluble phytate-metal complexes in the intestinal tract prevents metal absorption. The formation of these complexes is pH dependant. Rackis and Anderson (1977) reported that reduced availability of essential minerals by either phytate or phytate-protein complex in legumes and other protein foods depends on several factors such as the following:

- The ability of indogenous carrier in the intestinal mucosa absorbs essential minerals bound to phytate and other dietary substances.
- The concentration of the phytic acid in foodstuffs.
- The concentration of minerals in the foodstuffs.
- Phytase inhibition.
- The digestion or hydrolysis of phytase enzyme in the intestine.
- Processing of products or methods of processing.

2.9.2 **Tannin content:**

Tannins are polymeric phenolic compounds of medium to high molecular weight because of their ability to form complexes with proteins. Plant tannins are found responsible for the astringency of tannin-rich foods (Yagoub, 2003).

Tannins are believed generally, to play significant role in nutrition by binding the most essential nutrients, thus altering their chemical and functional properties. Chemically, they are diverse polyphenolic compounds that occur widely in the plant kingdom, in which they have a variety of biological activities based on their structural differences and adverse hydrolytic relativities. Tannins are broadly classified into two groups: hydrolysable and condensed. Tannins have been extensively investigated because of their harmful effects on growth of animal and protein utilization through formation of insoluble complexes with them. The latter process is suggested to be responsible of reducing the digestibility of protein, and consequently the protein value of food (Mcleod, 1974). As so many tannins have been found to exhibit the ability to inactivate several enzymes (Strumeyer and Malin, 1975). "Tannins have been implicated in adversely
effecting the digestibility of dietary protein and to a lesser extent that of available carbohydrates and lipids (Mosely and Griffiths, 1979).

A literature search on tannin content of *Cassia obtusifolia* leaves gives 2.34 % (Babiker, *et al.*, 1998) and 2.50 % (Abdalla, 1989).

**2.9.3 Total polyphenols:**

Polyphenol refers to a complex family of phenolic compounds, which are widely distributed in plants, and the level of them varies greatly even between the cultivars of the same spices. Genetic factors, as well as environmental conditions largely influence their presence in plant foods. In plants, several functions have been attributed to polyphenols; they have anti-pathogenic, anti-herbivore and allopathic properties (Brice and Morrision, 1982; Ray and Hastings, 1992). Soluble extractable polyphenols are low or intermediate molecular mass phenolics that are extracted easily using different solvents, such as water, methanol, or aqueous acetone. Non-extractable polyphenols are mainly condensed tannins of high molecular mass, which are bound to protein and fiber (Bravo, 1998). Polyphenols have different effects in the intestine depending on their solubility. Extractable polyphenols appear to be absorbed from the digestive tract and produce systemic effect, such as reduction of the metabolic utilization of absorbed amino acids and elevated plasma levels of growth hormones. Non-extractable polyphenols are not absorbed in the intestine and are recovered quantatively in feces (Taweel, 2003).
Polyphenols are classified into: phenolic acids (simple phenols) flavonoids and tannins-simple phenols and flavonoids are relatively low molecular weight compounds, representing the vast majority of plant phenolics that are mostly soluble (Yagoub, 2003).

Ousman et al., (2005) calculated that the total phenol compounds of Cassia obtusifolia leaves were 4.8 % on dry matter basis.
CHAPTER THREE
MATERIALS AND METHODS

3.1 Materials
The green leaves of *Cassia obtusifolia* and kawal were obtained from Nyala (western Sudan).

*Cassia obtusifolia* leaves and kawal were obtained in dry form after being sun dried and freed from foreign materials and powdered by hummer mill with same mesh size and was kept in clean bottles at room temperature for further use.

3.3 Methods

3.4 Proximate composition

3.4.1 Crude fiber:
The crude fiber content was carried out by the method of A.O.A.C (1984). Two grams of dried and defatted sample were transferred to a 600ml beaker with a few anti-bombing granules. The sample was digested with 200ml of 0.255 N sulphuric acids for exactly 30minutes, and the beaker was periodically swirled. The contents were removed and filtered through Buchner funnel, and washed with boiling water. The digestion was repeated using 200ml of 0.313N sodium hydroxide for 30minutes, and treated similarly as above. After that, the fiber was washed with 1% hydrochloric acid to neutralize the sodium hydroxide and then rinsed with distill water. After the last washing the sample was put in a dish, and dried in an oven at 103 °C for
one hour then cooled and weighed. The dried residue was ignited in muffle furnace at 500°C overnight, cooled and weighed. The crude fiber was calculated using the following equation.

\[ \text{CF\%} = \frac{w_1 - w_2 \times 100}{W_s} \]

Where:

CF\% = crude fiber.
WS = weight of sample.
W1 = weight of crucible with sample.
W2 = weight of crucible with ashed sample.

3.4.2 Fat content:

Total fat was determined according to the A.O.A.C method (1984). Two grams of sample were extracted with petroleum ether (BP 60-80 °C) for 8hr. In soxhlet apparatus. The fat content was calculated according to the following equation.

\[ \text{Fat\%} = \frac{w_2 - w_1 \times 100}{\text{Weight of sample}} \]

Where:

W1 = weight of empty flask
W2 = weight of flask with oil
3.4.3 Protein content:

The protein content of the samples was determined by the micro-kjeldahl technique according to the A.O.A.C method (1984): 0.2 g of sample was weighed accurately into micro-kjeldahl flask, two hundred milligrams of catalyst mixture and 3.5ml of concentrated sulphuric acid were added, the sample content were heated on an electric heater for 2hr and cooled, then the contents were placed into the distillation apparatus. Twenty milliliters of 40% NaoH were added and the ammonia evolved was received in 10ml of 2% boric acid solution. The trapped ammonia was titrated against HCl (0.02N) using universal indicator (methyl red + bromocresol green). The total nitrogen and protein were calculated using the following formula:

\[
N\% = \frac{\text{volume of HCl} \times N \times 14 \times 100}{\text{Weight of sample} \times 1000}
\]

\[
P\% = N\% \times 6.25
\]

Where:

N\% = crude nitrogen.

P\% = crude protein.

N = normality of HCl.

14 = equivalent weight of nitrogen.
3.4.4 Ash content:

The ash content of sample was measured according to the A.O.A.C method (1990) using muffle furnace (model Tipoformo ZA No. 18203 Gef Ran 1001): two grams of sample were weighed into porcelain crucible and placed in a temperature controlled furnace at 600°C for complete ashing, the crucible with ash was transferred directly to a desiccator, cooled, weighed and calculated as percent of original weight of sample.

\[
\text{Ash content (\%) = \frac{(W_1 - W_2) \times 100}{\text{Sample weight}}}
\]

Where:

\( W_1 \) = weight of crucible with ash.

\( W_2 \) = weight of empty crucible.

3.5 Protein fractionation due to solubility

The Mendel and Osborne (1914) technique for protein fractionation was used in this study.

3.5.1 Determination of water soluble proteins (Albumins):

A sample of 2.5 grams was taken from defatted seed flour for fractionation of total proteins. To this amount of the flour, 2 volumes of 50ml distilled water were added and the mixture was shaken for 30 minutes using mechanical shaker, then centrifuged at 300 rpm for 20 minutes to separate the insoluble part from the liquor. The extraction liquor was made up to 100 ml.
Ten ml were taken for protein estimation according to the micro-kjeldahl method. The following formula was used for calculating percentage of albumin:

\[
\text{Total albumin} = \frac{T \times 1.4 \times DF \times 6.25}{W \times 1000}
\]

T = titer reading.
DF = dilution factor.
W = weight of sample.

**3.5.2 Determination of salt soluble proteins (Globulins):**

The insoluble part obtained after extraction of albumin was re-extracted with 2 volumes of 50 ml NaCl (1M) for 30 minutes with continuous shaking. The mixture was then centrifuged at 3000 rpm for 20 minutes to separate the insoluble part. The extracted liquor was collected in 100 ml volumetric flask. Ten ml of the liquor were taken for estimation of soluble protein by the micro-kjeldahl method.

Percentage of globulins as following:

\[
\text{Total globulins} = \frac{T \times 1.4 \times DF \times 6.25}{W \times 1000}
\]

\[
\text{Globulins} = \frac{\text{total globulins} \times 100}{\text{Total proteins}}
\]
3.5.3 **Determination of alcohol soluble proteins (Prolamins):**

The insoluble parts obtained after extraction of Salt soluble proteins was re-extracted with 2 volumes of 50 ml 70% ethanol added to insoluble part with continuous shaking for 30 minutes in mechanical shaker. The peptized liquor was separated from the residue by centrifugation at 3000 rpm for 20 minutes. The peptized liquor was collected in 100 ml volumetric flask. Ten ml of the liquor were taken for protein determination by the micro-kjeldahl method. The percentage of alcohol soluble proteins was calculated from total proteins as following:

\[
Prolamin (\%) = \frac{\text{total prolamins}}{\text{Total protein}} \times 100
\]

3.5.4 **Determination of alkali-soluble proteins (Glutelins):**

The insoluble part obtained after the extraction of prolamin was re-extracted with 2 volumes of 50 ml NaOH (0.2%) for 30 minutes with continuous shaking. The insoluble part was separated by centrifugation at 3000 rpm for 20 minutes. The peptized liquor was collected in 100 ml volumetric flask and 10 ml taken for nitrogen determination.

3.5.5 **Protein content of insoluble fraction:**

The remaining insoluble part of the sample was digested in 10 ml concentrated sulphuric acid and used for estimation of insoluble nitrogen in digest.
3.6 **In vitro protein digestibility**

Determination of *in vitro* protein digestibility was carried out according to Saunder *et al.*, (1973) method. Two hundred milligrams of sample were placed into a 50 ml centrifuge tube, 15ml of 0.1M HCl containing 1.5mg pepsin were added, and the tube was incubated at 37 °C for three hours. The suspension was then neutralized with 0.5ml of NaOH (calculated 3.3ml), then treated with 4mg of pancreatin in 7.5ml of 0.2 M phosphate buffer (pH 8.0) containing 0.005 sodium azide. The mixture was then gently shaken and incubated at 37 °C for 24hours. After incubation the sample was treated with 10 ml 10% trichloroacetic acid and centrifuged at 5000×g for 20 min at room temperature.

Nitrogen in supernatant was estimated using micro-kjeldahl method. Digestibility was calculated using the formula

\[
\text{Protein digestibility} \% = \frac{N \text{ in supernatant} \times 100}{N \text{ in sample}}
\]

\[
N \text{ in supernatent} = \frac{T \times TV \times N \times 14 \times 100}{A \times B \times 1000}
\]

**Where:**

- **T** = titer reading.
- **TV** = total volume of the liquid extract.
- **N** = normality of acid.
3.7 Amino acid analysis

Samples containing 10mg protein were hydrolyzed with 6N HCl containing 0.1% phenol and 0.1% B-mercaptophenol at 110 °C for 24 hours in test tubes sealed under vacuum (Moore and Stein, 1963). The hydrolyzed material was flash evaporated, dissolved in 10 ml sodium citrate buffer (pH 2.2), and passed through Millipore filter. The filtrate, adjusted to contain 0.5 mg protein/ml, was analyzed with LKB biochrom 4150(alpha) automatic amino acid analyzer.

3.8 Determination of mineral content

Mineral content of each sample was extracted according to Pearson's method (1981). Two grams of sample were placed in muffle furnace at 550 °C for 4 hr, samples were cooled and 10 ml of 5N HCl were added, then boiled gently for 10 minutes using sand bath, diluted to volume (100 ml) with distilled water and taken for mineral determination by atomic absorption spectrophotometry (Perkin Elmer, 2380).

3.9 Determination of Antinutritionnal factors

3.9.1 Determination of phytic acid content:

Phytic acid content was determined by the method described by Wheeler and Ferrel (1971). Two grams of dried sample were weighted in 125 ml conical flask. The sample was extracted with 50 ml of 3% trichloroacetic acid (TCA) for 3 hr with mechanical shaking. The supernatant was centrifuged for 5 minutes. Ten milliliters aliquot of the supernatant was transferred to a 40 ml
tube and 4 ml of FeCl₃ (FeCl₃ solution containing 2 mg Fe^{3+} ion/ml 3% TCA) were then added to the aliquot. The tube was heated in a boiling water bath for 45 min. One or two drops of 3% sodium sulphate (Na₂SO₄) in 3% TCA were added. The tube was cooled and centrifuged for 10-15 min and the clear supernatant was decanted. The precipitate was washed by dispersing well in 25 ml 3% TCA, heated for 10-15 min in boiling water bath and then centrifuged again. Washing was repeated with distilled water, the washed precipitate was dispersed in few milliliters of distilled water enriched with 3 ml of 1.5 N NaOH, and the volume completed to approximately 30 ml with distilled water, heated in boiling water bath for 30 min and hot filtered using whatman No.2. The precipitate was washed with 60-70 ml hot water, and the washing was decanted. The precipitate from the filter paper was dissolved in 40 ml hot 3.2 N HNO₃ and placed in 100 ml volumetric flask. The paper was washed with hot distilled water and the washing was collected in the same flask then completed to volume. A 0.5 ml aliquot was taken from the above solution and transferred into 10 ml volumetric flask. Then 2 ml of 1.5 N KSCN (potassium thiocyanate) were added and completed to volume by water then immediately (within one minute) read at 480 nm using (SP6 Py Unieam) spectrophotometer.

A standard curve of different Fe(NO₃)₃ concentrations and corresponding optical densities was plotted to calculate the ferric ion concentration. The phytate phosphorus was calculated from the iron concentration assuming 4:6 iron to phosphorus molar ratio.
Phytate (mg/100g) = \( \frac{6/4 \times A \times C \times 20 \times 10 \times 50 \times 100}{1000 \times S} \)

**Where:**

A = optical density.

C = concentration corresponding to optical density.

S = weight of sample.

### 3.9.2 Determination of tannin content:

Tannin content (TC) of *Cassia obtusifolia* leaves and kawal samples were estimated using modified vanillin-HCl in methanol as described by Price et al (1978). 0.2g of ground sample was placed in 100ml conical flask. 10 ml of 1% HCl in methanol (v/v) were added, the contents were mechanically shaken for 20 min and centrifuged at 2500 rpm for 5min. One ml of supernatant was pipettes into a test tube and 5ml of vanillin-HCl reagent (mixing equal volume of 8% concentrated HCl in methanol and 1% vanillin in methanol) were added. The optical density was read using a colorimeter (Lab system Analyzer 9 filters, J, Mitra and Bros.Pvt .Ltd) at 500nm after 20min incubation at 30 °C, a blank sample reading was carried out with each run of samples. A standard curved was repeated expressing the result of tannic cid, i.e. amount of tannic (mg per ml) which gives color intensity equivalent to that given by tannin after correcting for blank.

**Calculation:**

\[
TC (\%) = \frac{C \times 10 	imes 100}{200}
\]
Where:

C = concentration corresponding to optical density.

10 = volume of extract in ml.

200 = sample weight in mg.

3.9.3 Total polyphenol (TP) determination:

Polyphenolic content of each sample was estimated using Prussian blue assay, as described by Price and Butler (1977). About 60mg of ground sample was extracted with 3ml methanol in a 50ml conical flask, and then poured into a filter paper. The tube was quickly rinsed with additional 3ml methanol and the content poured once more into the filter paper; the filtrate was diluted to 50ml with distilled water, mixed with 3ml 0.1M FeCl₃ in 0.1N HCl for 3min, followed by the time addition of 3ml 0.008M K₃Fe(CN)₆. The absorption was read after 10min at 720 nm on spectrophotometer (Corning, 259).

3.9.4 Standard curve preparation:

Tannic cid standard curve was prepared by dissolving 100mg tannic acid in distilled water in a 0.1 liter volumetric flask and made up to mark. This spread stock solution of 100ppm. Various standard concentrations (0, 2, 4, 6, 8 and 10) were repeated. The Prussian blue assay described above was then employed to the standard solution. The standard curve was obtained by plotting concentration against the corresponding absorbance reading, which gave linear relationship.

Calculation:
Total polyphenol (%) = \( C \times \frac{56}{60} \times 100 \)

**Where:**

\( C \) = concentration corresponding to optical density.

56 = total volume.

60 = weight of sample in milligrams.

### 3.10 statistical analyses

Each determination was carried out on three samples, analyzed in triplicate, and averaged. Data was assessed by the analysis of variance (ANOVA) (Snedecor and Cochran, 1987). Duncan's multiple range test was used to separate means. Significance was accepted at \( P \leq 0.05 \) (Duncan, 1955).
CHAPTER FOUR  
RESULTS AND DISCUSSIONS

4.1. Chemical composition of green leaves of *Cassia obtusifolia* and kawal.

Chemical composition of green leaves of *Cassia Obtusifolia* and kawal (as dry matter) is shown in Table 1.

**4.1.1 Fiber content:**

Fiber content of green leaves of *Cassia Obtusifolia* and kawal (as dry matter) is shown in Table 1. Fermentation was found to cause no significant change (p<0.05) in fiber content. The fiber content is decreased from 13.04 to 12.90 %.

The fiber content of green leaves of *Cassia Obtusifolia* and kawal obtained were in agreement with the values obtained by Dirar *et al.*, (1985) who reported 13.5 and 12.1 % for green leaves of *Cassia obtusifolia* and kawal, respectively.

**4.1.2 Fat content:**

The fat content of green leaves of *Cassia obtusifolia* and kawal (as dry matter) is shown in Table 1. Fermentation was found to cause no significant change (p≤0.05) in fat content. The fat content of green leaves of *Cassia obtusifolia* increased from 3.50 to 4.50 % in kawal.
The values obtained in this study were in agreement with the value obtained by Ousman et al. (2005) who reported that the fat content of green leaves of *Cassia obtusifolia* was 4.6% as dry matter, and higher than the values obtained by Dirar et al. (1985) who reported 2.5 and 3.8% for green leaves of *Cassia obtusifolia* and kawal, respectively.

### 4.1.3 Protein content:

Protein content of green leaves of *Cassia obtusifolia* and kawal (as dry matter) is shown in Table 1. Fermentation was found to cause highly significant increase ($p < 0.05$) in protein content. The protein content increased from 24.81 to 35.13%.

The protein content of green leaves of *Cassia obtusifolia* in this study were in agreement with value obtained by Dirar et al., (1985) who reported 24.3% and also in agreement with value obtained by Pirie (1983) who reported a range from 20 to 30%. But the value obtained in protein content of kawal in this study was higher than the value obtained by Dirar et al., (1985) who reported 26.2%.

El Tinay et al., (1979) and Zamora and Fields (1979) reported an increase in protein content in cereals and legumes due to fermentation. Dirar (1978) reported that during Merissa preparation (a fermented alcoholic beverage made from sorghum) total nitrogen showed a slight increase after 12 hours (pH 4.6) of fermentation. The increase in protein content can also be attributed to microbial synthesis of proteins from metabolic intermediates during their growth cycles (Zamora and Fields, 1979). Rose (1961) in his study of
microbial foods reported that microbial cell matter contain appreciable amount of protein. He found that the rate of protein synthesise in micro-organism is higher than in higher plants or animals.

4.1.4 Ash content:

The ash content of green leaves of *Cassia obtusifolia* and kawal (as dry matter) is shown in Table 1. Fermentation was to cause highly significant increase (p<0.05) in ash content. The ash content increased from 13.67 to 18.00 %. The ash content of green leaves of *Cassia obtusifolia* in this study were in agreement with values obtained by Dirar et al., (1985) who reported 12.6 and 19.6 % for green leaves of *Cassia obtusifolia* and kawal, respectively. Also this value agrees with that obtained by Abdella (1989) who reported that ash content of kawal was 19.5 %, but higher than the value obtained by Ousman et al., (2005) who reported that the ash content of green leaves of *Cassia obtusifolia* was 10.4%

4.2 Protein fractionation of green leaves of *Cassia obtusifolia* and kawal.

Protein fractionation of green leaves of *Cassia obtusifolia* and kawal is shown in Table 2.

4.2.1 Albumin content:

Albumin content of green leaves of *Cassia obtusifolia* and kawal is shown in Table 2. Fermentation was found to significantly increase (p≤0.05) albumin
Table 1: Chemical composition of green leaves of *Cassia obtusifolia* and kawal (as dry matter).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fat content %</th>
<th>Crude fiber %</th>
<th>Crude protein %</th>
<th>Ash content %</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cassia obtusifolia</em></td>
<td>3.50 a</td>
<td>13.04 a</td>
<td>24.81 b</td>
<td>13.67 b</td>
</tr>
<tr>
<td>Leaves</td>
<td>(+0.503)</td>
<td>(+1.323)</td>
<td>(+0.506)</td>
<td>(+1.528)</td>
</tr>
<tr>
<td>Dry Kawal</td>
<td>4.50 a</td>
<td>12.90 a</td>
<td>35.13 a</td>
<td>18.00 a</td>
</tr>
<tr>
<td></td>
<td>(+0.500)</td>
<td>(+0.361)</td>
<td>(+1.042)</td>
<td>(+1.000)</td>
</tr>
</tbody>
</table>

- Each value in an average of three values expressed on dry weight basis.
- Values are means (+ standard deviation).
- Means not sharing a common letter in a column are significant at p ≤ 0.05 as assessed by Duncan’s multiple range tests.
Content. The albumin content of green leaves of *Cassia obtusifolia* increased from 12.59 to 14.42 % from total protein in kawal.

### 4.2.2 Globulin content:

Globulin content of green leaves of *Cassia obtusifolia* and kawal is shown in Table 2. Fermentation was found to significantly increase (p<0.05) globulin content. The globulin content of green leaves of *Cassia obtusifolia* increased from 58.52 to 63.38 % from total protein in kawal.

### 4.2.3 Prolamin content:

Prolamin content of green leaves of *Cassia obtusifolia* and kawal is shown in Table 2. Fermentation was found to significantly increase (p<0.05) Prolamin content. The prolamin content of green leaves of *Cassia obtusifolia* increased from 8.69 to 13.83 % from total protein in kawal.

### 4.2.4 Glutelin content:

Glutelin content of green leaves of *Cassia obtusifolia* and kawal is shown in Table 2. Fermentation was found to significantly increase (p<0.05) glutelin content. The glutelin content of green leaves of *Cassia obtusifolia* increased from 5.03 to 8.32 % from total protein in kawal.
4.2.5 Insoluble protein content:

Insoluble protein content of green leaves of *Cassia obtusifolia* and kawal is shown in Table 2. Fermentation was found to significantly decrease (p<0.05) insoluble protein content. The insoluble protein content of green leaves of *Cassia obtusifolia* decreased from 17.81 to 5.41 % from total protein in kawal.

4.3 *In vitro* protein digestibility of green leaves of *Cassia obtusifolia* and kawal

*In vitro* protein digestibility of green leaves of *Cassia obtusifolia* and kawal is shown in Table 3. Fermentation was found to cause highly significant increase (p<0.05) in *in vitro* protein digestibility. It was increased from 49.433 to 61.867 %.

The values obtained in this study were in agreement with value obtained by Babiker *et al.*, (1998) who reported that, the *in vitro* protein digestibility of green leaves of *Cassia obtusifolia* was 52.6 %.

Fermentation is known to cause increase in *in vitro* protein digestibility due to microflora that may produce some proteolytic enzymes during fermentation, which may be responsible for increasing protein digestibility. Also Monawar (1983) stated that the reduction in pH during fermentation plays an important role in enhancing native proteolytic enzyme activity and consequently promotes the breakdown of protein to smaller polypeptides which are easily digested by enzymes, after consumption by humans.
Table 2: Protein fractionation of green leaves of *Cassia obtusifolia* and kawal (as dry matter).

<table>
<thead>
<tr>
<th>sample</th>
<th>Globulin %</th>
<th>Albumin %</th>
<th>Prolamin %</th>
<th>Glutelin %</th>
<th>Insoluble protein %</th>
<th>sum</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cassia obtusifolia</em></td>
<td>58.52 b</td>
<td>12.59 a</td>
<td>8.69 b</td>
<td>5.03 b</td>
<td>17.81 a</td>
<td>102.64</td>
</tr>
<tr>
<td>leaves</td>
<td>(+0.325)</td>
<td>(+1.139)</td>
<td>(+0.242)</td>
<td>(+0.162)</td>
<td>(+0.315)</td>
<td></td>
</tr>
<tr>
<td>Dry Kawal</td>
<td>63.38 a</td>
<td>14.43 a</td>
<td>13.83 a</td>
<td>8.32 a</td>
<td>5.41 b</td>
<td>105.37</td>
</tr>
<tr>
<td></td>
<td>(+0.317)</td>
<td>(+0.404)</td>
<td>(+0.078)</td>
<td>(+0.081)</td>
<td>(+0.239)</td>
<td></td>
</tr>
</tbody>
</table>

- Each value is an average of three values expressed on dry weight basis.
- Values are means (+ standard deviation).
- Means not sharing a common letter in a column are significant at $p \leq 0.05$ as assessed by Duncan’s multiple range tests.
Table 3: *In vitro* protein digestibility of green leaves of *Cassia obtusifolia* and kawal

<table>
<thead>
<tr>
<th>sample</th>
<th><em>in vitro</em> protein digestibility</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cassia obtusifolia</em></td>
<td></td>
</tr>
<tr>
<td>Leaves</td>
<td>49.433(^a) (+1.079)</td>
</tr>
<tr>
<td>Dry Kawal</td>
<td>61.867(^b) (+1.050)</td>
</tr>
</tbody>
</table>

-Each value in an average of three values expressed on dry weight basis.
-Values are means (± standard deviation).
-Means not sharing a common letter in a column are significant at p ≤ 0.05 as assessed by Duncan’s multiple range tests.
4.4 Amino acid content

The amino acid content of green leaves of *Cassia obtusifolia* and kawal is shown in Table 4. Fermentation was found to cause increase in valine, cysteine, methionine, isoleusine, leucine, glycine and alanine and decrease in threonine, tyrosine, phenylanine, lysine, histidine, arginine, aspartic acid, serine and glutamic acid.

4.5 Mineral content of green leaves of *Cassia obtusifolia* and kawal

Mineral content of green leaves of *Cassia obtusifolia* and kawal is shown in Table 5

4.5.1 Sodium content:

Sodium content of green leaves of *Cassia obtusifolia* and kawal is shown in Table 5. Fermentation was found to cause significant decrease (p<0.05) in sodium content.

The sodium content of green leaves of *Cassia obtusifolia* and kawal decreased from 1.423 to 0.880 g/kg.

The values obtained in this study were in agreement with values obtained by Dirar *et al.*, (1985) who reported 1.42 and 0.87 g/kg for green leaves and kawal, respectively.
Table 4: Amino acid content of green leaves of *Cassia obtusifolia* and kawal (on dry matter basis).

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>green leaves (mg/100g)</th>
<th>Kawal (mg/100g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Valine</td>
<td>946.600</td>
<td>1086.900</td>
</tr>
<tr>
<td>Cysteine</td>
<td>No peak</td>
<td>226.500</td>
</tr>
<tr>
<td>Methionine</td>
<td>127.188</td>
<td>47.588</td>
</tr>
<tr>
<td>Isoleusine</td>
<td>737.388</td>
<td>850.813</td>
</tr>
<tr>
<td>Leucine</td>
<td>1322.275</td>
<td>1474.613</td>
</tr>
<tr>
<td>Glycine</td>
<td>757.075</td>
<td>760.021</td>
</tr>
<tr>
<td>Alanine</td>
<td>1007.450</td>
<td>1590.113</td>
</tr>
<tr>
<td>Threonine</td>
<td>664.113</td>
<td>574.788</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>438.100</td>
<td>421.600</td>
</tr>
<tr>
<td>Phenylanine</td>
<td>768.225</td>
<td>96.313</td>
</tr>
<tr>
<td>Lysine</td>
<td>878.725</td>
<td>766.610</td>
</tr>
<tr>
<td>Histidine</td>
<td>525.700</td>
<td>407.025</td>
</tr>
<tr>
<td>Arginine</td>
<td>787.525</td>
<td>736.475</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>1855.388</td>
<td>1304.563</td>
</tr>
<tr>
<td>Serine</td>
<td>544.400</td>
<td>422.650</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>1579.025</td>
<td>1411.313</td>
</tr>
</tbody>
</table>
4.5.2 Calcium and phosphorous content:

Calcium and phosphorous content of green leaves of *Cassia obtusifolia* and kawal is shown in Table 5. Fermentation was found to cause significant increase \((p \leq 0.05)\) in calcium and phosphorus content.

Calcium content of green leaves of *Cassia obtusifolia* and kawal increased from 3.870 to 4.170 g/kg and phosphorus increased from 0.267 to 0.287 g/kg. The values obtained in this study were in agreement with values obtained by Dirar *et al.* , (1985) who reported from 3.85 to 4.13 g/kg calcium content and 0.26 to 0.28 g/kg phosphorus content.

4.5.3 Magnesium and manganese content:

Magnesium and manganese content of green leaves of *Cassia obtusifolia* and kawal is shown in Table 5. Fermentation was found to cause no significant change \((p \leq 0.05)\) in magnesium content.

The magnesium content of green leaves of *Cassia obtusifolia* and kawal increased from 0.367 to 0.457 g/kg. But manganese content was found to significantly increase \((p \leq 0.05)\); it increased from 75.33 to 112.333 mg/kg. The values obtained in this study were in agreement with values obtained by Dirar *et al.* , (1985) who reported from 0.3 to 0.42 g/kg magnesium content and 75 to 112 mg/kg manganese content.
4.5.4 Iron and zinc content:

Iron and zinc content of green leaves of *Cassia obtusifolia* and kawal is shown in Table 5. Fermentation was found to cause no significant decrease (p≤0.05) in iron content, and significant increase (p≤0.05) in zinc content. The iron content of green leaves of *Cassia obtusifolia* and kawal was decreased from 533.667 to 84.33 mg/kg. But Zinc content was increased from 536.0 to 84.667 mg/kg.

The values obtained in this study were in agreement with values obtained by Dirar *et al.*, (1985) who reported from 533.667 to 84.667 mg/kg iron content and from 32.0 to 84.0 mg/kg zinc content.

4.6 Anti-nutritional factors of green leaves of *Cassia obtusifolia* and kawal.

Anti-nutritional factors of green leaves of *Cassia obtusifolia* and kawal are shown in Table 6.

4.6.1 Phytic acid content:

Phytic acid content of green leaves of *Cassia obtusifolia* and kawal is shown in Table 6. Fermentation was found to cause significant decrease (p≤0.05) in phytic acid content. The phytic acid content decreased from 649.13 to 340.92 mg/100g.

Generally, fermentation is known to cause high reduction in phytic acid content due to the low pH of fermented dough which is considered to be optimum for the phytase activity.
Table 5: minerals content of green leaves of *Cassia obtusifolia* and kawal (as dry matter).

<table>
<thead>
<tr>
<th>sample</th>
<th>Na</th>
<th>Ca</th>
<th>P</th>
<th>Mg</th>
<th>Mn</th>
<th>Fe</th>
<th>Zn</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cassia obtusifolia</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leaves</td>
<td>1.423 a (±0.120)</td>
<td>3.870 b (±0.11)</td>
<td>0.267 b (±0.32)</td>
<td>0.367 a (±0.023)</td>
<td>75.333 b (±0.33)</td>
<td>533.667 b (±0.23)</td>
<td>536.007 a (±0.25)</td>
</tr>
<tr>
<td>Dry Kawal</td>
<td>0.880 b (±0.012)</td>
<td>4.170 a (±0.13)</td>
<td>0.287 a (±0.23)</td>
<td>0.457 a (±0.33)</td>
<td>112.333 a (±0.12)</td>
<td>84.33 a (±0.41)</td>
<td>84.667 b (±0.61)</td>
</tr>
</tbody>
</table>

- Each value in an average of two values expressed on dry weight basis.
- Values are means (± standard deviation).
- Means not sharing a common letter in a column are significant at p ≤ 0.05 as assessed by Duncan's multiple range tests.
4.6.2 Tannin content:

Tannin content of green leaves of *Cassia obtusifolia* and kawal is shown in Table 6. Fermentation was found to cause significant decrease (p<0.05) in tannin content. The tannin content decreased from 2.39 to 2.24 %.

The values obtained in this study were in agreement with the value obtained by Babiker *et al.*, (1998) who reported that the tannin content of green leaves of *Cassia obtusifolia* was 2.34 %, but lower than the value obtained by Abdella (1989) who reported 2.50% for tannin content of green leaves of *Cassia obtusifolia*.

Fermentation was found to cause degradation of tannin content and this may be due to the action of enzymes.

4.6.3 Total polyphenols:

The total polyphenol content of green leaves of *Cassia obtusifolia* and kawal is shown in Table 6. Fermentation was found to cause significant decrease (p<0.05) in total polyphenol content. The total polyphenol content decreased from 4.77 to 3.80%.

The total polyphenol content of green leaves of *Cassia obtusifolia* in this study is in agreement with the value obtained by Ousman *et al.*, (2005) who was reported 4.8%.

Reduction in polyphenols may be due to activation of polyphenol oxidase (Dhankher and Chauhan, 1987).
Table 6. Anti-nutritional factors of green leaves of *Cassia obtusifolia* and kawal (as dry matter).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Tannin %</th>
<th>Phytic acid mg/100g</th>
<th>Polyphenol %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cassia obtusifolia Leaves</td>
<td>2.39 a (+0.012)</td>
<td>649.13 a (+7.137)</td>
<td>4.77 a (+0.252)</td>
</tr>
<tr>
<td>Dry Kawal</td>
<td>2.24 b (+0.021)</td>
<td>340.92 b (+5.952)</td>
<td>3.80 b (+0.200)</td>
</tr>
</tbody>
</table>

- Each value in an average of three values expressed on dry weight basis.
- Values are means (± standard deviation).
- Means not sharing a common letter in a column are significant at p ≤ 0.05 as assessed by Duncan’s multiple range tests.
CHAPTER FIVE
CONCLUSION AND RECOMMENDATIONS

5.1 Conclusions:

From the results of the present investigation, it can be concluded that:

1- Fermentation of Cassia obtusifolia leaves caused an increase in fat content, fiber content, crude protein and ash content.
2- The protein was fractionated according to solubility; the result indicated that fermentation resulted in an improvement of globulin, albumin, prolamin and glutelin but reduced insoluble protein fraction.
3- Fermentation activated some enzymes eventually leading to improvement in protein digestibility.
4- Fermentation increased Ca, Mg, P and Mn but reduced Na, Fe and Zn.
5- The unfermented leaves contain much higher values of the essential amino acids than fermented leaves.
6- Fermentation resulted in reduced levels of anti-nutritional factors (phytic, tannin, and total polyphenols).
5.2 Recommendations:

1- The acute shortage of meat and animal proteins in developing countries has made it necessary for consumer to rely heavily on protein from legumes (especially kawal) which are rich in protein, B-vitamins, dietary fiber and mineral content.

2- Attention should be directed toward novel methods for kawal processing and utilization.

3- More research should be conducted for reducing anti-nutritional factors, improving \textit{in vitro} protein digestibility and improving kawal protein by reducing amino acid losses.

4- More detailed nutrition studies are needed to investigate the effect of kawal fermentation on vitamins, flavor components, protein functionality and toxic constituents.
REFERENCES


