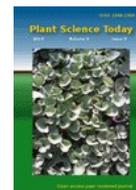




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Review Article

Cassava: meeting the global protein need

Vasavi Rama Karri* and Nirmala Nalluri

Department of Biotechnology, G.I.T, GITAM University, Visakhapatnam 530 045, India

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Corresponding Author

Vasavi Rama Karri

✉ vasavi8@gmail.com

Abstract

Cassava (*Manihot esculenta* Crantz) is a nutty flavored, starch-tuber perennial woody shrub originated from tropical America belongs to Euphorbiaceae family of plants. After rice and maize, it is considered as the third largest source of carbohydrate food in the tropics and its sweet, chewy underground tuber is one of the popular edible root-vegetables. It is ranked as a 21st century crop, as it acknowledges to the universal economy trends and climatic changes. Currently, use of cassava leaves as a potential source of protein, vitamins and minerals was reviewed. The effect of malnutrition on health and development of people and its control by using cassava leaves as a protein rich source were briefly discussed. Cascade use of cassava leaves, in industrial applications like natural filler for potential reinforcement of polypropylene based composites was also presented. Although, cassava leaves are vital source of essential nutrients, their anti-nutrients and cyanogenic glucosides content limits their consumption, which can be overcome by the development of an efficient, simple and low-cost processing methods for protein extraction from cassava leaves. There are supporting evidences for efficacy of cassava leaf protein in reducing the effect of malnutrition by the intake of protein rich cassava leaves, fortified with various common food items. So consumption of cassava leaves enriched with high protein, vitamin and mineral contents with the development of suitable processing technology to remove anti-nutrients can be an alternative source to meet the global protein demand.

Keywords

Cassava leaves; High protein; Nutrients; Anti-nutrients; Detoxification; Human consumption

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Introduction

Cassava (*Manihot esculenta* Crantz) is a perennial woody shrub originated from tropical America and its sweet, chewy underground tuber is one of the popular edible root-vegetables. The estimated production of cassava is more than 263 million tons and is extensively grown in 105 tropical and subtropical countries. The other names of cassava are yuca, manioc and tapioca. The height of the cassava plant is around 2.75 meters (9 feet) and the leaves are divided into 3–7 lobes (Fig. 1 & Fig. 2). It is an annually grown shrub, which is propagated through stem cuttings after the tubers have been harvested. It is an essential source of carbohydrates, vitamin B, C, calcium and other

essential minerals. Nutritional composition of this crop varies due to different factors like, variety and age of the harvested crop, nature of soil, climatic conditions and other environmental factors during cultivation.

Mostly, this crop is cultivated in various countries like Africa, Asia and Latin America with worldwide production of 250 million tons per year. According to previous reports, more than half of the total world cassava production is from African countries, the rest is from Asia and Latin America. Nigeria is world-leading producer of cassava, with its annual production of 52 million tons. By 2020 cassava production is predicted to be estimated around 291 million tons (Scott *et al.*, 2000). It is

mainly cultivated for starchy roots (Fig.3). In addition to root, other parts (stem, leaves and petioles) of cassava are edible and are extensively used as food in Africa (Lancaster & Brooks, 1983), which are available throughout the year (Moyo *et al.*, 1998).



Fig 1: Morphology of healthy cassava leaves

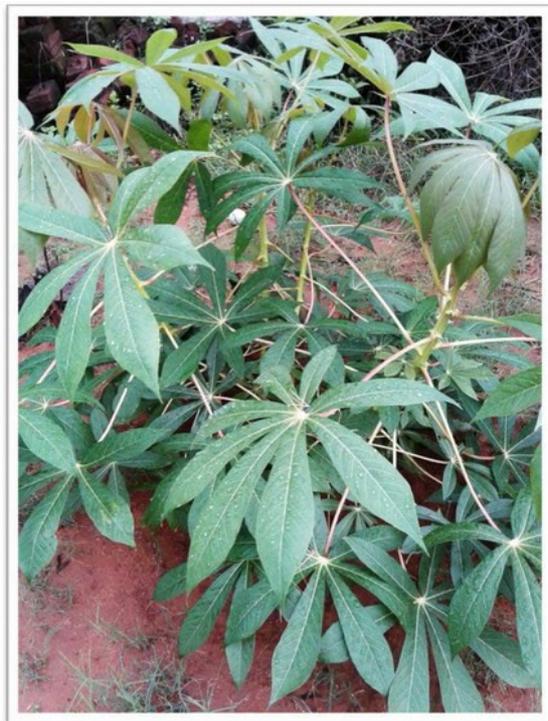


Fig 2: Two months old cassava plant

After processing, roots of cassava provide flour, which is used in preparation of bread, cakes and ice cream cones for human consumption (Fig.4 (a) & (b)). Cassava root starch is a highly valuable product and used in different brewing, textile, pharmaceutical, paper and oil industries. Cassava starch is also used as an alternative source in the production of ethanol and certain chemicals. By physical, chemical or enzymatic processes, native form of starch is modified into another form of starch, which is used in different

applications like, preparation of various types of foods, animal feed, high-quality paper and textile sizing. Biodegradable plastics are prepared by using both starch and modified starch. Various types of sweeteners are produced by hydrolysis of cassava starch with acidic and enzymatic substances or by using both. Cassava starch is used to prepare acetic acid, citric acid and itaconic acids, which are used to produce different products in food industry. In addition to this, products like synthetic resins, plastics and rubber are also produced from cassava starch. Leaves of cassava are used as feed for silkworms in silk industry and the ground stems are used to grow mushrooms. Finally, the waste obtained from cassava field and its processing industry is used in biogas production.

Nutritional value of cassava leaves

Nutritional composition of cassava depends on several factors like, age, variety, tissue (root or leaf) and environmental conditions. Compare to cassava leaves, carbohydrate content is rich in roots, where as, its leaves and by-product of root harvest are rich in protein (around 14–40 % of dry matter (DM)), minerals, vitamins B1, B2 and C, and carotenes (Adewusi and Bradbury, 1993). Compared to other green foods, the leaves are considered as vital protein source. According to the previous reports, the cassava leaf protein contains low methionine, lysine and isoleucine contents, but it's the amino acid profile responds positively with those of milk, cheese, soybean, fish and egg (Devendra, 1977). Even though, the cassava leaves are rich source of macro and micronutrient contents, the techniques used for extraction process leads to huge loss of nutrients (Hahn, 1988). To promote cassava production on large scale, the Nigerian Government has launched a campaign to utilize produced cassava for various aspects like export, for the production of glucose syrup and to use as an inclusion in wheat flour for bread baking. After this program government of Nigeria has developed some improved cultivars like TMS 30572, TMS 30555, MS 6 and the local cultivar of Idileruwa and distributed to farmers to increase cassava production.

Carbohydrate and Protein content of cassava leaves

The crude protein (5 to 7 g/100g), crude fat (1 to 2 g/100 g) and mineral (2 g/100 g) contents of cassava leaves are more than those of the legumes and leafy legumes, except soybean. Mostly, leaf protein of cassava ranges from 14 % to 40 % of DM in different varieties (Eggum, 1970). Cassava leaf crude protein content is comparable to that of fresh egg (10.9 g/100g) and its amino acid profile is well balanced compared to that of the egg (Jacquot, 1957) except for methionine, lysine and slightly isoleucine contents. Furthermore, essential amino



Fig 3: Cassava roots harvested after 8 months



(a)

(b)

Fig 4: (a) & (b): Processed form of cassava root

acid content of cassava leaves is higher than that of soybean protein content.

Vitamins and minerals content of cassava leaves

Cassava leaves are rich source of calcium, magnesium, manganese, iron and zinc (Wobeto *et al.*, 2006). When compared to liver (121 mg/kg FW) and egg yolk (58.7 mg/kg FW), cassava leaf meal is rich in iron (259 mg/kg), although iron content from plant origin is generally less compared to animal food sources. Similarly, cassava leaves are rich in thiamine content compared to legumes except for soybeans. The leaves have high thiamine content than several animal foods including fresh egg, cheese and 3.25 % fat whole milk. The content of riboflavin (vitamin B2) in cassava leaves (0.60 mg/100 g) is greater than that of legumes, leafy legumes, soybean, cereal, egg, milk and cheese. The vitamin A content of cassava leaves is comparable with that of carrots and exceeds those reported for legumes and leafy legumes. The vitamin C content (60 to 370 mg/100

g) of cassava leaves is high, compared to values reported for other vegetables. Thus, the overall vitamin content of the cassava leaves is compared and reported that it is better than legumes, leafy legumes, cereals, egg, milk and cheese.

Fiber content in cassava leaves

The content of fiber in cassava leaves is higher compared to the content of fiber in legumes and other leafy legumes and it ranges between 1 and 10 g/100 g FW. Dietary fiber plays an important role in healthy diet and can reduce problems related to constipation and also helps in prevention of colon cancer (Rock, 2007). The rich fiber nature of cassava may facilitate intestinal peristalsis and bolus progression (Favier, 1977, Baer *et al.*, 1996), but if the content of fiber is too high, it leads to negative effect in humans.

Anti-nutritional factors of cassava leaves

The anti-nutritional compounds like phytates, polyphenols, oxalates, nitrates and saponins

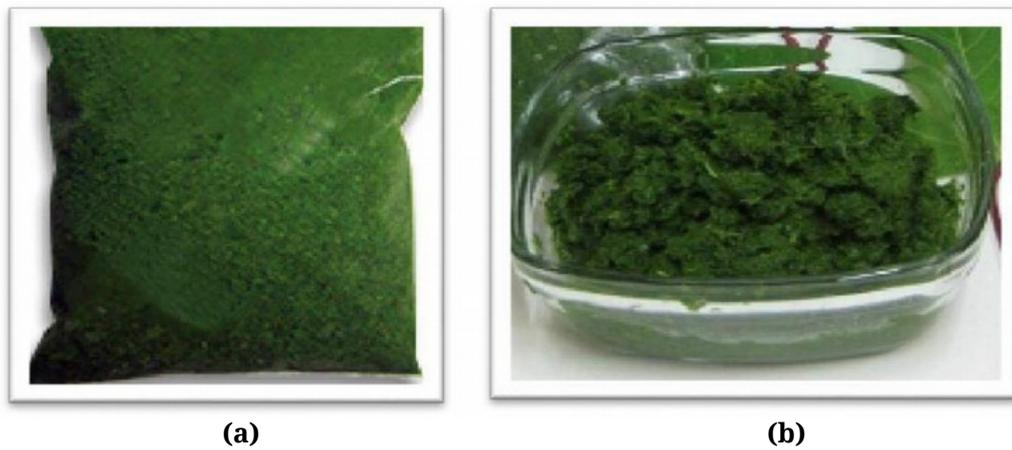


Fig 5: (a) & (b): Cassava leaves grounded to powder and paste

present in cassava leaves affect the digestion and the absorption of nutrients in the body. To combat this problem, different processing techniques are being developed to denature the anti-nutritional factors and to increase the nutritional value of the leaves and roots (Montagnac *et al.*, 2009b).

Based on earlier studies, it was reported that the polyphenols or tannin content of cassava leaves (2.1-120 mg/100g dry weight) (Wobeto *et al.*, 2007) interfere with the uptake of non heme-iron in consumers, decrease in absorption of thiamin, digestibility of starch (Silva and Silva, 1999), lipid and protein (Bravo, 1998). Cassava leaves contains 1.35 - 2.88 g/100g dry weight (Wobeto *et al.*, 2007; Correa, 2000;) of oxalates and are found to interfere with calcium uptake (Wobeto *et al.*, 2007).

Making use of protein rich cassava leaves as nutritional food

As the main target setup by the bio-economy is to make better and most diversified use of plants from the existing resource, there is a need to analyze cascade uses of cassava. The post harvesting process of cassava causes severe environmental problems due to generation of large amount of waste in the form of peel, pulp, wastewater and leaves, which can be converted into valuable products and energy in an environmentally friendly manner. Compared to different type of wastes produced during processing of cassava, leaves are the most essential ones, as they are highly rich in protein and nutrients, which are to be properly utilized in order to overcome the world problems like food insecurity and malnutrition prevailing in developing countries. Therefore, much attention is required on the cassava leaves to use them as an economical and sustainable source of protein and nutrients to meet the universal protein requirement.

Due to population growth global protein demand is increasing excessively. According to the

UN Food and Agriculture Organization (FAO), worldwide consumption of meat is estimated to reach 463 million tons by 2050, which requires a huge amount of feed protein, which will be difficult to produce in an eco-sustainable way. Most of the populations rely on plant source to meet the requirement of food protein, but it is not meeting the global protein demand. In order to overcome this problem, it is necessary to search for an alternative protein source like cassava leaves. But they are generally considered as waste or as a lesser by-product and are consumed in small quantities by humans.

Cassava leaves are a rich source of protein (17.7–38.1 % on dry weight basis), vitamins (B₁, B₂, C) as well as carotenoids and minerals. The total essential amino acid content of cassava leaf protein is similar to that of a hen's egg and greater than that of spinach leaf, soybean, oats and rice. The Congolese consider cassava as 'all-sufficient' as they can get bread from the roots and 'meat' from the leaves. The total share of cassava leaves in Congo is more than 60 % of all vegetables consumed and is available throughout the year.

Cassava leaves are consumed as a vegetable in almost 60 % of countries in sub-saharan, Africa and in some Asian countries like Indonesia, Philippines and Malaysia (Bokanga, 1994; Ngudi *et al.*, 2003b; Achidi *et al.*, 2008). In most of the African countries like Senegal to Mozambique, which are cultivating cassava, leaves are consumed as alternative source of protein and micronutrients, depending on the variety and recipes. In addition to the above mentioned countries, other countries like Tanzania, Kenya, Malawi and Madagascar also grow cassava for leaves in addition to roots, which are consumed as a vegetable (Dahniya, 1994; Moyo *et al.*, 1998).

Cassava leaves are consumed by pregnant women to increase breast milk production in Sierra Leone and Liberia (Aregheore, 2012). In Sierra Leone a typical cassava leaf dish is prepared with fish, capsicum, groundnut and onions. In

Indonesia, the young leaves are eaten as gulai daun singkong (cassava leaves in coconut milk), urap (javanese salad) and as a main ingredient in buntill (javanese vegetable rolls). In Sri Lanka, a side dish called as "Malluma" is prepared using cassava leaves. In Brazil, detoxified manioc is grounded and cooked to a dry, hard or crunchy meal known as farofa, which is used as a condiment and can be toasted in butter or eaten alone as a side dish. In Indonesia, the production of cassava leaves for human consumption is estimated around 0.5-0.7 million tonnes/year (Wargiono *et al.*, 2002). But, the exact quantitative survey of cassava leaves consumption is required.

Importance of cassava leaves as a potential reinforcement of polypropylene based composites

For so many years, to improve the mechanical properties and to obtain the characteristics required in actual application, polymer composites are prepared and combined with various types of natural reinforcing fillers. A lot of attention was focused in research and industrial sectors to use natural filler as a potential reinforcement of the composites in industrial applications. Use of natural fillers is more beneficial and advantageous over synthetic fillers and fibers, due to their lightweight, low cost, ability to reduce abrasion of machinery and its renewable & biodegradable nature. Now a days a lot of work has been done by researchers on the use of natural fillers like wood, wheat, barley, hemp, flax, coconut fruit crust and sugar cane bagasse as potential reinforcement of composites. Nowadays, natural fibers are emerging fast as potential alternatives to inorganic or synthetic materials for various applications as building materials and automotive components.

One of the natural filler with great potentials is a processed cassava leaf, which has continued to attract the interest of researchers in the areas of surface treatment of steel. Steel has attracted the interest of researchers due to the high percentage of carbon and nitrogen that is present in the composition of materials. Based on previous reports of Adetunji *et al.* (1991) cyanide solution from cassava can be used for the extraction of gold.

Based on the previous work, no research has been focused on processed cassava leaves as reinforcement in polymer matrix composites. Cassava leaves are considered as significant potential polymer filler because of their composition and its property of containing high amount of carbon and nitrogen in addition to cellulose and hemicellulose. A natural polymer is more advantageous because of its high polarity, due to the presence of large amounts of hydroxyls in its macromolecules, which interact with lignocellulosic fibers that result in improved mechanical properties. The use of polypropylene as a matrix polymer in composites has been

studied extensively due to their excellent mechanical, physical and thermal properties. Processed cassava leaves are expected to be a potential reinforcement in polymer matrix composite, because these are biomass by-product from cassava firm that can be obtained locally. Some researchers done work on this and they concluded that the tensile strength and hardness for the composites containing 7 % wt cassava leaf powder was observed to be higher than the other composites and pure polypropylene. The impart strength of the composites increase as percentage of cassava leaf powder is increases.

Treating cassava leaves with ammonia for protein extraction

Cassava is considered as one of the four top priority crops in Venezuela, which leads to increase in the consumption of cassava leaves. Even though, the leaves are rich in vitamins, proteins (14–40 %), minerals and carotenoids, but after cropping they are left in the field after harvesting the roots. Commonly, humans use cassava roots as food, animal feed and also as a source of starch for the chemical industries. On the other hand, cassava leaf meal has been used for feeding of animals like swine. At industrial scale, present techniques used to obtain protein concentrates from cassava leaves were failed due to low extraction yields and high content of tannins and left over fiber has poor nutritional value and low digestibility as ruminant feed.

To counteract the above problem, cassava leaves are treated with ammonia, which enables the separation of grass proteins from the fibers and reduces the tannin content that increases the potential of the treated leaves for animal feeding, especially for swine and poultry. While protein extraction, a solid fraction rich in cellulose is remained as a byproduct, which can be converted into sugars with higher yields and further converted into ethanol by fermentation.

During the process of ammonia treatment, the chemical composition of the leaves will not be effected. The extraction of white proteins from ammonia treated cassava leaves with the following conditions, pH 10, 90°C, and 1:10 solid/liquid ratio for 30 min rendered the maximum yield of 29.10 %. Leaves of cassava are used as high quality feed for poultry and swine because of its protein rich amino acid profile. Compared to the untreated sample (~50 %), the extraction yield is low in treated sample due to high lignin content (16.78 %).

In treated leaves, the yield of sugar was 54.72 %, which is 3.4 times higher compared to that of the untreated leaves and the yield can be further increased by loading ammonia. Different types of sugars produced during this process are glucose, xylose, and arabinose. Yield of white protein and sugars can be effectively increased with ammonia treatment.

Invention of appropriate advanced method for protein extraction from cassava leaves

Cassava leaves are the main source of protein in various cassava growing countries, where there is no alternative source of protein. The limiting factors for consumption of cassava leaves as food are toxicity and anti-nutrients. The toxicity of cassava leaves is due to cyanogenic glucosides (linamarin and lotaustralin), which are 5–20 times higher compared to roots, which may cause cyanide poisoning showing symptoms like headache, dizziness, nausea, diarrhea and vomiting. Because of the above said health problems, before consumption these anti-nutritional and toxic factors must be addressed properly during processing step.

Different traditional methods developed for cassava leaf processing are drying, pounding and long periods of boiling (Fig. 5 a & b). Among these methods, pounding cassava leaves in a wooden mortar and pestle for 15 minutes, followed by boiling in water for 10 - 120 minutes is the most frequently used method. Earlier studies reported that, there is a considerable loss of vitamin A and B contents and reduction in vitamin C content (60 %), even by ten minutes of boiling. The process of boiling may also denature the native enzymes like, linamarase and hydroxy nitrile lyase, which are involved in the breakdown of toxic compounds (linamarin and acetone cyanohydrin, respectively) present in the leaves. Currently used methods of processing are not able to completely detoxify the cassava leaves to the safe consumption level. Hence, there is a necessity to develop efficient, simple and low-cost processing methods to improve the nutritional quality of cassava leaves by reducing toxicity, anti-nutritional factors, low digestibility and bad taste.

The other limiting factors for the use of cassava leaf protein are high levels of chlorophyll, xanthophylls and fiber, which can be reduced by extraction of juice from the leaves to obtain leaf protein concentrate (LPC) by coagulation through steam injection. According to the previous studies, during LPC preparation, a variation was observed for different factors like extraction efficiency, nutritional value, methionine and lysine content, which might be because of different extraction methods and tannin content in various cassava varieties and cultivars. But, finally the efforts to prepare LPC on industrial scale was not succeeded due to low protein recovery along with high tannin content and poor digestibility of the residue fiber.

Further research is required to develop innovative technologies to improve protein recovery and valuable by-products. Normally, cassava leaf meal (CLM) or cassava pellets are prepared from whole leaves or stems by reducing the moisture content (15–20 %) either with sun drying or mechanical pressing.

According to the United Nations Food and Agriculture Organization, it is estimated that one in nine or 795 million people out of 7.3 billion people in the world were suffering from chronic malnutrition

during the period of 2014-2016. With the development of an efficient cassava leaf protein extraction methods we could provide an alternative protein and nutritional source to the majority of the people to increase the food and nutritional security.

Preparation of nutritional diets from cassava leaf protein

Cassava based dishes are highly consumed in areas of its cultivation, where they have regional, national or ethnic importance. Different modified traditional foods or nutritionally balanced foods (snacks) can be prepared with cassava leaves to provide sufficient amount of protein, minerals and vitamins in their diet, based on consumer preferences. In this orientation, efforts are made in Brazil to reduce the effect of malnutrition in pregnant women and children. In this process a food supplement called as 'multimistura' has been prepared by using cassava leaf powder as one of the ingredients. To reduce poverty, these type of food products need to be promoted in order to encourage cassava leaf consumption as a vital component of the diet. Common food items prepared with nutrient rich proteinaceous cassava leaves can be a possible cost effective and sustainable approach to deal protein and micronutrient deficiencies in millions of people around the world.

Improvement of the nutritional value of cassava through biofortification and processing methods

Because of its importance as a staple crop, cassava needs biofortification. Since 2005, the Bill and Melinda Gates foundation has promoted a global effort to develop cassava germplasm enriched with bio-available nutrients (BioCassava Plus [<http://biocassavaplus.org/>]). This initiative is called as Bio-Cassava Plus and has 6 major objectives like:

- (1) To increase the mineral content of zinc and iron
- (2) To increase the protein content
- (3) To increase vitamins A and E content
- (4) To decrease the content of cyanogens
- (5) To delay the post-harvest deterioration
- (6) To develop virus-resistant varieties.

Protein value of biofortified cassava

By employing cross breeding of wild varieties, researchers tried to improve the nutritional content of cassava. Compared to typical cassava cultivars, two hybrids developed by this process exhibited enhanced levels of protein content. The interspecific hybrid (UnB 033) and *Manihot dichotoma* developed through cross breeding showed high protein content (26.4 %) in the leaves, compared to other cassava cultivars (24.25 %) (Nassar *et al.*, 2004). Compared to the typical cassava cultivars (EB01), the above mentioned hybrid showed 5-times more manganese and zinc content and the content of cyanide was also moderate, *i.e.*, 128.5 ± 11.7 mg cyanide/kg FW

(Nassar *et al.*, 2004). A second valuable hybrid was developed by crossing with *Manihot oligantha* (Nassar and Dorea, 1982), in which the content of protein was double in the roots compared to typical cassava cultivars. The interspecific hybrid had high protein content (4.5 %), than the normal cassava cultivars (0.9 % to 1.4 %). Moreover, the peel of developed cassava hybrid had higher protein content (8.06 %) than typical cassava cultivars (from 1.11 % to 2.09 %) (Nassar and Dorea, 1982). Compared to common cultivar, the developed interspecific cassava hybrid had an improved amino acid profile with enhanced lysine (10 times more) and methionine (3 times more) contents.

Through transgenic approach, efforts are made to reduce the cyanogens content in cassava by suppressing cyanogen synthesis or accelerating cyanogen breakdown (Siritunga and Sayre, 2007). Alternatively, Siritunga and Sayre (2007) reported that, cyanogen content can be reduced by enhancing cyanogen detoxification and cyanide volatilization during processing (Siritunga and Sayre, 2007), which impart additional benefit by increasing the protein content of the roots.

Postharvest processing to enhance cassava protein

The protein content and quality of edible cassava products can be enhanced by adopting suitable postharvest processing techniques. During the process of solid-state fermentation via *Aspergillus niger* (Iyayi and Losel, 2001), crude protein content of cassava root and leaf by-products can be increased along with decreasing the cyanogen content up to 95 % (Birk *et al.*, 1996). Similar type of study was reported by Smith *et al.*, 1986 by solid-state fermentation via *Sporotrichum pulverulentum* to improve the protein content of cassava roots. In the above process, the fungus was able to produce 30.4 g of high-quality protein per 100 g of dry cassava in 48 h at 45°C. The bio-availability of protein in fermented cassava leaves was similar to that of soybean pressed cake diets delivered to ruminants. So, fermented cassava leaves can replace soybean as a source of protein (Bakrie *et al.*, 1996).

The protein value of cassava meal could be enhanced by the development of cassava leaf protein concentrates with low fiber. Compared to cassava leaf meal (22 %), crude protein content of cassava leaf protein concentrate was twice (42 % to 43 %) (Castellanos *et al.*, 1994). Indeed, Fasuyi and Aletor (2005) reported that cassava leaf protein concentrates had high crude protein, fat and gross energy content and low crude fiber and ash contents than those of cassava leaf meals. According to FAO/WHO reports (1973), even though methionine (2.48 g/16 g N) and cysteine were limited in protein concentrates, the amino acid analysis determined that lysine (6.80 g/16 g N), leucine (9.65 g/16 g N), valine (6.30 g/16 g N) and tryptophan (2.31 g/16 g N) contents exceeded those of other protein sources like soybean, fish and egg. In addition, the water

absorption capacity (181.5 % ± 45.4 %), fat absorption capacity (19.2 % ± 1.2 % to 40.8 % ± 1 %), emulsion capacity (32.5 % ± 8.3 %), stability (42.9 % ± 2.9 %), the least gelation concentration (12.5 % ± 3.4 %), the foaming capacity (32.1 % ± 7.7 %, stability (10.2 ± 4.1 cm³), and the solubility of cassava leaf protein concentrates in acid and alkaline media support the nutritive potential of cassava leaf protein concentrate as an indispensable source of vital elements (Fasuyi and Aletor, 2005). Different viscous products like soups, protein-rich carbonated beverages and curds; or as additives for gel formation in food products can be prepared from the cassava leaf protein concentrate formulations because of their above specified properties. These properties also enable cassava leaf protein concentrate to form and stabilize emulsions in food products (Fasuyi and Aletor, 2005). Based on growth bioassays in rats, it was reported that even though cassava leaf protein concentrate is a potential source of protein, it should be fed with another viable protein source, to provide di-methionine supplements to support growth (Fasuyi and Aletor, 2005). Therefore, leaf protein concentrate of cassava would act as a potential alternative protein source for human and animal nutrition with other sources of protein (to provide amino acids which are absent in cassava leaf protein concentrate) to meet the global protein requirement.

Conclusion

Cassava root is an important source of carbohydrate rich food among the tropics. In addition to carbohydrates, roots also contain low content of crude protein, vitamins and minerals. On the other hand, cassava leaves contain high content of essential amino acids compared to soybean protein, which enable them to use as an important source of protein to reduce the effect of malnutrition in majority of the countries caused due to protein deficiency. Even though, cassava leaves are an important source of essential amino acids, the limiting factor for their consumption is presence of anti-nutrients (cyanides, polyphenols, oxalates and saponins etc.), which need to be removed properly during the process of protein extraction. With the development of suitable technology to eliminate anti-nutrients during protein extraction, cassava leaves can be opted as one of the possible source of high protein and nutrients to meet the global protein demand for economical and sustainable development.

Competing Interest

The authors declare that they have no competing interests.

Authors' contributions

KVR carried out literature search on the effect of malnutrition on human health and selected this topic to combat this problem. NN collected literature related to this topic and prepared the manuscript. KVR coordinated NN in finalizing the manuscript by interpreting the data in

correct orientation. Both KVR and NN made significant efforts in completing this manuscript.

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