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Fermentation of Cassava Leaves Improves Provitamin A Carotenoid Bioefficacy in Mongolian gerbils (*Meriones unguculatus*)

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Authors' contributions

This work was carried out in collaboration between all authors. Authors LZ, CD and ST designed the study and wrote the protocol. Authors LZ and CD managed the analyses of the study and performed the statistical analysis. Author LZ managed the literature searches and wrote the first draft of the manuscript. Authors CD, SN and ST read and revised the first draft of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The aim of this study was to evaluate the effect of fermented cassava leaves used as diet on provitamin A carotenoid bioefficacy.
Study Design: Carotenoid analysis of fermented (F) and non-fermented (NF) cassava leaves, feeding Mongolian gerbils with F and NF leaves and β-carotene bioconversion evaluation.
Place and Duration of Study: Felix Houphouet-Boigny University, Abidjan (March to August 2015) and University of Wisconsin-Madison, USA (March to June 2016).
Methodology: Fermented cassava leaves were fed to Mongolian gerbils (*Meriones unguculatus*) and compared with non-fermented leaves and controls. Gerbils (32 days old, *n* = 46) were vitamin A (VA)-depleted for 3 weeks. After depletion, baseline gerbils (*n* = 6) were killed and remaining gerbils (*n* = 40) were weight-matched to 4 groups (*n* = 10/group) in the following treatments: VA-free feed (VA-); non-fermented leaves (NF); fermented leaves (F); and VA-free feed with daily oral doses of retinyl acetate dissolved in oil (VA+). The feeds were prepared using F and NF leaves at 3.53 and 4.27%, respectively, to equalise daily theoretical VA intake at 35 nmol β-carotene/g feed. Serum and livers were analysed using UPLC®.

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Results: The daily feed intake from the F and NF groups did not differ (4.38 ± 0.40 g). Serum retinol concentrations did not differ among groups, but the VA+ group had higher liver retinol (1.39 ± 0.32 µmol/liver) than the F and NF groups ($P < 0.05$). The calculated bioconversion factors were 13 and 37 µg β-carotene equivalents to 1 µg retinol for the F and NF groups, respectively.
Conclusion: This study showed that the provitamin A carotenoids from small quantities of F and NF leaves were effective at maintaining VA status of gerbils when assessed by liver stores.

Keywords: Cassava leaves; carotenoid bioefficacy; fermentation; Mongolian gerbils.

1. INTRODUCTION

Vitamin A (VA) is a fat-soluble vitamin including retinol, retinal, and retinoic acid [1]. This essential nutrient plays a vital role in numerous biological functions such as the visual system, immune response, cellular differentiation, and embryonic development [2,3]. The lack of this nutrient in the human body, usually referred to VA deficiency (VAD), is a global nutritional problem that impairs the health of infants, children, and pregnant and lactating women, especially in developing countries [4]. Indeed, VAD causes varying degrees of xerophthalmia, from reversible night blindness to irreversible total blindness [5]. Faced with VAD and its physiological disorders (e.g., anaemia, xerophthalmia, growth retardation, morbidity, and mortality), dietary diversification remains a sustainable intervention [6].

The dietary based strategy for VAD mitigation in tropical Africa was supported by FAO and consists of increasing production and consumption of provitamin A-rich plant foods because they are cheaper and more available than animal food products [7,8]. Provitamin A-rich foods are those which have relatively high concentrations of β-carotene, β-cryptoxanthin, or α-carotene, which can be converted by humans to VA. Leafy vegetables, including cassava leaves, are considered good sources of provitamin A-carotenoids for approximately 60% of African populations. They are particularly good sources of β-carotene [7,9]. Although cassava leaves provide provitamin A carotenoids, they have potential toxicity due to cyanogenic glucosides if not prepared in ways to destroy these compounds. Furthermore, the leaves contain some antinutritional factors, such as tannins, oxalate, and phytic acid, which may affect nutrient bioavailability [10,11]. Traditional preparation techniques, such as cooking in water after shredding or chopping, followed by sun-drying, pounding, and washing, can remove more than 90% of the cyanogens contained in cassava leaves before consumption [9,12].

Another method used for cassava leaf detoxification is fermentation. Indeed, some populations of West and Central Africa ferment cassava leaves for 3 to 4 days to prepare dishes with a distinct flavour [13]. In addition, fermentation can enhance the nutritional quality and improve the digestibility of vegetables by producing beneficial byproducts [14]. All these processing methods (i.e., cooking, pounding, washing, sun-drying, and fermentation) may influence the bioaccessibility and bioavailability of provitamin A carotenoids as demonstrated by studies related to biofortified cassava roots [15–18]. With the exception of a study conducted in VAD Wistar rats fed dried and powdered cassava leaves [19], the bioavailability of provitamin A carotenoids from traditionally fermented cassava leaves has not been documented. Thus, this work was undertaken in order to assess the impact of fermentation on the bioefficacy of provitamin A carotenoids from cassava leaves by feeding Mongolian gerbils (*Meriones unguculatus*) either fermented or non-fermented leaves with appropriate controls. Mongolian gerbils are an appropriate animal model to study the bioefficacy of provitamin A carotenoids to retinol [20].

2. MATERIALS AND METHODS

2.1 Cassava Leaves and Fermentation Processing

Cassava leaves were collected at maturity from a peri-urban farmland (latitude: 5°19′14″ North; longitude: 4°22′59″West) located in Abidjan District (Ivory Coast). The leaves were authenticated by the National Floristic Center (University Felix Houphouët-Boigny, Abidjan). The leaves were removed from the plants, washed several times with distilled water and drained at ambient temperature. Leaves were cut into small pieces and separated into two portions of 250 g each. The first portion was wrapped in clean papaya leaves for 4 days to induce natural fermentation in a covered plastic box. Afterwards, the fermented (F) leaves were oven-

dried (50°C/3 days) and ground into a powder with a laboratory crusher. The second portion was not subjected to the fermentation step but was used as the non-fermented (NF) leaves. All the dried and powdered samples were stored at -18°C in airtight containers before being transported to the University of Wisconsin-Madison, WI, USA, for further experimentation.

2.2 Proximate Analysis of Leaves

Moisture, ash, protein, and lipid were determined using established methods and expressed on a dry matter basis [21]. Carbohydrates were calculated by difference and vegetable energy conversion factors [22] were used for the calculation of energy as indicated by the following formulae:

Carbohydrates: 100 – [weight in grams (moisture + proteins + lipids + ash + fibers)]

Energy: (proteins x 2.44) + (carbohydrates x 3.57) + (lipids x 8.37)

2.3 Carotenoids Analysis of Leaves

Total carotenoids of fermented (F) and non-fermented (NF) leaves were extracted using a previously reported method [3] with slight modifications. Powdered samples (0.05 g) were mixed with 5 mL ethanol containing butylated hydroxytoluene (0.1%, w/v) and heated in a water bath at 85°C for 5 min. Then, 400 µL KOH in water (80% w/v) was added for saponification and the suspension was mixed using a vortex for 20 s and heated in a water bath at 85°C for 5 min. The tubes containing the reaction mixture were placed in ice after introducing 3 mL deionised water and carotenoids were extracted three times with 4 mL of hexanes. β-*apo*-8'-carotenal was used as an internal standard and was added after saponification to account for mechanical losses. The combined extracts were dried under nitrogen and reconstituted in 1 mL 50:50 methanol-dichloroethane. For the carotenoid identification and quantification, 25 µL extract was injected into an HPLC system (Waters Corporation; Milford, MA, USA) consisting of a 717 autosampler, 1525 binary pump, and a 2996 photo-diode array detector. The column used was a C30 YMC carotenoid column (4·6 x 250 mm, 3 mm; YMC America; Allentown, PA, USA). The HPLC solvent gradient included methanol-water (92:8, v/v) with 10 mM ammonium acetate (solvent A) and 100% methyl tertiary-butyl ether (solvent B). Samples were analysed at 1 mL/min with a 30-min linear gradient from 70 to 40% solvent A. Lutein, β-carotene (including all-*trans*, 13-*cis*, and 9-*cis*), and α-carotene were identified and quantified using HPLC-purified standards. Chromatograms were generated at 450 nm.

2.4 Animal and Procedures

Male Mongolian gerbils (32 days old, *n* = 46) were obtained from Charles River Laboratories (Kingston, NY, USA) and housed in groups of 2 or 3 in plastic cages and immediately fed a VA-free rodent mix from Envigo-Teklad (Madison, WI, USA). The animals were given free access to feed and water. Room temperature and humidity were held constant with a 12 h light/dark cycle. The gerbils were weighed daily for the first week and then three times per week for the remainder of the study. After 3 weeks of VA depletion, gerbils (*n* = 6) were euthanised by exsanguination while under isoflurane anaesthesia to determine baseline serum and liver VA concentrations. The remaining gerbils (*n* = 40) were weight-matched and sorted into 4 groups (*n* = 10/group) corresponding to the treatments described below. After a 5-week treatment period, the remaining gerbils were exsanguinated and blood samples were centrifuged at 2200 X *g* for 15 min in BD Vacutainer Gel and Clot Activator tubes (Becton Dickinson, Franklin Lakes, NJ, USA) for serum isolation. Livers were excised and stored at -80°C. Animal handling procedures were approved by the College of Agriculture and Life Sciences Animal Care and Use Committee at the University of Wisconsin-Madison.

2.5 Experimental Design

Dietary treatments were based on equalising the provitamin A content in the NF and F cassava leaf groups as indicated in Table 1. Two control groups were fed VA-free feed with daily oral doses of VA (retinyl acetate) dissolved in cottonseed oil (VA+ group) or a placebo dose (VA- group). The NF and F groups also received a placebo cottonseed oil dose to match the volume of the VA+ group. Due to the difference in total carotenoids contents of F and NF cassava leaves, the feeds were prepared using F and NF cassava leaves at 3.53 and 4.27% respectively, to provide 35 nmol/g feed to the gerbils daily. The concentration of VA (retinyl acetate) in cottonseed oil was 0.98 nmol/µL, and the amount to be fed was based on 12 µg β-carotene equivalents to 1 µg retinol.

Table 1. Composition of gerbils diets used for experimentation

Components	VA free diet (g/kg feed)	NF diet (g/kg feed)	F diet (g/kg feed)
Casein (VA free)	200	191.46	192.94
L-cysteine	3	2.8719	2.8941
Sucrose	360.5	345.1067	347.7744
Maltodextrin	120	114.876	115.764
Maize starch	150	143.595	144.705
Cottonseed oil	60	57.438	57.882
Cellulose	60	57.438	57.882
Mineral mix	35	33.5055	33.7645
Magnesium oxide	1.8	1.72314	1.73646
Calcium phosphate (dibasic)	2	1.9146	1.9294
Vitamin mix	5	4.7865	4.8235
Vitamin E acetate	0.2	0.19146	0.19294
Vitamin D3	0.004	0.003829	0.003859
Choline bitartrate	2.5	2.39325	2.41175
Non-fermented cassava leaves	0	42.7	0
Fermented cassava leaves	0	0	35.3
Total (g)	1000.004	1000.004	1000.004

VA: Vitamin A; F: Fermented cassava leaves; NF: Non-fermented cassava leaves

2.6 Liver and Serum Analysis

Vitamin A and β-carotene of serum and liver were analysed using modified procedures [23]. Serum (250-500 µL) was aliquoted, proteins denatured with ethanol, extracted three times with hexanes (1 mL), and dried under nitrogen. Samples (0.5 g) of liver were ground with 3-5 g anhydrous sodium sulfate, followed by several extractions with dichloromethane and filtered into 50 mL volumetric flasks. A 5 mL aliquot of the extract was dried under nitrogen. C_{23}-β-*apo*-carotenol was used as an internal standard [24]. Serum and liver extracts were reconstituted in methanol-dichloromethane (100 µL, 50:50; v/v) and 2 µL was injected into the UPLC® system (Waters Corporation; Milford, MA, USA). Liver retinol was the sum of retinol and all identifiable retinyl esters. Chromatograms were generated at 325 nm for retinoids and 450 nm for carotenoids.

2.7 Statistical Analysis

Values were reported as means ± SD. Data were analysed using XLStat 2016.2 (Addinsoft, NY, USA). Carotenoid composition, gerbil weights, feed intakes, and serum and liver VA and β-carotene concentrations were compared using one-way ANOVA. Differences among processed leaves and treatment groups were determined using least significant differences (LSD) at $P < 0.05$.

3. RESULTS AND DISCUSSION

3.1 Proximate and Carotenoid Composition of Cassava Leaves

The proximate composition of F and NF cassava leaves is presented in Table 2. The nutritive parameters differed ($P < 0.05$) except for moisture, protein, and carbohydrate contents. Ash content ranged from 6.59 ± 0.01% (F leaves) to 8.46 ± 0.58% (NF leaves). There was variation in the fibre contents ranging from 24.87 ± 0.35% (NF leaves) to 30.22 ± 0.50% (F leaves). The mean values of proteins and carbohydrate contents of NF and F cassava leaves were 21 and 34%, respectively. The highest energy value (229.0 ± 4.62 kcal/100 g) was reported for NF cassava leaves.

Carotenoid contents and their chromatographic profile are given in Table 3 and Fig. 1, respectively. The identified carotenoids were lutein, 13-*cis*-β-carotene, α-carotene, all-*trans*-β-carotene, and 9-*cis*-β-carotene. Lutein and all-*trans*-β-carotene were the major carotenoids while α-carotene, 13-*cis*-β-carotene and 9-*cis*-β-carotene represented less than 20% of total carotenoids. Fermentation resulted in a higher total β-carotene contents and consequently higher theoretical VA value. In green leaves, carotenoids components are organised in

pigment-protein complexes located in cell chloroplasts [25] and fermentation processing may make carotenes easily extracted by breaking down carotenoids-protein bounds. The examination of the carotenoid profile showed that the β-carotene contents of dried cassava leaves (311 – 377 nmol/g) were higher than results reported in a previous gerbil study [7]. Many factors affect the carotenoid content of various leafy vegetables, such as variety, location, cultivation, and post-harvest handling practices [26].

Table 2. Proximate composition of dried fermented (F) and non-fermented (NF) cassava leaves

Nutritive parameters	NF	F
Moisture (%)	4.08 ± 0.00[a]	4.24 ± 0.14[a]
Ash (%)	8.46 ± 0.58[a]	6.59 ± 0.01[b]
Fibre (%)	24.87 ± 0.35[b]	30.22 ± 0.50[a]
Lipids (%)	6.29 ± 0.01[a]	5.31 ± 0.03[b]
Proteins (%)	21.73 ± 0.19[a]	20.12 ± 0.00[a]
Carbohydrates (%)	34.55 ± 1.13[a]	33.51 ± 0.68[a]
Energy (kcal/100 g)	229.0 ± 4.62[a]	213.2 ± 2.68[b]

Data are presented as means of triplicate analyses ± SD. Means with the same superscript letter in the same raw for a parameter are not different at P > 0.05. F: Fermented cassava leaves; NF: Non-fermented cassava leaves

Table 3. Carotenoid composition of dried non-fermented (NF) and fermented (F) cassava leaves

Carotenoid (nmol/g)	NF	F
Lutein	543 ± 12.3[a]	558 ± 2.83[a]
α-carotene	1.30 ± 0.50[b]	2.84 ± 0.08[a]
13-*cis*-β-carotene	36.6 ± 3.63[a]	41.5 ± 7.95[a]
9-*cis*-β-carotene	61.0 ± 5.58[b]	75.0 ± 1.23[a]
All-*trans*-β-carotene	311 ± 34.2[b]	377 ± 6.66[a]
Total β-carotene	408 ± 36.2[b]	493 ± 6.71[a]
Theoretical VA[1]	819 ± 73.0[b]	989 ± 13.5[a]

Data are presented as means of triplicate analyses ± SD. Means with the same superscript letter in the same raw for a parameter are not different at P > 0.05. F: Fermented cassava leaves; NF: Non-fermented cassava leaves.
[1]The theoretical VA value was determined as twice the amount of total β-carotene + α-carotene

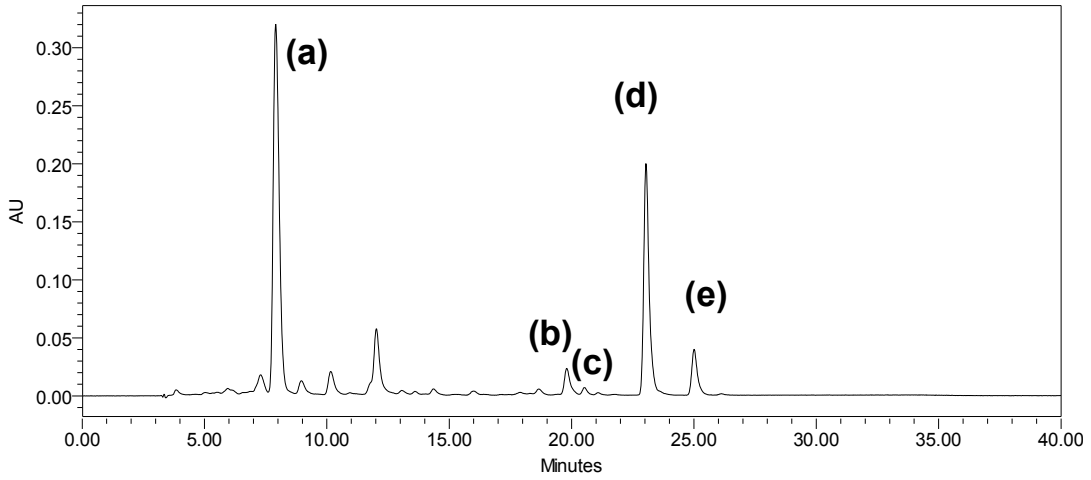

Fig. 1. Chromatographic profile of carotenoids from cassava leaves. (a): lutein, 7.8 min; (b): 13-*cis*-β-carotene, 20 min; (c): α-carotene, 20.7 min; (d): all-*trans*-β-carotene, 23.3 min; and (e): 9-*cis*-β-carotene, 25.3 min

3.2 Gerbil and Liver Weights and Feed Intake

The purpose of this study was to measure the bioefficacy of provitamin A carotenoids from F and NF cassava leaves by feeding Mongolian gerbils. Gerbils are considered a more accurate predictor of provitamin A bioefficacy than rats and mice [27]. Indeed, these animals show similar metabolism of α- and β-carotene to humans [16,20]. During this study, the gerbils continued to gain weight but the final weight did not differ among groups. The final body weights of gerbils ranged from 61.4 ± 4.4 g at baseline to 65.0 ± 5.0 g in the VA+ group. This result could be corroborated to the small percentage of leafy vegetables added to the diets. In addition, the depletion period did not impair the growth rate because the initial liver retinol provided the VA necessary for maintaining normal growth [28]. The group fed F leaves had higher liver weight (2.55 ± 0.29 g) compared with the NF group (2.22 ± 0.30 g). The daily feed intake of gerbils from the F and NF groups did not differ with values of 4.35 and 4.42 g/day, respectively. The positive control group (VA+ group) received 26 ± 6.0 nmol retinyl acetate/day, which was equivalent to 1 µg retinol to 12 µg β-carotene equivalents fed.

3.3 Serum and Liver Vitamin A Contents

Liver and serum vitamin A contents are depicted in Fig. 2. The VA+ group had higher liver retinol (0.62 ± 0.15 µmol/g liver and 1.39 ± 0.32 µmol/liver) than the NF group but did not differ from the F group ($P < 0.05$) (Fig. 2A and 2B). Indeed, many factors such as matrix meal effect, nutrient and genetic status of the host, effectors of absorption and bioconversion may affect in a

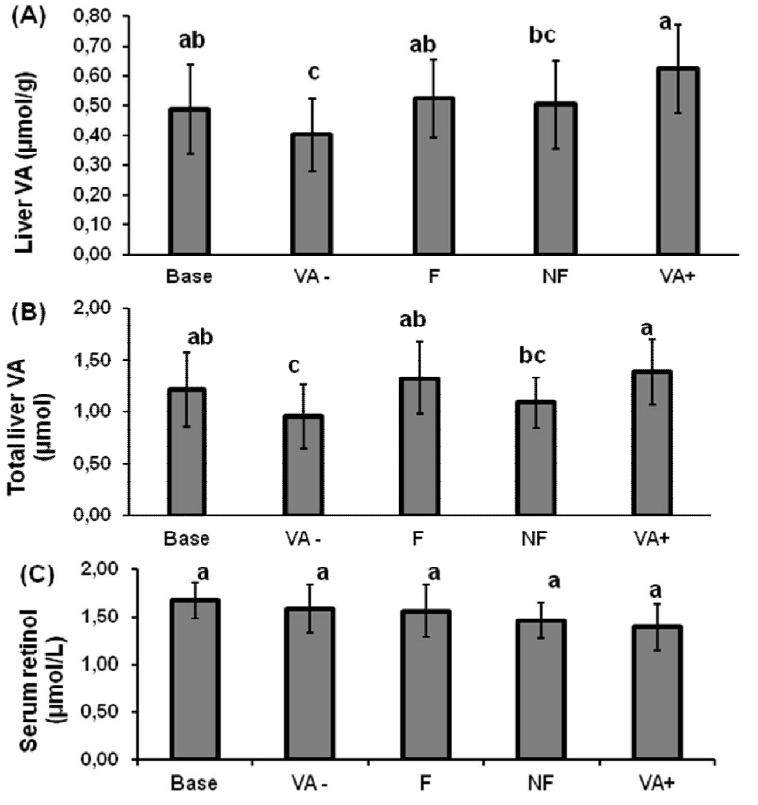

Fig. 2. (A) Liver retinol concentration (µmol/g liver); (B) Total retinol liver (µmol/liver) and (C) Serum retinol concentration (µmol/L) of gerbils fed fermented and non-fermented cassava leaves. Liver retinol includes retinol and all identifiable retinyl esters. Means with uncommon letters are significantly different from each other ($P < 0.05$). Base: Baseline group; VA-: Vitamin A free fed control group without a daily oral dose of retinyl acetate; VA+: Vitamin A free fed control group with a daily oral dose of retinyl acetate; F: Fermented cassava leaves diet group; NF; Non-fermented cassava leaves diet group

different way the bioavailability of carotenoids [4]. Both the F and NF groups maintained baseline retinol concentrations. Serum retinol concentrations did not differ among the groups and values ranged from 1.40 ± 0.24 µmol/L (VA+ group) to 1.68 ± 0.19 µmol/L (Baseline) (Fig. 2C). Serum retinol concentrations were normal (>0.7), and this result is not surprising because serum retinol is under homeostatic control and only decreased when the liver is severely VA deficient [29]. Thus, the best method to assess VA status remains the direct measurement of liver reserves of VA as demonstrated by many studies [3,30, 31]. β-carotene was the only carotenoid detected in the livers with a mean value of 6.41 nmol/liver.

3.4 β-carotene to Retinol Bioconversion Factors

The calculated bioconversion factors were 13 and 37 µg β-carotene equivalents to 1 µg retinol for the F and NF groups, respectively. During metabolism, provitamin A carotenoids are converted by oxidative cleavage to all-*trans*-retinal that is reduced to all-*trans*-retinol. The retinol is then stored in the esterified form in the liver and other tissues, such as lung and fat [32]. The gerbils replete with cassava leaves (especially the F group) showed liver retinol content higher than the VA- group indicating evidence for the bioavailability of carotenoids from cassava leaves and globally for dark green vegetables. It is important to mention that carotenoids were not detected in serum as discussed in previous studies [23,33]. Bioconversion factors used to convert provitamin A carotenoids to retinol were calculated by comparing VA stored in the liver of the F and NF groups with that in the VA+ group. In our study, the bioconversion factor for the F group was similar to that (12:1) used by the Institute of Medicine [34]. However, this calculated bioconversion factor is lower than those (26-28:1) of studies conducted with dark green leafy vegetables [35] demonstrating the positive impact of fermentation on provitamin A carotenoid bioavailability. This result could be linked to the positive effect of fermentation processing on digestibility [14]. Indeed, bioavailability depends on the processing of the food matrix, which can improve nutrient absorption through the gastrointestinal tract [19]. Both F and NF cassava leaves were used in small quantities (3.53 and 4.27%) compared with the amounts usually consumed by populations in combination with starchy foods. Additionally, cassava roots are considered a food security crop. By increasing the consumption of cassava leaves, especially after fermentation, VA status of children could be improved as demonstrated in some human studies feeding dark green leafy vegetables [36].

4. CONCLUSION

In conclusion, this study showed that the provitamin A carotenoids from small quantities of F and NF cassava leaves were effective at maintaining VA status of Mongolian gerbils as assessed by liver stores. Because cassava roots are widely consumed, the leaves could be used as an affordable source of provitamin A carotenoids to improve intake by poor populations in order to alleviate hidden hunger and particularly VAD. For this, public policy based on the promotion of native leafy vegetables to improve the nutritional status of the population will be required.

ETHICAL APPROVAL

As per international standard or university standard was written ethical approval has been collected and preserved by the authors.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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