European Journal of Nutrition & Food Safety

12(11): 90-103, 2020; Article no.EJNFS.63512
ISSN: 2347-5641

In vitro Protein Digestibility and Iron Bioavailability According to Agro-Ecological Zone and Stage of Maturity of *Moringa oleifera* Lam Leaves

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Authors' contributions

This work was carried out in collaboration among all authors. Author AAJA designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors FNE and MCM managed the analyses of the study. Author MCM managed the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/EJNFS/2020/v12i1130325
Editor(s):
(1) Dr. Rasha Mousa Ahmed Mousa, University of Jeddah, Saudi Arabia.
Reviewers:
(1) Jeje, Oludiya Ayorinde, Federal Polytechnic Auchi, Nigeria.
(2) Tiziana Maria Sirangelo, CREA - Council for Agricultural Research and Agricultural Economy Analysis, Italy.
Complete Peer review History: http://www.sdiarticle4.com/review-history/63512

Original Research Article

Received 27 September 2020
Accepted 02 December 2020
Published 19 December 2020

ABSTRACT

This study aims to determine the influence of agro-ecological zone and stage of maturity on *in vitro* protein digestibility (IVPD) and iron bioavailability from *Moringa oleifera*.leaves. The young and mature leaves were collected from three farms in each agro-ecological zone in Cameroon and processed in powders. Nutrient contents (proteins, total and free iron), bioactive compounds (total fiber, total polyphenols, phytates), IVPD, and iron bioavailability were determined. The stage of maturity affects significantly the variations of total iron contents (90.10%) while the agro-ecological zone affects significantly the variations of total polyphenol (48.28%), total fiber (80.97%), and phytates (46.48%) contents. Total protein contents were affected by the interaction of both

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(35.88%). Mature leaves have higher total iron, bioactive compounds contents in the mean compared to young leaves, and whatever the agro-ecological zone and stage of maturity of leaves, the IVPD is near casein, and bioavailability of iron is low. The young leaves have higher IVPDs and lower bioavailability of iron while mature leaves have lower IVPDs and higher bioavailability of iron. To alleviate protein malnutrition and iron deficiency through the utilization of *M. oleifera* leaves, it's necessary to consider the effects of stage of maturity and agro-ecological zone on bioactive compounds contents and their anti-nutritional properties.

Keywords: Moringa oleifera leaves; agro-ecological zone; stage of maturity; protein digestibility and iron bioavailability.

1. INTRODUCTION

Moringa oleifera also is known as "tree of life" is a crop originated from Asia and widely grown in Africa and particularly in all agro-ecological zones in Cameroon [1]. Their leaves are rich in protein, calcium, iron, zinc, can be eaten as raw salad, cooked in sauces, or stored in the form of powder for several months without any loss of nutrients [2]. Their leaves also contain high contents of the bioactive compounds such as polyphenols, tannins, phytates, and fibers [3] which significantly vary with several factors such as origin and stage of maturity [4]. Agamou et al. [1] report that whatever the origin of *M. oleifera* in Cameroon, young and mature leaves have high contents in protein, iron, polyphenols, tannins, phytates, and fibers. However, no correlation was made between these high contents of bioactive compounds with the digestibility of protein and bioavailability of iron in those studies. Both nutritional properties are generally associated with the richness in proteins and iron of *M. oleifera* leaves, which justifies their utilization to alleviate protein malnutrition and iron deficiency [5,6]. Even if the bioactive compounds possess many properties such as antioxidant, anti-inflammatory, anti-obesity, antidiabetic, anticancer and antimicrobial [7,8], they can also be anti-nutritional factors, due to their interaction with proteins and iron [9]. Indeed, mainly depending on the content of bioactive compounds in food, the formation of insoluble and non-digestible complexes with proteins and iron leads to low digestibility of proteins and the non-bioavailability of iron [10, 11]. Though, this important nutritional aspect has been somewhat neglected over the past decade to benefit the therapeutic properties and health benefits associated to high contents of bioactive compounds in the leaves [6,8,12]. Very few authors have dwelled on the relationship between factors that influence nutrient and bioactive compounds contents and their consequences on nutritional properties of leaves. Agamou et al. [1] were reported previously the effects of origin on component contents of young and matures leaves but nothing about the influence of the agro-ecological zones on those contents and their consequences on nutritional properties. Thus, the present study aims to determine the influence of the agro-ecological zone and stage of maturity of *M. oleifera* leaves on the digestibility of proteins and bioavailability of iron.

2. MATERIALS AND METHODS

2.1 Sampling

Young (bright green colored leaves at the apex of the branch) and mature (dark green-colored leaves at the base of the branch) leaves of *Moringa oleifera* trees (3 to 4 age) were harvested from three farms (each at least 1000 m^2), well-kept (irrigate, weed) and without fertilizer application, in each agro-ecological zone of Cameroon in August 2012. The altitude, climatic conditions, and soil type of the five agro-ecological zones are presented in Table 1.

2.2 Preparation of Powders

After harvesting, leaflets were detached from the leaf, washed with clean water and dried at 55°C in an electric dryer (Riviera & Bar QD105A, Paris, France) for 5 hours [1]. The dried leaves were ground (Zaiba ZB-2225, China) and sieved through a 1mm mesh sieve to obtain a powder. The powders were then stored in airtight bottles at 4°C before analysis.

2.3 Determination of Moisture, Protein and Iron Contents

Moisture content was determined by drying 1 g of powder in a ventilated oven at 105ºC for 24 hours. Total protein content (N x 6.25) was obtained using the Kjeldahl method from 0.5 g of powder after mineralization [13]. Free iron

Table 1. Altitude, climatic conditions and soil type from different farms in each agro-ecological zone

Agro-ecological zone	Altitude (m)	Precipitation (mm)	Temperature (ºC)	Soil type
Zone I : Soudano-Sahelian Zone	184-294	137.1 – 197	25.05 – 36.33	Halomorphic
Zone II : Guinea Savannah High Zone	902	242.4 - 252.8	18.97 – 26.22	Vertisol
Zone III : Western Highland Zone	1457	250.4 – 260.8	15.71- 23.18	Ferralitic
Zone IV : Rain Forest with Single Mode	19	114.5 – 224.2	24.26 – 29.51	Yellow soil
Zone V : Rain Forest with Two Mode	610-732	181.2 – 333.1	20.63 -27.04	Ferralitic

Source: CMIP 5, MPI-ESM-LR RCP

content was determined by extraction from 0.5 g of powder in 0.03 N chloride acid solution for 3 hours at 37ºC [14], total iron from ashes in diacid mixture after an incineration of 2 g of powder, by atomic absorption spectrophotometry [15].

2.4 Determination of Bioactive Components Contents

2.4.1 Total fibers contents

Total fibers was determined by the method described by Wolff [16] with little modifications. The method consists in treating 5 g of powder by boiling in sulphuric acid, and then in sodium hydroxide. The residue obtained is then dried, then calcined and weighed.

2.4.2 Total polyphenols contents

Total polyphenols were determined according to Makkar et al. [17] with some modification. Ten (10 ml) of ethanol (70%) was added to 0.5 g of powder and the mixture stirred for 2 hours (using a Prolabo 54 433 agitator, Paris, France) at 220 rpm, to extract total polyphenols. After centrifuging (DL 6000 mark, rotor 15 cm, Japan) at 3000g for 20 min at 4°C, 0.02 ml of the supernatant was mixed with 0.2 ml of Folin-Ciocalteu reagent diluted (1/16), 0.4 ml of sodium carbonate (20%) and 1.38 ml distilled water. The mixture was vortexed and incubated in a water bath at 40°C for 20 minutes in darkness. Gallic acid (0.2g/l) was used as standard and absorbance was read at 725 nm.

2.4.3 Total tannins contents

Total tannins were determined according to Makkar et al. [17]. Polyphenol extract (1 ml) and 1ml of distilled water were precipitated with 0.1 g of PVPP (polyvinyl polypyrrolidone) to remove total tannins (100 mg PVPP is sufficient to bind 2 mg of tannins). Total polyphenols in the supernatant was determined as previously described in section *2.4.2* and the total tannins content obtained by difference with the total polyphenols before precipitation.

2.4.4 Phytates contents

Phytates were determined as described by Vaintraub & Lapteva Vaintraub & Lapteva [18] with some modifications. Phytates were extracted from 0.5 g of powder suspended in 10 ml of 3.5% HCl for 2 hours using a prolabo 54 433 agitator Paris, France. The extract was centrifuged (DL 6000, rotor 15 cm, Japan) at 1000 g for 20 minutes at 10°C. The supernatant was treated with 1 g sodium chloride and kept at 4°C for 24 hours. This was later centrifuged at 1000 g for 10 minutes at 10°C. A 3 ml aliquot of the supernatant was added to 1 ml of modified Wade reagent (0.03% $FeCl_3$ $6H_2O$ + 0.3% sulfo-salicylic acid) and centrifuged at 1000 g for 10 min at 10°C. The absorbance was read at 500 nm. Sodium phytate solution (40 µg/ml) was used as standard (100 g of sodium phytates equals 59.9 g of phytic acid).

2.5 Nutritional Properties

2.5.1 *In vitro* protein digestibility

The *in vitro* digestibility of proteins was evaluated by the pH-Stat method. A multiple enzymes solution containing 22 704 units of trypsin from bovine pancreas (type IX, 16 700 units/mg proteins EC: 232-650-8, Sigma-Aldrich) and 52 units of peptidase from bovine intestinal (102 units/g dry solid, EC: 232-875-1, Sigma-Aldrich) was prepared in buffer solution at pH 8 at 37ºC just before the analysis and stored in ice. The sample was prepared at 1 mg N/ml and stored at 4ºC at least one hour before the analysis. The multiple enzyme solution was added in the ratio of 1/10 to the sample in phosphate buffer whose pH was adjusted to 8. Casein was used as a reference protein during the quantification. The quantity of sodium hydroxide (X) necessary to

maintain the pH of the reaction medium at 8 during 10 minutes was noted by the device (842-titrando, metrohm, Swiss-made). The *in vitro* protein digestibility (IVPD) was calculated by Eq (1): IVPD = 76.14 + 47.77 X [19]. The results were expressed based on the percentage of casein digested (%cd).

2.5.2 Bioavailability of iron

The Phytates/iron molar ratio (Phy/Fe), an indirect estimation method of the bioavailability of iron, was calculated according to the Eq (2) [20]:

$$(Phy/Fe) = (n_{Phy}/n_{Fe}) = (m_{Phy}/M_{Phy})\ x\ (M_{Fe}/m_{Fe}) = Q \quad (2)$$

n_{Phy} = number of mole of phytates; n_{Fe}= number of mole of iron; m_{Phy}= mass of phytates
m_{Fe} = mass of iron; M_{Phy} = Molar mass of phytates (IP_6); M_{Fe}= Molar mass of iron
Q < 1: bioavailable; Q > 1: low bioavailability; Q > 10: non bioavailability and probably iron deficiency in consumers.

2.6 Statistical Analysis

Analyses were carried out in triplicates. Microsoft Excel 2013 software was used for calculation of means and standard deviations; Stat-graphic centurion 15.2 software (StatPoint Technologies, Inc, Warrenton, Virginia, USA) for the multiple-way analysis of variance and means separated using the Duncan multiple range test at *P = .05*. Sigma plot 11.0 software (Systat Software, Inc. 1735 Technology Drive, Suite 430 San Jose, CA 95110 USA) was used to plot the graphs and XL-stat 2020 software for Principal Component Analysis (PCA) to group the leaves from agro-ecological zone according to their nutritional properties.

3. RESULTS AND DISCUSSION

3.1 Moisture Content

Table 2 reports the moisture content of young and mature leaves of *M. oleifera* from the five agro-ecological zones. The stage of maturity has not affected the variations of moisture content. These content vary significantly (*P= 0.0001*) the agro-ecological zone to others from 8.25 ± 0.56 to 13.00 ± 0.56 g/100 g DM in mean. Its variations could be due to moisture content contained initially in the leaves according to climatic conditions of each agro-ecological zone. In that effect, the zones with the highest precipitations and lowers temperatures (Table 1) have higher moisture contents (Zone V and Zone III). However, the moisture content values obtained in that study allow *M. oleifera* leaves powders to be kept dry and to limit all microbial activities and biochemical deterioration which can reduce their nutritional potential during the study period. Similar ranges were reported (8.83 to 13.53 g/100 g DM) by Jongrungruangchok et al. [4] on samples of *M. oleifera* leaves collected from eleven localities in Thailand.

3.2 Total Protein Contents

The stage of maturity and agro-ecological zones of leaves does not affect significantly the total protein contents. The interaction of both influences significantly (35.88%, *P= 0.002*) the variations of total protein contents (Table 2), and range from 22.47 ± 0.64 to 24.68 ± 0.64 g/ 100 g DM in the mean. The zone having the lowest temperature and precipitation average has the highest total protein content (Western Highland Zone or Zone III). In the same manner, the zone with the highest temperature and lowest precipitation has low total protein (Soudano-Sahelian Zone or Zone I). These results because of the effect of temperature on the enzymes which are the proteins with catalytic properties and participants to the protein synthesis reactions. The increasing of temperature beyond the plant requirement can limit or inhibit their activities. This protein synthesis depends also on the need of the leaves which increases with the stage of maturity [1]. Similar results have been reported by Asante et al. [21] on *M. oleifera* leaves from two agro-ecological zones in Ghana with a range (25.34 to 26.98 g/100 g DM) near those obtained in that study. *M. oleifera* leaves are an important source of protein that can be used to fight against protein malnutrition and do not present significant correlations with anti-nutritional factors (Table 3) which could limit their assimilation by the body.

3.3 Total Iron and Free Iron Contents

Total iron contents vary significantly (*P= 0.005*) the agro-ecological zone to the other from 16.24 ± 0.59 to 18.35 ± 0.59 mg/100g DM in the mean (Table 2). Meanwhile, the stage of maturity affects significantly (90.10%, *P= 0.0001*) the systematic increase of total iron contents which are from young to mature leaves 12.86 ± 0.37 to 22.11 ± 0.37 mg/100g DM in the mean. These variations are probably due to the important role

Table 2. Moisture, proteins (g/100g DM) and iron (mg/100g DM) contents of young and mature leaves powders of *M. oleifera* from agro-ecological zones

Agro-ecological zone	Moisture content		Total proteins		Total iron	
	YL	ML	YL	ML	YL	ML
Zone I	9.75 ± 1.06^{Aab}	8.5 ± 1.70^{Aab}	23.22 ± 1.15^{Aab}	22.44 ± 1.4^{Aab}	12.97 ± 1.13^{Ab}	23.56 ± 1.21^{Bb}
Zone II	8.5 ± 0.70^{Aa}	9.00 ± 1.41^{Aa}	22.12 ± 0.91^{Aab}	24.95 ± 0.93^{Aab}	12.24 ± 0.84^{Aab}	21.62 ± 0.96^{Bab}
Zone III	12.5 ± 0.90^{Ac}	13.5 ± 1.07^{Ac}	25.81 ± 0.95^{Ab}	23.55 ± 0.98^{Ab}	13.83 ± 0.95^{Ab}	22.87 ± 1.08^{Bb}
Zone IV	7.00 ± 1.41^{Aa}	9.5 ± 1.7^{Aa}	22.70 ± 1.54^{Aab}	24.46 ± 1.05^{Aab}	12.78 ± 1.02^{Aab}	22.57 ± 1.55^{Bab}
Zone V	10.16 ± 1.15^{Ab}	11.16 ± 1.57^{Ab}	21.25 ± 1.79^{Aa}	23.69 ± 1.31^{Aa}	12.52 ± 2.20^{Aa}	19.96 ± 2.54^{Ba}

Values are means ± standard deviation (n=3). Means in the same line and column for each compound, with different uppercase and lowercase superscripts, are significantly different from each other respectively (P =.05). YL: Young Leaves; ML: Mature Leaves; Zone I : Soudano-Sahelian Zone ; Zone II : Guinea Savannah High Zone ; Zone III : Western Highland Zone; Zone IV : Rain Forest with Single Mode; Zone V : Rain Forest with Two Mode

Table 3. Pearson correlation between components of *M. oleifera* leaves

	Total tannins	Free iron	IVPD	Moisture content	Phy/Fe	Phytates	Total fiber	Total iron	Total polyphenol	Total protein
Total tannins	1									
Free iron	-0.336(0.341)	1								
IVPD	0.169(0.638)	0.450(0.191)	1							
Moisture content	-0.148	-0.156	-0.088	1						
	(0.681)	(0.665)	(0.808)							
Phy/Fe	-0.1063	0.925	0.389	(-0.110)	1					
	(0.770)	(0.0001)	(0.265)	(0.761)						
Phytates	0.3025	-0.446	-0.421	0.557	-0.1810	1				
	(0.395)	(0.196)	(0.225)	(0.094)	(0.616)					
Total fiber	0.3633	-0.903	-0.610	-0.060	-0.850	0.372	1			
	(0.302)	(0.0003)	(0.060)	(0.868)	(0.002)	(0.288)				
Total iron	0.201	-0.958	-0.515	0.217	-0.951	0.441	0.914	1		
	(0.575)	(0.0001)	(0.127)	(0.547)	(0.0001)	(0.201)	(0.0002)			
Total polyphenol	0.943	-0.324	0.049	0.103	-0.074	0.467	0.345	0.217	1	
	(0.0001)	(0.360)	(0.892)	(0.777)	(0.838)	(0.173)	(0.328)	(0.546)		
Total protein	-0.170	-0.366	-0.259	0.411	-0.392	0.049	0.104	0.317	-0.131	1
	(0.637)	(0.297)	(0.469)	(0.237)	(0.262)	(0.892)	(0.774)	(0.371)	(0.716)	

Correlation coefficient (p-value) in bold are significant at P = .05

of iron in several plant reactions. It's a constituent of many enzymes and participates in photosynthesis [1]. The intensity of this reaction depends on the quantity of chlorophyll, usually more concentrated in the mature leaves [22]. These high iron content of *M. oleifera* leaves are close to those (26.83 mg/100g DM) reported by Asante et al. [21] and confirm the importance granted to her in the fight against iron deficiencies [23]. However, a high negative significant correlation between total iron and free iron contents (r= -0.958, *P= 0.0001*, Table 3) show that the increase of total iron contents could indicate a lower his bioavailability. In that effect, the Fig. 1 report that the free iron contents in young leaves (16.88 ± 0.44 %) are higher than mature leaves (5.06 ± 0.44 %) in the mean significantly (*P= 0.006*). This result is probably because the compounds capable to chelate iron (fiber, total polyphenol, phytates) are lower in young leaves as compared to mature leaves. The high negative significant correlation between free iron contents and total fibers content (r= -0.903*, P= 0.0003*, Table 3) is under this issue. Most authors have reported the anti-nutritional effect of higher contents of some bioactive compounds on iron content and consequently on his bioavailability [24,25,26]. The young leaves of *M. oleifera* would be more appreciated for his supply in the soluble iron and able to be assimilated.

3.4 Bioactive Compounds Contents

3.4.1 Total fiber contents

Table 4 displays the total fiber contents of *M. oleifera* leaves from the five agro-ecological zones. We observed that the stage of maturity influence significantly (80.97%, *P=0.0002*) the variations of total fiber contents which increase systematically the young to mature leaves from 21.95 ± 0.23 to 26.74 ± 0.23 g/100 g DM in the mean. Indeed, fibers are more elaborated in mature leaves (made up of primary and secondary cell wall) compared to young leaves (only primary cell wall). Fry [26] reports that the primary cell wall is made up of cellulosic microfibrils while the secondary cell wall more rigid, is also constituted of several fibers such as cellulose, lignin, and hemicellulose. Agamou et al. [1] were reported that the stage of maturity is the most important factor which influences significantly (74.49%) the variations of total fiber contents in *M. oleifera leaves*. This is to follow our results which show that the agro-ecological

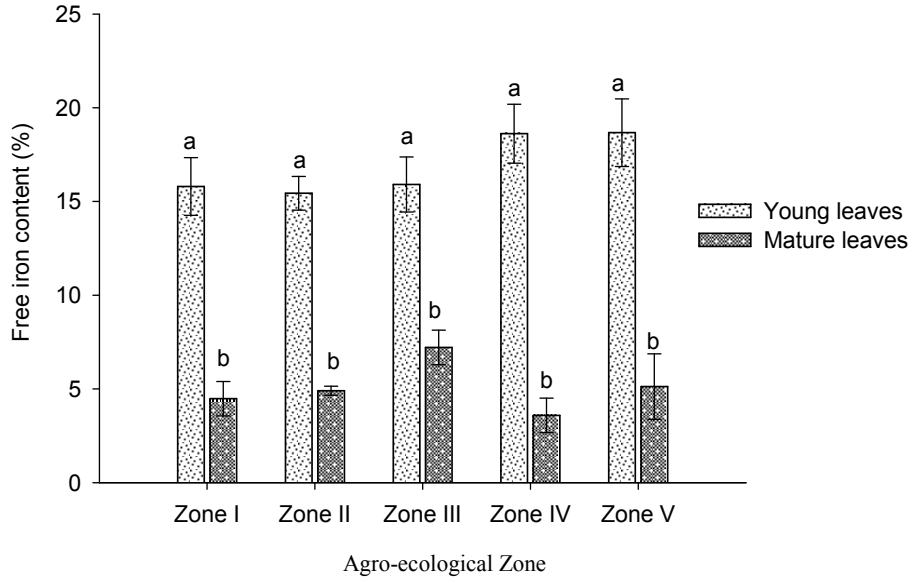

Fig. 1. Free iron content in young and mature leaves of *M. oleifera* from agro-ecological zone (Zone I: Soudano-Sahelian Zone; Zone II: Guinea Savannah High Zone; Zone III: Western Highland Zone; Zone IV: Rain Forest with Single Mode; Zone V: Rain Forest with Two Mode; columns with differing letters are significantly different at *P= .05*)

Table 4. Bioactive components contents in young and mature leaves powders of *M. oleifera* from agro-ecological zones (g/100 DM)

Agro-ecological zone	Total fiber		Total polyphenols		Total tannins		Phytates	
	YL	ML	YL	ML	YL	ML	YL	ML
Zone I	20.59 ± 0.75^{Aab}	27.24 ± 0.48^{Bab}	0.98 ± 0.04^{Aa}	1.05 ± 0.03^{Aa}	0.28 ± 0.02^{Ab}	0.38 ± 0.06^{Bb}	0.26 ± 0.02^{Aab}	0.33 ± 0.01^{Bab}
Zone II	22.82 ± 0.60^{Abc}	26.43 ± 0.68^{Bbc}	1.24 ± 0.01^{Ac}	1.21 ± 0.02^{Ac}	0.50 ± 0.02^{Ad}	0.53 ± 0.02^{Bd}	0.30 ± 0.02^{Aabc}	0.31 ± 0.01^{Babc}
Zone III	20.72 ± 0.60^{Aa}	26.14 ± 0.79^{Ba}	1.02 ± 0.01^{Aab}	1.09 ± 0.02^{Bab}	0.22 ± 0.01^{Aa}	0.22 ± 0.01^{Ba}	0.32 ± 0.01^{Abc}	0.33 ± 0.01^{Bbc}
Zone IV	23.40 ± 0.73^{Ac}	27.60 ± 0.49^{Bc}	0.89 ± 0.01^{Ab}	1.31 ± 0.04^{Bb}	0.15 ± 0.01^{Ac}	0.68 ± 0.02^{Bc}	0.26 ± 0.01^{Aa}	0.29 ± 0.01^{Ba}
Zone V	22.23 ± 1.62^{Aab}	26.30 ± 1.52^{Bab}	1.34 ± 0.04^{Ac}	1.22 ± 0.08^{Ac}	0.66 ± 0.05^{Ae}	0.52 ± 0.02^{Be}	0.32 ± 0.03^{Ac}	0.35 ± 0.02^{Bc}

Values are means ± standard deviation (n=3). Means in the same line and column for each compound, with different uppercase and lowercase superscripts, are significantly different from each other respectively (P = .05). YL: Young Leaves; ML: Mature Leaves; Zone I : Soudano-Sahelian Zone ; Zone II : Guinea Savannah High Zone ; Zone III : Western Highland Zone; Zone IV : Rain Forest with Single Mode; Zone V : Rain Forest with Two Mode

zone influence less (6.89%, *P= 0.043*) the variation of total fiber contents which ranged from 23.43 ± 0.37 to 25.5 ± 0.37 g/100g DM in the mean. These contents are in the range 19.01 and 29.16 g/100g DM reported by Cuellar-Nuñez et al. Cuellar-Nuñez et al. [27] and Castillo-López et al. [28] respectively. Fibers are the bioactive compounds having multiple health benefits (reduction of constipation, satietogenic power, diabetes, cardiovascular disease) [29, 30, 31]. However, the positive significant correlation with total iron (r= 0.914, *P= 0.0002*) and negative significant correlation with molar ratio Phy/Fe (r= -0.850, *P= 0.0002*), show that the high total fiber contents in the *M. oleifera* leaves studied can contribute to limit the bioavailability of iron. The low bioavailability of mineral in the foods rich in fiber has been attributed to the ability of these to chelate minerals and leading the increase of their elimination in the feces [31].

3.4.2 Total polyphenol contents

The total polyphenol contents increase significantly an agro-ecological zone to the other (48.28%, p=0.0018) from 1.01 ± 0.01 to 1.28 ± 0.01 g/100g DM in the mean, according to the stage of maturity (7.93%, *P= 0.006*) from 1.09 ± 0.01 (young leaves) to 1.17 ± 0.01 g/100g DM (mature leaves) in the mean (Table 4), and the interactions of both (36.62%, *P= 0.003*). These variations of polyphenol contents are probably due to the influence of environmental factors on the synthesis of secondary metabolites [32]. The secondary metabolites (polyphenols, tannins) are a large group of compounds that are synthesized by the plant in response to external attacks inherent to the environment (temperature, precipitations, agricultural practices, herbivores, pathogens agents) [33]. Makkar et al. Makkar et al. [17] report that these compound are involved in the regulation of symbiosis, control of seed germination, and chemical inhibition of competing plant species. The polyphenol contents in that study are lower than the range (3.65 to 4.02 g/100 g DM) obtained by Chelliah et al. [34]. This difference because the environmental factors previously mentioned varies from the location to the others. However, the absence of negative significant correlations (Table 3) between the polyphenols, proteins, and iron, could indicate low anti-nutritional activity of polyphenol on these nutrients. This is explained by the low polyphenol contents in the *M. oleifera* leaves studied, which is a real nutritional advantage. Though the polyphenols are known for their multiples health benefits [35], their anti-nutritional effects on the nutrients, due to their high contents in the food, have been reported by many authors [25,26].

3.4.3 Total tannins contents

The total tannin contents increase significantly the young to mature leaves from 0.36 ± 0.01 to 0.46 ± 0.01 g/100g DM in mean, and an agro-ecological zone to the other from 0.22 ± 0.01 to 0.59 ± 0.01 g/100g DM in the mean. These increases of total tannins contents (Table 4) are due to the influence of agro-ecological zone (51.56%, *P= 0.0001*), the stage of maturity (8.12 %, *P= 0.0020*), and the interactions of both (38.63%, *P= 0.0017*) on *M. oleifera* leaves. Meanwhile, these contents are lower than those (2.07 g/100g DM) reported by Maizuwo et al. [5]. The high positive significant correlation between the total tannins and total polyphenols (r= 0.943, *P= 0.0001*, Table 3) shows that these two compounds have the same behavior in the leaves. Knowing that tannins are also secondary metabolites, more particularly polyphenol compounds, the differences observed between his contents are due to the influence of the environmental factors on *M. oleifera* leaves such as previously mentioned for the total polyphenols. Nothing negative significant correlations are observed with proteins (r= - 0.170, *P= 0.637*) and iron (r= - 0.336, *P= 0.341*) (Table 3). It shows that the anti-nutritional activity of total tannins contents is low on these nutrients. Similar results showing the low anti-nutritional activity of tannins due to their low contents in *M. oleifera* leaves have been reported by Agbaire [36].

3.4.4 Phytates contents

Table 4 presents the phytates contents of young and mature leaves of *M. oleifera* from the agro-ecological zones. The contents of these compounds increase significantly from 0.27 ± 0.01 to 0.33 ± 0.01 g/100g DM in the mean, the agro-ecological zone to the other and from 0.29 ± 0.01 (young leaves) to 0.32 ± 0.01 g/100g DM (mature leaves) in the mean. These variations are due to the influence of the agro-ecological zone (46.48%, p=0.001), the stage of maturity (22.93 %, *P= 0.002*), and the interactions of both (12.23%, *P=0.003*). Phytates or myoinositol (1,2,3,4,5,6) hexakisphosphate acid is the storage form of phosphorus in plants that is fixed from the soil by the roots. They are found in the soluble form inside the plant cell and walls where they form insoluble complexes with divalent

cations [9]. Environmental factors such as soil type, pathogen agents, herbivores, temperature, or precipitations are capable to influence the synthesis of phytates and could explain the variations observed between values. The phytates contents of that study are lower than 2.23 g/100g DM obtained by Stevens et al. [37]. These differences are probably because the environmental factors are different from a location to the other. The lower phytates contents of that study could limit their anti-nutritional activities on iron or proteins [36]. The absence of negative significant correlations with these nutrients (Table 3) comes to confirm it.

3.5 Nutritional Properties

3.5.1 *In vitro* protein digestibility

The *in vitro* protein digestibility (IVPD) is a parameter for measuring the nutritional quality of a protein. It's used as an indicator of the bioavailability of amino acids of a protein [38]. The IVPD of young and mature leaves of that study is near those casein (98.01 ± 0.38%) (Fig. 2). Casein is a good biological value milk phosphoprotein (containing all the essential amino acids) used as the reference protein for his high digestibility [39]. Though the IVPD of leaves is near that casein, we observe that it decreases the young to mature leaves from 92.24 ± 0.17 to 89 ± 0.17 cd% in the mean and increase from 89.84 ± 0.27 to 93.21 ± 0.27 cd% an agro-ecological zone to the other. The IVPDs of Zone III to Zone V and Zone I to all the others present the significant differences between them. Meanwhile, only the IVPDs of young and mature leaves of Zone II do not present significant differences. These differences are due to the influence of interactions of the stage of maturity and agro-ecological zones (39.23%, *P= 0.0001*), the agro-ecological zones (28.96%, *P= 0.0012*), and the stage of maturity (25.57%, *P= 0.002*) on the anti-nutritional factors contents capable to chelate the proteins and reduce their digestibility. However, we note that from young to mature leaves, the IVPD decrease while the anti-nutritional factors contents increases. In the same manner, the IVPD increase from the zones where the precipitations are average (250.4 mm) with lower temperatures (15.71 ºC) to the lower precipitations (137.1 mm) with higher temperatures (36.33 ºC). These results show that the increase of anti-nutritional factors contents in the leaves, contributes to limit the digestibility of proteins; even if there are no negative significant correlations between proteins and anti-nutritional factors. These findings are in agreement with those of many authors who have reported the important anti-nutritional activity of bioactive compounds correlated to their high contents in the food [40]. However, the hot environments of leaves could favor the digestibility of protein when their contents are low. *M. oleifera* leaves are a source of good quality protein and that justifies all interest accorded to that leaves in the fight against protein malnutrition.

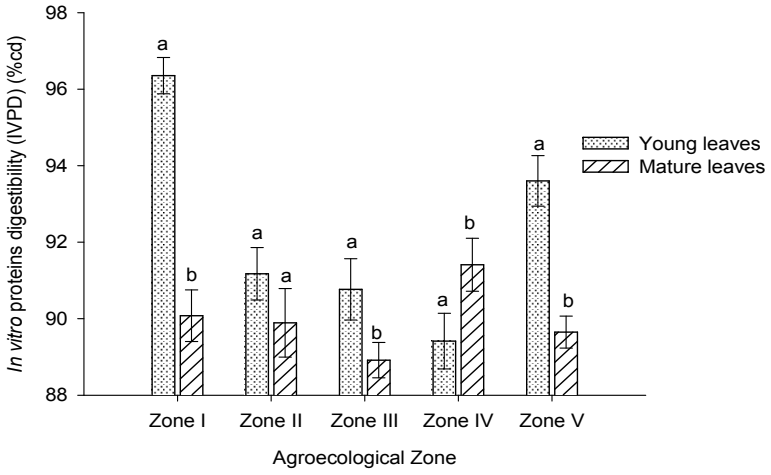

Fig. 2. *In vitro* protein digestibility of young and mature leaves of *M. oleifera* from agro-ecological zones (Zone I: Soudano-Sahelian Zone; Zone II: Guinea Savannah High Zone; Zone III: Western Highland Zone; Zone IV: Rain Forest with Single Mode; Zone V: Rain Forest with Two Mode; Columns with differing letters are significantly different at *P= .05*)

3.5.2 Bioavailability of iron

Fig. 3 reports the molar ratio of Phytates/Iron of young and mature leaves of *M. oleifera* from agro-ecological zones. The molar ratios (Q) varies from 1 to 10 and indicates the low bioavailability of iron whatever agro-ecological zone and stage of maturity of leaves. This result expressed the presence of insoluble phytates-iron complexes due to high phytates contents and which limit the assimilation of iron of *M. oleifera* leaves. The negative significant correlation (r= -0.951, *P= 0.0001*) between phytates and total iron (Table 3) show that the increase of phytates contents reduce the iron ability to be available. That confirms the presence of phytates-iron complexes responsible for low bioavailability of iron in the *M. oleifera* leaves studied. Indeed, the phytic acid myoinositol (IP6) is the main form of storage of phosphorus. It binds to minerals and forms with them insoluble phytate-mineral complexes. The formation of these complexes depends on the number of phosphate groups carried by the myoinositol [9]. This explains why the IP6 is one of the most important chelators of minerals [32]. The results obtained in that study are in agreement with those reported by Tedom et al. [41] on a leafy vegetable consumed in Cameroon. A reduction of anti-nutritional factors contents, more precisely Phytates, before the use of *M. oleifera* to fight against iron deficiency, whatever origin or stage of maturity of leaves, should be considered.

3.6 Grouping of *M. oleifera* leaves According to IVPD and Bioavailability of Iron

The important variations were observed in the nutritional properties of young and mature leaves of *M. oleifera* from five agro-ecological zones studied. The principal component analysis was carried out to group *M. oleifera* leaves according to similarities in their nutritional properties. The first principal component (F1) represents 46.21% of the variation between variables while the second principal component (F2) represents 21.32%, and the both contain 67.53% of the information (Fig. 4). We observe two main groups of variables. The first group consists of mature leaves from the five agro-ecological zones is composed of the higher bioactive compounds, total protein, total iron contents, with the lower IVPDs and the higher bioavailability of iron. However, the zones characterized by the lower precipitations (Zone I, III) involve higher total protein, total iron contents while those characterized by higher precipitations (Zone II, IV, V) involve higher bioactive compounds contents (total polyphenol, total tannins, phytates).

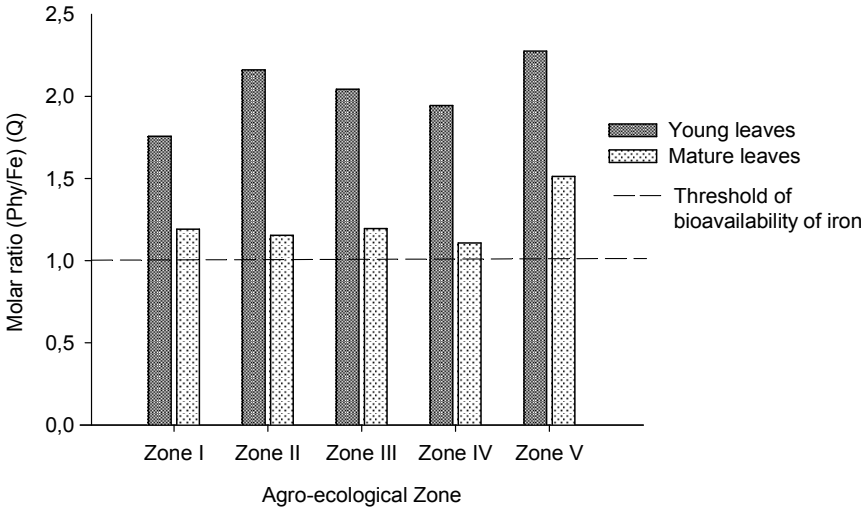

Fig. 3. Molar ratio Phytates/Iron (Phy/Fe or Q) of young and mature leaves of *M. oleifera* from agro-ecological zones (Zone I: Soudano-Sahelian Zone; Zone II: Guinea Savannah High Zone; Zone III: Western Highland Zone; Zone IV: Rain Forest with Single Mode; Zone V: Rain Forest with Two Mode)

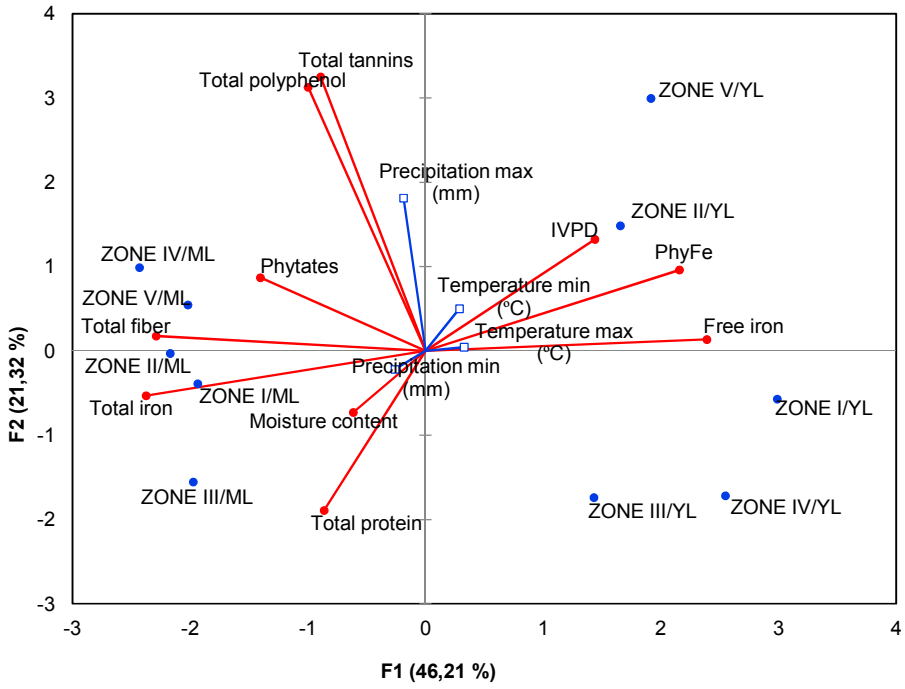

Fig. 4. Grouping of young (YL) and mature (ML) leaves of M. oleifera according to nutritional properties (precipitation and temperature are supplementary variables) (Zone I: Soudano-Sahelian Zone; Zone II: Guinea Savannah High Zone; Zone III: Western Highland Zone; Zone IV: Rain Forest with Single Mode; Zone V: Rain Forest with Two Mode)

The second group made up young leaves from the five agro-ecological zones is constituted of the lower bioavailability of iron and the higher IVPDs and free iron contents. Meanwhile, the zones characterized by the lower temperatures (Zones II, V) favor the highest IVPDs and the lowest bioavailability of iron over the three others (Zones I, III, IV) characterized by the higher temperatures with higher free iron contents. We noted that the soil type has no significant effects on the nutritional properties of the leaves. These findings show that the IVPD and bioavailability of iron of *M. oleifera* leaves depends on many factors such as climatic conditions, bioactive compounds contents, and stage of maturity.

4. CONCLUSION

In vitro protein digestibility (IVPD) and bioavailability of iron varies according to agro-ecological zones and stage of maturity of *M. oleifera* leaves. Whatever the agro-ecological zone and stage of maturity of leaves, the IVPD is near casein, and bioavailability of iron is low. The young leaves have higher IVPDs and lower bioavailability of iron while mature leaves have lower IVPDs and higher bioavailability of iron. The agro-ecological zones of high precipitations and low temperatures favor the high contents of bioactive compounds. Their anti-nutritional properties about proteins and iron limit the assimilation of these nutrients and should be considered before using the leaves of *M. oleifera* to alleviate protein malnutrition and iron deficiency.

ACKNOWLEDGEMENT

The authors would like to thank Drs William DJEUKEU and Wilfried NGAHA for their collaboration during the written of this paper.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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