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Studies on the Proximate, Anti-Nutritional and Antioxidant Properties of Fermented and Unfermented *Kariya* (*Hildergardia barterii*) Seed Protein Isolates

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Abstract

The study prepared protein isolates from fermented *Kariya* seeds. Nutritional, anti-nutritional and antioxidant properties of the fermented (FKI) and unfermented (UKI) isolates were evaluated. Results showed that fermentation increased the protein content of the isolates were between 90.71% to 93.91%. The processing treatments was found to reduce the levels of some anti-nutrients in the protein isolates from 3.29 mg, 1.26 mg and 0.05 mg/100 g in unfermented isolate to 1.32 mg, 0.55 mg and 0.02 mg/100 g in fermented isolate for oxalate, tannin and saponin respectively. The result of antioxidant properties revealed that FKI had better antioxidant properties than UKI and the anti-oxidative properties of the samples increased with increasing sample concentration. The study concluded that fermented *Kariya* seeds protein isolates could find applications as potential food ingredient.

Keywords: Anti-nutrients; Fermentation; Isolates; Antioxidants

Introduction

A wide range of oil-bearing seeds exist in the forest of many African countries which are underutilized. Some of these oil seeds have been shown to be functional foods. Functional foods are important ingredients of a balanced human diet in many parts of the world due to their high protein content [1]. Plant proteins play significant roles in human nutrition, particularly in developing countries where average protein intake is less than the required [2]. It is worthy of note that plant protein products are gaining increasing interest as ingredients in food systems in many parts of the world and the final success of utilizing plant proteins as food additives depends greatly upon the functional characteristics that they impart to foods [3]. Since oil-seeds are valuable sources of proteins, many studies on protein functionality of major and minor oilseeds such as soybean [4] peanut, winged bean and ground nut have been reported [5]. Many of the vegetable proteins require processing techniques to provide food material with acceptable functional properties, such as emulsification, fat and water absorption, texture modifications, colour control and whipping properties, which are attributed primarily to the protein characteristics.

Kariya seeds (*Hildergardia barterii*) are consumed mostly in West African countries as raw or roasted nuts having a flavour like that of peanuts and it is grown for the ornamental nature. According to Hildergaia [6], the flowers, which are usually borne on leafless branches, mature into one-seeded pods, each about 50 mm in length, having a peanut–like seed in a nutshell. When the pods are completely matured and dry, they drop from the tree and are disposed as refuse in many parts of the world where they are found. Only in few West African countries are the kernels used in preparing traditional foods as condiments, eaten raw or roasted like peanut. Studies by Ogunsina et al. [7] showed that *Hildergardia barterii* kernel contains 17.5, 37.5, 2.8 and 6.5% of crude protein, crude fat, ash, and crude fibre respectively. In view of the high level of crude protein (17.5%) in *Kariya* seed, processing the whole flour to protein rich products such as protein isolate could enhance its utilization as a food ingredient.

However, consumption of most of the oil seeds found in the world is limited because in their raw state, they contain high levels of anti-nutrients which are potentially toxic [8]. *Kariya* seed is not an exception, the concentration of these anti-nutrients in plant protein sources vary with the species of plant, cultivar and post-harvest treatments (processing methods) [9]. Khare [10] revealed that processing treatments such as soaking, cooking and fermentation are capable of reducing the anti-nutrient in legumes and oilseeds. The nutritional values of many plant foods can be enhanced through fermentation as it improves the nutritional properties of plant foods, prolongs shelf life and increases the protein content and carbohydrate accessibility and reduce anti-nutrients of plant foods [8]. Adebayo et al. [11] worked on the physicochemical and functional properties of *Kariya* flours. Previous studies have also suggested that fermentation improved the properties of oil seeds [12]. There is no reported work in the literature on the protein isolates of fermented *Kariya* seeds. This work therefore, aimed at fermenting *Kariya* seed; isolate its proteins and then evaluating the effect of fermentation on the physic-chemical, functional anti-nutritional and anti-oxidant characteristics of the protein isolate with a view to increasing its utilization as food ingredients.

Materials and Methods

Collection and preparation of plant materials

Dried *Kariya* pods were gathered from various ornamental *Kariya* trees in Obafemi Awolowo University, Ile-Ife, Nigeria. The nuts extracted from the pods were sorted to remove extraneous materials such as stones and leaves. The kernels were obtained by shelling the nuts manually which were cleaned to remove chaffs and immature kernels.

Fermentation and preparation of samples

Kariya kernels were rinsed with tap water and drained. The samples were divided into two portions; a portion was soaked for 24 h with warm water at 50°C and the water was changed every 6 h interval. The

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soaked seeds were then transferred into different calabash pots, lined uniformly with banana leaves (up to 5 layers) and allowed to ferment inside the incubator (30°C). The fermented seeds were taken out after 96 h and oven dried at 60°C to terminate the fermentation process. The second portion was neither soaked nor fermented. The fermented and the unfermented samples were milled separately using Kenwood® grinder (PM-Y44B2, England) and sieved through 200 µm sieve. The resulting flours of the two samples were subsequently defatted using n-hexane in a sohxlet extraction apparatus. The defatted flours were desolventized by drying in a fume hood and the dried flours finely ground to obtain homogenous defatted flours. The flour samples were packaged in an air-tight polythene bags for further processing.

Preparation of protein isolates

Kariya protein isolate was prepared by the method described by Gbadamosi [13]. A known weight (100 g) of the defatted flours (fermented and non-fermented) was dispersed in 1000 ml of distilled water to give a final flour to liquid ratio of 1:10 in separate containers. The suspension was gently stirred on a magnetic stirrer for 10 min. The pH of the resultant slurry was adjusted to the point at which the protein was most soluble (pH 10.0) and the extraction was allowed to proceed with gentle stirring for 4 h keeping the pH constant. Non-solubilized materials were removed by centrifugation at 3500 × g for 10 min. The proteins in the extracts were then precipitated by drop wise addition of 0.1 N HCl with constant stirring until the pH was adjusted to the point at which the protein was least soluble (pH 4.0). The mixture was centrifuged (Harrier 15/80 MSE) at 3500 × g for 10 min in order to recover the protein. After separation of proteins by centrifugation, the precipitate was washed twice with distilled water. The precipitated protein was re-suspended in distilled water and the pH was adjusted to 7.0 with 0.1 M NaOH prior to freeze-drying. The freeze-dried protein was later stored in air-tight plastic container at room temperature for further use.

Proximate composition of fermented and unfermented *Kariya* isolates

Moisture content determination: Moisture content was determined by the standard [14] official method by weighing 1 g (W_1) of the samples in moisture cans and drying in a hot air-oven (Uniscope, SM9053, England) at 105 ± 1°C until to constant weight (W_2) was obtained. The samples were removed from the oven, cooled in a desiccator and weighed. The results were expressed as percentage of dry matter as shown in the equation below:

$$Moisture\ content\ (\%) = \frac{W_1 - W_2}{W_1} \times 100$$

Where,

W_1 = Weight of flour before drying,

W_2 = Weight of flour after drying,

Ash content determination: Ash content was determined by the official [14] method using muffle furnace (Carbolite AAF1100, UK). Two grams (W_3) of the sample were weighed into already weighed (W_2) ashing crucible and placed in the muffle furnace chambers at 700°C until the samples turned into ashes within 3 h. The crucibles were removed, cooled in a desiccator and weighed (W_1). Ash content was expressed as the percentage of the weight of the original sample.

$$Ash\ content\ (\%) = \left(\frac{W_1 - W_2}{W_3}\right) \times 100$$

Where,

W_1 = Weight of crucible + ash

W_2 = Weight of empty crucible

W_3 = Weight of sample

Protein content determination: The total protein content was determined using the Kjeldahl method [14]. The protein isolates (0.20 g) was weighed into a Kjeldahl flask. Ten milliliter of concentrated sulphuric acid was added followed by one Kjeltec tablet (Kjeltec-Auto 1030 Analyzer, USA). The mixture was digested on heating racket to obtain a clear solution. The digestate was cooled, and made up to 75 ml with distilled water and transferred onto kjeldahl distillation set up followed by 50 ml of 40% sodium hydroxide solution, the ammonia formed in the mixture was subsequently distilled into 25 ml, 2% boric acid solution containing 0.5 ml of the mixture of 100 ml of bromocresol green solution (prepared by dissolving 100 mg of bromocresol green in 100 ml of methanol) and 70 ml of methyl red solution (prepared by dissolving 100 mg of methyl red in 100 ml methanol) indicators. The distillate collected was then titrated with 0.05M HCl. Blank determination was carried out by excluding the sample from the above procedure

$$(\%)\ protein = \frac{1.401 \times M \times F\ (ml\ titrant - ml\ blank)}{sample\ weight}$$

Where,

M = Molarity of acid used = 0.05

F = Kjeldahl factor = 6.25

Carbohydrate content: Carbohydrate was expressed as a percentage of the difference between the addition of other proximate chemical components and 100% as shown in equation below;

Carbohydrates = 100 - (protein crude fat + ash + fibre + moisture)

Anti-nutritional properties of *Kariya* protein isolates

Determination of tannins: The concentration of tannin in the kariya protein isolates was determined using the modified vanillin–hydrochloric acid (MV – HCl) method of Price [15] was used.

Various concentrations (0.0, 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml) of the catechin standard solution was pipetted into clean dried test tubes in duplicate. To one set was added 5.0 ml of freshly prepared vanillin – HCl reagent prepared by mixing equal volume of 4% (w/v) vanillin/ MeOH and 16% (v/v) HCl/ MeOH and to the second set was added 5.0 ml of 4% (v/v) HCl/methanol to serve as blank. The solutions were left for 20 min before the absorbance was taken at 500 nm. The absorbance of the blank was subtracted from that of the standards. The difference was used to plot a standard graph of absorbance against concentration.

Kariya protein isolate was extracted separately with 10 ml of 1.0% (v/v) HCl–MeOH. The extraction time was 1 hour with continuous shaking. The mixture was filtered and made up to 10 ml mark with extracting solvent. Filtrate (1.0 ml) was reacted with 5.0 ml vanillin–HCl reagent and another with 5.0 ml of 4% (v/v) HCl–MeOH solution to serve as blank. The mixture was left to stand for 20 min before the absorbance was taken at 500 nm.

$$\text{Tannin}\ (\text{mg/g}) = \frac{x\ (mg/ml) \times 10ml}{0.2\ (g)} = 50x\ (\text{mg/g})$$

Where,

x = value obtained from standard catechin graph

Determination of oxalate: Oxalate was determined using titrimetric method by Falade [16]. Two grammes of the sample was weighed in triplicate into conical flasks and extracted with a 190 ml distilled water and 10 ml 6M HCl. The suspension was placed in boiling water for 2 h and filtered and made up to 250 ml with water in a volumetric flask. To 50 ml aliquot was added 10 ml of 6M HCl and filtered and the precipitate washed with hot water. The filtrate and the wash water combined and titrated against conc. NH_4OH until the salmon pink colour of the methyl red indicator changed to faint yellow. The solution was heated to 90°C and 10 ml 5% (w/v) $CaCl_2$ solution was added to precipitate the oxalate overnight. The precipitate was washed free of calcium with distilled water and then washed into 100 ml conical flask with 10 ml hot 25% (v/v) H_2SO_4 and then with 15 ml distilled water. The final solution was heated to 90°C and titrated against a standard 0.05M $KMnO_4$ until a faint purple solution persisted for 30 s. The oxalate was calculated as the sodium oxalate equivalent.

1 ml of 0.05M $KMn0_4$ =2 mg sodium oxalate equivalent/g of sample

Determination of saponin: The spectrophotometric method of Brunner [17] was used for saponin analysis. 1 g of finely ground sample was weighed into 250 ml beaker and 100 ml of isobutyl alcohol was added. The mixture was shaken for 2 h to ensure uniform mixing. Thereafter the mixture was filtered through a Whatman No. 1 filter paper into a 100 ml beaker and 20 ml of 40% saturated solution of magnesium carbonate was added and the mixture made up to 250 ml. The mixture obtained with saturated $MgCO_3$ was again filtered through a whatman No. 1 filter paper to obtain a clear colourless solution. 1 ml of the colourless solution was pipette into a 50 ml volumetric flask and 2 ml of 5% $FeCl_3$ solution was added and made up to mark with distilled water. It was allowed to stand for 30 min for blood red colour to develop. Saponin stock solution was prepared and 1-10 ppm standard saponin solutions were prepared from saponin stock solution. The standard solution was treated similarly with 5% of $FeCl_3$ solution as done for 1 ml of sample above. A dilution of 1 to 10 was made from the prepared solution. The absorbances of the samples as well as that of the standard solution were read after colour development in a 752S Spectrum lab UV, VIS Spectrophotometer at a wavelength of 380 nm.

$$Saponin = \frac{absorbance\ of\ sample \times dil.\ factor \times gradient\ of\ standard\ graph}{sample\ weight \times 10{,}000.}(mg/g)$$

Antioxidant properties of protein isolates

DPPH radical scavenging activity assay: The free radical scavenging ability of the extract was determined using the stable radical DPPH (2, 2-diphenyl-2-picrylhydrazyl hydrate) as described by Pownall [18]. To 1 ml of different concentrations (0.5, 1.0, 1.5, 2.0 and 2.5 mg/ml) of the extract or standard (vitamin C) in a test tube was added 1 ml of 0.3 mM DPPH in methanol. The mixture was mixed and incubated in the dark for 30 min after which the absorbance was read at 517 nm against a DPPH control containing only 1 ml methanol in place of the extract. The percent of inhibition was calculated from the following equation:

$$Inhibition(\%) = \frac{(Acontrol - Asample)}{Acontrol} \times 100$$

Where $A_{control}$ is the absorbance of the control reaction (containing all reagents except the test compound) and A_{sample} is the absorbance of the test compound. Inhibition concentration leading to 50% inhibition (IC_{50}) was calculated from the graph plotting inhibition percentage against extract concentrations.

Metal chelating ability assay: The metal-chelating activity of the isolates was carried out according to the method described by Singh [19]. Solutions of 2 mM $FeCl_2 \cdot 4H_2O$ and 5 mM ferrozine was diluted 20 times (1 ml of each of the solutions made up to 20 ml with distilled water separately). An aliquot (1 ml) of different concentrations (6.25, 12.5, 25.0, 50.0 and 100.0 mg/ml) of sample extract was mixed with 1 ml $FeCl_2 \cdot 4H_2O$. After 5 min incubation, the reaction was initiated by the addition of ferrozine (1 ml). The mixture was shaken vigorously and after a further 10 min incubation period the absorbance of the solution was measured spectrophotometrically at 562 nm. The percentage inhibition of ferrozine–Fe^{+2} complex formations was calculated using the formula:

$$Chelating\ effect = \frac{Acontrol - Asample}{Acontrol} \times 100$$

Where,

$A_{control}$ = absorbance of control sample (the control contains 1 ml each of $FeCl_2$ and ferrozine, complex formation molecules) and

A_{sample} = absorbance of a tested samples.

Determination of ferric reducing antioxidant power (FRAP): The FRAP assay uses antioxidants as reductants in a redox-linked colorimetric method with absorbance measured with a spectrophotometer The principle of this method is based on the reduction of a colourless ferric-tripyridyltriazine complex to its blue ferrous coloured form owing to the action of electron donating in the presence of antioxidants [20]. A 300 mmol/L acetate buffer of pH 3.6, 10 mmol/L 2,4,6-tri-(2-pyridyl)-1,3,5-triazine and 20 mmol/L $FeCl_3.6H_2O$ was mixed together in the ratio of 10:1:1 respectively, to give the working FRAP reagent. A 50 µl aliquot of the extract at concentration (0.0, 0.2, 0.4, 0.0.6, 0.8 and 1 mg/ml) and 50 µl of standard solutions of ascorbic acid (20, 40, 60, 80, 100 µg/ml) was added to 1 ml of FRAP reagent. Absorbance measurement was taken at 593 nm exactly 10 minutes after mixing against reagent blank containing 50 µl of distilled water and 1 ml of FRAP reagent.

The reducing power was expressed as equivalent concentration (EC) which is defined as the concentration of antioxidant that gave a ferric reducing ability equivalent to that of the ascorbic acid standard.

Statistical analysis: All the analyses were conducted in triplicate and subjected to statistical analysis using analysis of variance (ANOVA). Means were separated using Duncan's multiple range test.

Results and Discussion

Proximate composition of fermented and unfermented *Kariya* protein isolates

The results of the effect of fermentation on the proximate composition of Fermented kariya isolates (FKI) and unfermented kariya protein isolates (UKI) presented in Table 1. The results showed that UKI had higher moisture content of (3.39 ± 0.09) than FKI (2.35 ± 0.11). The protein content of FKI was (93.91 ± 1.93) was higher than the value obtained for UKI (90.71 ± 1.61) and this values was significantly different ($p>0.05$) from each other. Ash content of 0.49 ± 0.03 was recorded for FKI and the value was lower than the value (0.67 ± 0.06) obtained for UKI. Crude fibre was not detected in the samples and the value obtained for carbohydrate content in sample UKI was higher than the value obtained for FKI. The values obtained for UKI and FKI in this work were higher than the value reported for conophor nut isolates (80.00%) by Gbadamosi [13] and compared favourably with the values reported by Samruan et al. [21] for sunflower protein isolates (90.1%).

Proximate composition	UKI	FKI
Moisture content	3.39 ± 0.09[a]	2.35 ± 0.1[b]
Crude fibre	ND	ND
Ash	0.67 ± 0.06[a]	0.49 ± 0.03[b]
Crude fat	0.98 ± 0.02[b]	1.09 ± 0.21[a]
Protein	90.71 ± 1.61[b]	93.91 ± 1.93[a]
Carbohydrates	4.43 ± 0.98[a]	1.98 ± 0.51[b]

Values reported are means ± standard deviation of triplicate determinations. Mean values with different superscript within the same row are significantly (P<0.05) different.

Table 1: Proximate compositions of fermented and unfermented *Kariya* seed protein isolates.

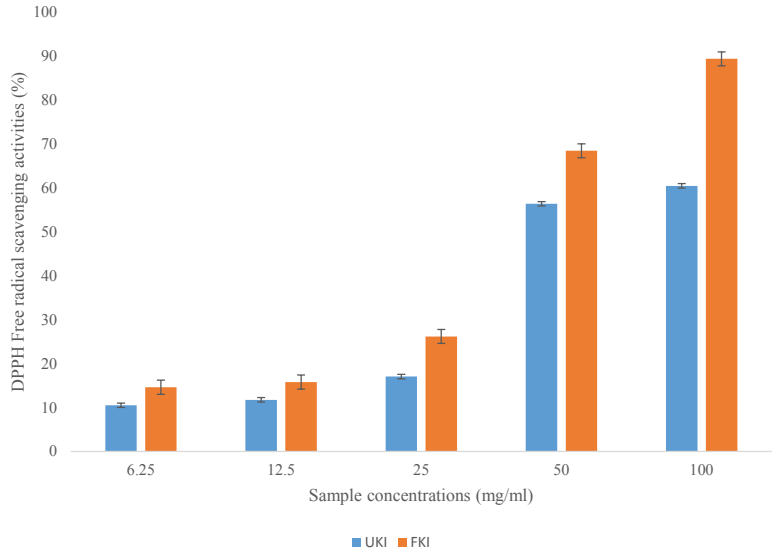

Figure 1: DPPH free radical scavenging abilities of fermented and unfermented *Kariya* protein Isolates. Error bars showing the standard deviation of triplicate determinations.

The results reveal that *Kariya* seed protein isolates has the potential to satisfy the protein needs of the ever increasing population. Ash content is an indication of the mineral contents of the samples. The ash content of the samples indicated the usefulness of this under-utilized seed to satisfy the macro and micro elements need of the consuming populace in the developing world.

Anti-nutritional properties of fermented and unfermented *Kariya* seed protein isolates

The effect of fermentation on some anti-nutritional properties (oxalate, tannin and saponin) of fermented *Kariya* isolates (FKI) and unfermented *Kariya* isolates (UKI). The study showed that UKI contained 3.29 mg, 1.26 mg and 0.05 mg/100 g for oxalate, tannin and saponin respectively while FKI contained 1.32 mg, 0.55 mg and 0.02 mg/100 g for oxalate, tannin and saponin respectively and these values were significantly different ($p<0.05$). The values represented about 61.70%, 56.35% and 60% reduction in the levels of the oxalate, tannin and saponin, respectively in unfermented *Kariya* protein isolates. Defatting was employed as processing technique on UKI while soaking, fermentation and defatting were employed as processing techniques on FKI. The significant reduction in the levels of the anti-nutrient could be attributed to soaking and fermentation processes carried out during the processing of FKI. Factors such as soaking, deffating and fermentation applied during sample preparation could be responsible for degrading the anti-nutrients in these samples. Oxalates bind minerals like calcium and magnesium and interfere with their metabolism, which leads to muscular weakness and paralysis [22]. Tannins have been reported to affect nutritive value of food products by chelating metals such as iron and zinc and reduce the absorption of these nutrients and also forming complex with protein thereby inhibiting their digestion and absorption [22]. Saponins have been found to cause haemolytic activity by reacting with the sterols of erythrocyte membrane The levels of these tested anti-nutrients in UKI and FKI were low and were within the tolerable (safe) levels for man (12.0, 1.5 and 100 mg /100 g, for oxalate, tannin and saponin respectively) [23]. This study however revealed that soaking, fermentation and defatting could be employed separately or in combination in the processing of *Kariya* seeds to significantly reduce the levels of anti-nutrients isolates in *Kariya* protein isolates.

Antioxidant properties of fermented and unfermented *Kariya* protein isolates

The effect of fermentation on the 1,1-diphenyl-2 picrylhydrazyl (DPPH) free radical scavenging abilities of FKI and UKI is shown in Figure 1. The results showed that the free radical scavenging capacities of the samples as measured by DPPH assay increased as the concentration of the sample extract increased from 0.5-2.5 mg/ml. The increase was significant when the FKI was compared with UKI at each of the concentration considered. At the highest concentration of the sample extracts (2.5 mg/ml), the inhibition percentage of the sample extracts for FKI was 69.44% and this value was higher than 61.35% obtained for UKI at the same concentration (2.5 mg/ml). The result clearly showed that fermentation enhanced the free radical scavenging capability of the isolates by about 13.00% than the unfermented isolates. Table 2 shows the potency of the samples in terms of IC_{50} value. The results

SAMPLE	DPPH IC_{50} (mg/ml)	MC IC_{50} (µg/ml)	FRAP (AAEµg/g)
FKI	1.73 ± 0.2^{b}	1.85 ± 0.17^{b}	0.93 ± 0.3^{b}
UKI	2.21 ± 0.77^{a}	1.93 ± 0.04^{a}	1.50 ± 0.12^{a}
Ascorbic acid	0.67 ± 0.03^{c}	-	-
EDTA	-	0.08 ± 0.06^{c}	-

Values reported are means ± standard deviation of triplicate determinations. Mean values with different superscript within same column are significantly (P<0.05) different. FKI: Fermented *Kariya* Isolate, UKI: Unfermented *Kariya* isolate.

Table 2: IC_{50} values fermented and unfermented *Kariya* seed protein isolates.

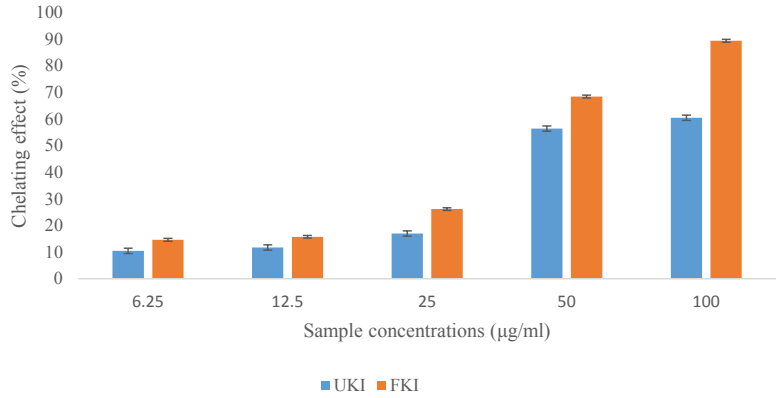

Figure 2: Metal chelating effect of fermented and unfermented *Kariya* protein Isolates. Error bars showing the standard deviation of triplicate determinations.

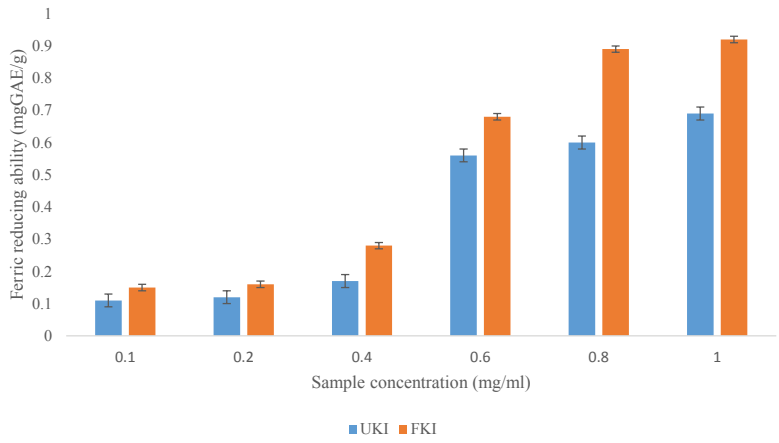

Figure 3: Ferric reducing power of fermented and unfermented *Kariya* protein isolate. Error bars showing the standard deviation of triplicate determinations.

revealed lower value of FKI when compared with UKI. The lower value of FKI indicates better radical scavenging than UKI. Similar results were reported by Je [23] on the fermentation of soybeans proteins.

The chelating effects of the isolates as influenced by fermentation are shown in Figure 2. The results showed that sample FKI had better chelating effect than sample UKI at each of the concentration considered. Just like the DPPH, the chelating effect of the samples increased with an increase in the sample concentration. At the highest concentration of 100 µg/g, chelating percentage for sample FKI was 78.69. The value was higher than 70.9% obtained for UKI. Considering the IC_{50} values of the samples, it was observed that 1.93 was obtained for FKI. The value was higher than 1.85 recorded for sample unfermented kariya isolate 1.95. The trend observed in this work was in agreement with the observation of Je [24] on the fermentation of soy proteins.

Ferric reducing effect of the samples as a function of fermentation is presented in Figure 3. The results revealed the positive influence of fermentation on the ferric reducing ability of the samples. Results showed progressive increase in the ferric reducing abilities as sample concentration increased. Isolates produced from fermented kariya seed (FKI) was found to have higher chelating effect than the unfermented sample (UKI). The results also revealed the potential of FKI as having better ferric reducing than UKI. The result was in line with the observation of Samruan [21] on the ferric reducing abilities of rapeseed proteins.

The results of the anti-oxidant properties of the fermented isolate clearly showed the beneficial effects of fermentation in positively influencing the free radical scavenging abilities, chelating metals and in reducing ferric ions of *Kariya* protein isolates.

Conclusion

The study investigated the effect of fermentation on physicochemical, functional, anti-nutritional properties of *Kariya*

protein isolates. Fermentation increased emulsifying properties, water and oil absorption capabilities, *in-vitro* protein digestibility of *Kariya* seed isolates. On the other hand, fermentation decreased bulk density and foaming properties. The processing methods employed (fermentation) significantly reduced the levels of tested anti-nutrients (tannin, saponin and oxalate) below the tolerance levels. Fermentation was also observed to increase the levels of some antioxidant properties isolates produced from fermented *Kariya* seed. The study revealed that fermented *Kariya* isolates could find application as functional ingredient in food systems.

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