

Effect of Heating on, Lactobacillus Fermentation and Enzyme Treatment on the Content of Phytic Acid in Kernels of *Adansonia digitata* and *Sclerocarya birrea*

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Abstract: The nutritional value of diets is enhanced by traditional oilseeds, fruits and vegetables. They add taste and flavour to the food, improve palatability and help to balance protein, vitamin and mineral intakes. Seeds and kernels are good sources of protein and energy, valuable supplements in children's diets and also useful in preparing snack foods. *Adansonia digitata* and *Sclerocarya birrea* fruits are widely distributed throughout Sub-Saharan Africa. These oilseeds are commonly eaten in rural areas of Mozambique as snacks or in food preparation. They show to contain high amounts of several minerals, for example iron, magnesium and zinc. However, they have been shown to contain anti-nutrient such as phytic acid, which may decrease the absorption of minerals from the diet, especially zinc and iron, and to a lesser extent, calcium and magnesium. In this study it was analysed the content of phytic acid in kernels of *Adansonia digitata* and *Sclerocarya birrea* using high-performance ion chromatography after enzyme (phytase) treatment and incubation with *Lactobacillus plantarum*. The results show that the amount of phytic acid in both kernels can be reduced from 4.4 g/100 g to 1.6 g/100 g by various processing techniques such as heating or autoclaving followed by incubation with *Lactobacillus plantarum* or by enzyme (phytase) treatment. The content of phytic acid was lower after autoclaving than after boiling. Treatment with phytase reduced 20 to 30% of phytic acid content by after 15 minutes treatment. The means of the mineral content are presented in dry matter; Fe (5.0 mg/100 g), Mg (666 mg/100 g) and Zn (5.5 mg/100 g) in *Adansonia digitata* kernels and in *Sclerocarya birrea* 4.0 mg/100 g, 391 mg/100 g and 4.5 mg/100 g respectively for Fe, Mg and Zn. Around half the content of these minerals in the kernels was found in the supernatant after 15 minutes' enzyme treatment.

Keywords: *Adansonia digitata*, *Sclerocarya birrea*, Kernel, Enzyme, Phytic Acid, Phytase, Fermentation

1. Introduction

Kernels from wild fruits are commonly eaten in rural areas of Mozambique. They provide essential nutrients to the diet and are available in the dry season when other foods are scarce [1, 2]. The oilseeds from the *Adansonia digitata* (*A. digitata*) fruit are commonly eaten fresh, roasted, or dried and ground into flour which can be added to soups and stews as a thickener. The roasted and ground kernels can be processed into a paste, or boiled for long, fermented and then dried, for use in several preparations [3, 4]. Kernel sauce is

prepared by roasting the kernels followed by grinding into powder. The powder is used as protein concentrates in several spiced sauces such as tomato [5]. The kernels from the *Sclerocarya birrea* fruit (*S. birrea*) are very tasty and widely eaten. They are removed from the hard shell dried and eaten alone, or cooked and eaten together with a mixture of dried peanut extract, red pepper, salt and other spices in the form of a "meat bundle" [6].

According to Magaia et al. [7], kernels of *A. digitata* and *S. birrea* have shown high content of several minerals, such as, iron, magnesium and zinc, as reported in several studies, ex:

[8-11]. Nonetheless, these kernels have shown to contain phytic acid (refer to Table 3), which is considered to decrease the absorption of minerals from the diet [12]. Phytic acid, also known as inositol hexaphosphate, has six PO_4 groups and is a strong chelator to divalent ions, especially zinc and iron and to a lesser extent, calcium and magnesium [13], and as the main storage form of phosphorus in many plant tissues, especially bran and seeds [14].

Removal of phosphate groups from the inositol ring decreases the mineral binding strength of phytic acid and thus improves the nutritional value [15]. Domestic food preparation techniques, such as boiling, can reduce the phytic acid content to some extent, but soaking in an acid medium, lactic acid fermentation, sprouting, and the use of enzymes (phytase) are more effective methods of reducing phytic acid [16, 17]. Fermentation, either spontaneous or with a starter culture, is a simple and efficient procedure of reducing the phytic acid content in foods. At suitable pH (4.8 to 5.6) native plant phytase is activated, which removes phosphate groups from the phytic acid [18]. Microbial phytase can be produced by *Lactobacillus* bacteria at the optimal pH is 5 to 6 [16, 19]. *Lactobacillus* strains have been reported to produce extracellular phytase [20].

Results from preliminary experiments (data not shown) indicated that the content of phytic acid was about 5.5% in kernels of *A. digitata* and 3.4% in *S. birrea* kernels. This prompted us to study how the content of phytic acid in kernels of *A. digitata* and *S. birrea* could be influenced by heating, fermentation with *Lactobacillus* bacteria or incubation with enzyme phytase. Furthermore, we determined the content of some minerals in the water solution after the enzyme treatment.

2. Materials and Methods

2.1. Samples

Kernels from two wild fruit species were studied: *Adansonia digitata* (Family of Bombacaceae, local name n'buvo or malambe), and *Sclerocarya birrea* (Family of Anacardiaceae, local name n'canhi). The kernels, were sub-samples from another study [21]. The hard shell of the *Adansonia digitata* fruit was removed by crushing, and the kernels were collected, vacuum-packed in different batches and stored at -18°C . Before analysis, *Adansonia digitata* kernels were milled in a coffee grinder (TEFAL, Type 8100, PreP'Line, China) and sieved (500 μm mesh). The *Sclerocarya birrea* kernels were removed from the hard shell also by crushing, and grounded with a mortar and pestle.

2.2. Chemicals

All chemicals were of analytical grade and de-ionised water was used for all experiments. Hydrochloric acid and $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ were obtained from Fluka (Sigma-Aldrich, Steinheim, Germany) and sodium phytate from BDH Chemicals Ltd (UK). Wheat phytase (0.01-0.004 units/mg) was obtained from Sigma Chemical Co. (Stockholm,

Sweden). For the experiments with *Lactobacillus* bacteria, the chemicals were obtained from Merck (Darmstadt, Germany), Sigma-Aldrich Co. (Steinheim, Germany), and VWR-International (Stockholm, Sweden), and *Lactobacillus plantarum* 299v (DSM 9843, Probi AB, Lund, Sweden).

2.3. Processing

The samples of kernels of *Adansonia digitata* and *Sclerocarya birrea* were subjected to heating until boiling and autoclaving followed by fermentation with *Lactobacillus* bacteria and other samples by incubation with phytase and without pre-treatment.

2.3.1. Lactobacillus Plantarum Preparation

One capsule of *Lactobacillus plantarum* 299v was suspended in sterile MRS medium and incubated for 24 hours at 37°C . The cells were washed 3 times with small portions of 0.9% (w/v) NaCl and centrifuged at 4000 rpm for 10 min. *Lactobacillus plantarum* cells were suspended in 50 ml 0.9% (w/v) NaCl and subjected to serial dilution. From each dilution tube 0.1 ml was plated onto MRS plate count agar. After incubation for 48 hours at 37°C , the number of colonies was counted to evaluate the cell level in the undiluted suspension; the result being 12×10^8 colony-forming units (CFU) per ml.

2.3.2. Heating

Approximately 1 g sample of each processed kernels (*A. digitata* and *S. birrea*) was taken in the glass, added 5 ml distilled water thoroughly homogenized for pH measurement then boiled for 15 minutes to inactivate possible intrinsic phytase, cooled down at temperature 25°C . 1 ml bacteria solution (12×10^8 CFU/ml) was inoculated in the samples and fermented for 48 hours at 37°C , and at the end of fermentation the pH was measured in the sample to see the complete lactic fermentation.

2.3.3. Autoclaving

Approximately 1g sample of each grounded kernels (*A. digitata* and *S. birrea*) was taken in the glass, added 5 ml distilled water thoroughly homogenized for pH measurement. The homogenized solutions were autoclaved for 15 minutes also to inactivate possible intrinsic phytase, cooled down at temperature 25°C , then were inoculated with 1 ml bacteria solution (12×10^8 CFU/ml), fermented for 48 hours at 37°C and measured the pH to control complete fermentation. Both samples (heated and autoclaved) were freeze-dried and stored at -18°C until for phytic acid analysis.

2.3.4. Enzyme Treatment

On these series of experiments, a stock solution of wheat phytase (10 mg/ml) was prepared. For each processed kernels (*A. digitata* and *S. birrea*), were prepared glasses and 1g of sample were suspended in 9 ml of pure water and the pH adjusted to 5 (optimal pH for phytase). Then 1 ml of wheat phytase solution (10mg/ml) was added to each glass and incubated in a water bath at 55°C under magnetic stirring for 2, 15, 30, 60 and 240 minutes.

In addition to see the effect of wheat phytase in the samples, was prepared the concentration of 50 mg/ml of wheat phytase solution. The same amount of samples (1 g of *A. digitata* and *S. birrea*) were taken, mixed in 5 ml of pure water in glasses and 5 ml of the wheat phytase solution added, the pH adjusted to optimal for phytase and incubated only for 60 minutes according to the conditions above. All samples, after incubation, were removed from the water bath and immediately boiled for 5 minutes to inactivate the enzyme. As control, samples without phytase were boiled for 5 minutes. Then, the samples (*A. digitata* and *S. birrea*) cooled at room temperature, were separated in two groups. One group of each samples were freeze-dried and stored at -18°C until phytic acid analysis. The other samples were centrifuged at 18000 rpm for 20 minutes. The supernatants were collected and determination of the mineral content performed.

2.4. Dry Mater Analysis

The dry matter content was determined after drying 2 g of each sample in an oven at 105°C until constant weight [22].

2.4.1. Phytic Acid Analysis

Phytic acid was analysed as inositol hexaphosphate [23], in Laboratory of Food Science, Chalmers University of Technology, Goteborg-Sweden. Samples were extracted with 20 ml 0.5 M HCl for 3 hours at 20°C under magnetic stirring. The extracts were frozen overnight, thawed and centrifuged at 12000 rpm for 10 minutes, and an aliquot (300 µl) of the supernatant was injected into a high-performance ion chromatography (HPIC) instrument (Waters Associates Inc, Milford, MA) equipped with a guard-column (PA-100, 4 x 50 mm i.d., Dionex Corp., Sunnyvale, CA, USA) and an analytical column (HPIC OmmiPac PA-100, 4 x 250 mm i.d.). The column was eluted isocratically with 80% HCl (1 M) and 20% water. Inositol hexaphosphate was identified and quantified after post-column reaction with $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$, and the absorbance was monitored at 290 nm (Waters 486, tuneable absorbance detector). Sodium phytate was used as an external standard.

2.4.2. Mineral Analysis

The mineral content in the supernatant was determined after the addition of 65% nitric acid, corresponding to 1% of the sample volume. The samples were filtered and an aliquot was analysed using inductively coupled plasma-atomic emission spectrometry (ICP-AES, Perkin Elmer, OPTIMA 3000 DV).

2.5. Statistical Analysis

Microsoft® Excel was used for statistical evaluation. Student's t-test was performed to determine significant differences. A value of $p < 0.05$ was considered to indicate statistical significance.

3. Results and Discussion

During the sample preparation, the pH was controlled before addition of bacteria solution (12×10^8 CFU/ml) and after the 48 hours' fermentation at 37°C. At the beginning,

the variation of pH in the samples was 6.5 – 7 and after fermentation, the pH had decreased to 4 and 5.

For determination of phytic acid was analysed as inositol hexaphosphate, the calibration curve was linear for the concentration range 0.1 to 0.6 µmol used in the analysis.

3.1. Boiled and Autoclaved Samples

The results of the determinations of phytic acid content in the series of experiments with boiling and autoclaving followed by addition of *Lactobacillus* and fermentation, are given in Table 1. The content of phytic acid on the samples of *A. digitata* and *S. birrea* are lower after autoclaving than after boiling.

Table 1. Phytic acid in *A. digitata* and *S. birrea* kernels pre-treated by boiling or autoclaving for 15 minutes before incubation with *L. plantarum* for 48 hours at 37°C.

Sample	Addition of <i>L. plantarum</i>	Phytic acid (g/100 g dry matter)	
		<i>A. digitata</i>	<i>S. birrea</i>
Boiled	No	4.4±0.6	2.2±0.1
Boiled	Yes	4.2±0.0	1.9±0.2
Autoclaved	No	3.6±0.1	1.8±0.0
Autoclaved	Yes	3.3±0.0	1.6±0.0

The reduction of the amount of phytic acid after fermentation was significant for the autoclaved samples, but not for the boiled samples. Based on the analyses of phytic acid conducted using HPIC, the results of the present study for boiled *A. digitata* kernels (3.6 – 4.4 g/100 g) generally agree with the results reported from other studies using other methods (Table 3). The findings of this study are, however, lower than results from previous studies [24, 25], and higher than in others developed studies [26-29]. In addition, there is one report showing markedly lower values of phytic acid [30]. The results regarding the amounts of phytic acid in boiled *S. birrea* kernels (1.8 – 2.2 g/100 g) are around five times higher than that found in another study [31].

The addition of *L. plantarum* bacteria had a significant effect on the phytic acid content in the kernels, which had been autoclaved before incubation.

3.2. Enzyme Treatment

The Table 2 gives the amount of phytic acid in the kernels after treatment with wheat phytase for different times.

Table 2. Phytic acid content in *A. digitata* and *S. birrea* kernels incubated at 55°C with phytase for different times and then boiled for 5 minutes.

Phytase (mg/g sample)	Incubation time (min)	Phytic acid (g/100 g dry matter)	
		<i>A. digitata</i>	<i>S. birrea</i>
0	0	4.0±0.1	2.4±0.2
10	2	3.9±0.2	1.9±0.3
10	15	2.9±0.7	2.0±0.1
10	30	3.2±0.4	2.1±0.3
10	60	3.3±0.0	2.3±0.2
10	240	2.5±0.2	1.0±0.1
50	60 ¹	1.9±0.1	0.8±0.2

¹50mg/ml of phytase

For *A. digitata* kernels, the phytic acid content after 2

minutes' incubation was only somewhat lower than in the untreated kernels, but a slight decrease was observed after 15 minutes. This value was maintained up to 60 minutes. However, after 4 hours' incubation, the content had decreased to around 63% of the original value, and this decrease was significant. Similar results were obtained for *S. birrea* kernels, and after 4 hours' incubation the phytic acid content was only about 42% of the original value; this decrease was significant.

The lowest phytic acid contents were seen after 60 minutes' incubation with a 5-fold higher enzyme concentration, when the value decreased significantly to 48% and 33% of the initial value in *A. digitata* and *S. birrea* kernels, respectively.

Few data are available in the literature on phytic acid in the kernels studied, and are summarized in Table 3. It has not found any data of phytic acid in these kernels from Mozambique.

Table 3. Literature data on the phytic acid content of the seeds of *A. digitata* and kernels of *S. birrea*.

Phytic acid (%)	Based on	Reference
<i>A. digitata</i>		
6.66 and 7.13	- ¹	Adubiaro et al., 2011
4.903*	DW ²	Mitchikpe et al., 2008
1.75 and 0.62	-	Saulawa et al., 2014
1.40*	-	Proll et al., 1998
1.20	-	Ezeagu, 2005
0.20	-	Nkafamiya et al., 2007
0.0730*	FW ³	Osman, 2004
0.18 and 0.16 ⁴	-	Nnam and Obiakor, 2003
<i>S. birrea</i>		
0.423*	DW	Muhammad et al., 2011

*=recalculated

¹=not given; ²DW=dry weight. ³FW=fresh weight; ⁴=units not stated.

From the Table 2, during soaking or enzyme incubation of the samples of *A. digitata* and *S. birrea*, phytic acid is transferred to the surrounding liquid. The addition of phytase to the medium reduced the phytic acid content in the supernatants by 40-60% after 4 hours' treatment, but a five-fold higher enzyme concentration was more efficient.

Results from a previous study showed that the phytic acid content in *A. digitata* kernel flour decreased by 65.57% after boiling for 1 hour, and changing the water at 20 minutes'

intervals [29]. This also suggests that the ratio of solid to liquid is an important factor for the reduction of phytic acid in the kernels. Furthermore, this indicates that boiling for prolonged period may decrease the phytic acid content. Changing the water may reduce the content of important minerals, as can be observed that the amounts of iron, magnesium and zinc (Table 4) in the supernatant increased after enzyme incubation (wheat phytase). This would probably also be the case in boiling.

Table 4. Contents of iron, magnesium and zinc in the supernatant from *A. digitata* and *S. birrea* kernels incubated at 55°C with or without phytase for different times.

Phytase (mg/g sample)	Incubation time (min)/Sample	Fe (mg/100 g dry matter)	Mg	Zn
	<i>A. digitata</i>	4.0-6.0*	626-706*	5.2-5.7*
0	0	0.9±0.09	109±5.51	4.5±0.04
10	2	4.4±0.14	329±5.65	1.3±0.00
10	15	3.0±0.28	404±23.9	1.1±0.08
10	30	2.6±0.16	373±21.9	0.9±0.00
10	60	2.1±0.12	359±17.1	1.2±0.01
10	240	2.1±0.08	409±24.6	1.4±0.00
	<i>S. birrea</i>	4.0*	346 - 436*	4.5*
0	0	0.2±0.03	49±0.03	0.7±0.00
10	2	2.3±0.10	299±20.2	2.6±0.00
10	15	2.4±0.11	289±17.3	2.8±0.02
10	30	2.4±0.09	311±20.0	2.7±0.01
10	60	2.1±0.09	310±16.2	2.2±0.02
10	240	2.1±0.11	344±21.8	2.4±0.02

*Results from a previous study on mineral content in the kernels (Magaia et al., 2013).

As shown on Table 4, almost 50% of the original mineral content can be found in the supernatant after only a few minutes' enzyme incubation, apart from zinc in *A. digitata* (20%). The results show that the amount of minerals in the supernatant was higher than in the samples not incubated, apart from zinc for the *A. digitata* sample. The time for

enzyme incubation did not seem to have a major influence on the mineral content, although there was a tendency for the longest incubation time to result in an increase in the concentration of magnesium in the supernatants from both kernels.

4. Conclusion

The data of this study will make a substantial contribution to the knowledge concerning phytic acid content in kernels from these two wild fruits. The results show that the presence of phytic acid in the kernels can be reduced by various processing techniques such as boiling, autoclaving followed by incubation with *Lactobacillus plantarum* or by enzyme (phytase) treatment. The fermentation with bacteria shown significant effect on the phytic acid content in the autoclaved kernels. Enzyme incubation reduced the phytic acid content by 20-30% after 15 minutes, and a higher enzyme concentration was found to be more efficient. Almost 50% of the estimated original content of minerals were found in the supernatant after a few minutes' enzyme incubation. This suggests that the water used for boiling should be included in the dishes if the kernels are to be used as a dietary supplement. From the present study, it was observed a weak relation between the content of phytic acid and minerals in the supernatant of the enzyme-treated samples, although the incubation time had no significant effect on the mineral content. One explanation of this could be that part of the enzyme was bound to the kernel matrix, reducing the effect. Future experiments should include different ratios of solids to liquid and different enzyme concentrations. Studies need to be conducted to ascertain the reduction of phytic acid by soaking and cooking for more time and also studies to evaluate the level of other antinutrients in *Adansonia digitata* and *sclerocarya birrea* kernels.

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