Effects of cooking and fermentation on the antinutrients, total phenolic contents and antioxidant properties of sandbox *(Hura crepitans)* seeds

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Abstract

The effects of cooking and fermentation periods on the antinutrients (oxalates, tannins and saponin), antioxidants (DPPH, metal chelating, and ferric reducing power) and total phenolic contents (TPC) of *Hura crepitans* (Sandbox) seeds flour samples were investigated. Raw unfermented sandbox (RUS) seeds had the highest levels of antinutrients (oxalate 8.35 mg/100g; tannin 1.44 mg/100 g; and saponin 0.18 mg/100 g). The sample that was cooked and fermented for 96 h (CFS96) showed the lowest values for all the antinutrients (oxalate: 6.58 mg/100 g; tannins: 1.18 mg/100 g; saponin: 0.10 mg/100 g). CFS96 also had the highest total phenolic contents and DPPH radical scavenging ability (IC$_{50}$: 0.14 mg/ml) while RUS showed the highest metal chelating ability (IC$_{50}$: 0.09 mg/ml). The best ferric reducing antioxidant power was exhibited by raw fermented sandbox (RFS) seeds. Therefore cooking and fermentation processes reduced the antinutrients and improved the antioxidant potential of sandbox seeds.

Introduction

Recently, concerted efforts are being directed to adequately evaluate underutilized oil seeds as an alternative protein source in the diets, particularly in developing countries. Africa and indeed most tropical and sub-tropical countries are blessed with numerous seeds and nuts, many of which are yet to be fully exploited due to dearth of information on their chemical composition and functional characteristics. *Hura crepitans* commonly referred to as sandbox tree fall into this category of such seed-bearing plants (Muhammed *et al*., 2013).

Hura crepitans Linn. is a tropical plant belonging to the family Euphorbiacea. It is known as 'Odan Mecca' by the Kabba people of Kogi State, Nigeria and "Aroyin" by the Ijesha people of Osun State, Nigeria. *Hura crepitans* is often planted in towns and villages as a cover tree. The tree has short, densely crowned spines on the trunk and branches; it also has long-stalked leaves with prominent, closely-parallel pinnate nerves and highly distinctive leaves. This tree flowers usually at the beginning and end of rainy season. One nut is a flattened and fluted disc with 5–10 lobes about 2.5 cm deep and 7.5 cm wide on a stout stalk. The capsule splits explosively releasing one flattened circular seed about 18 mm across from

each chamber (Fowomola and Akindahunsi, 2007).

Previous reports have shown that *Hura crepitans* seed contains high level of good quality protein and oil with a range of 22 to 37.64% and 43.52 to 53.81%, respectively (Fowamola and Akindahunsi, 2007; Olatidoye *et al*., 2010; Okolie, 2012; Muhammed *et al*., 2013). Similarly, the presence of antinutrients such as saponin, oxalate, tannin etc as well as effect of fermentation on these antinutrients have been documented (Fagbemi and Atun, 2001; Fowamola and Akindahunsi, 2008). Fermented plant proteins are important to both the Oriental and African continents. Fowomola and Akindahunsi (2008) also reported fermentation to have played a major role in the improvement of nutritional quality, structural properties, shelf–life as well as reduction of antinutrients present in plant foods.

According to Soetan and Oyewole (2009), the introduction of new plant varieties into our diets may expose humans and animals to new toxic factors with unsuspected biological effects. The knowledge that these compounds elicit both toxic and advantageous biological responses has given rise to several investigations in recent times as to their possible physiological implications in various biological systems. These anti-nutritional factors are increasingly recognized as significant items of the

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diet of humans and animals (Osagie, 1998). Thus, they affect the overall nutritional value of foods and feeds.

In order to prolong the storage of foods, several synthetic antioxidants such as butylated hydroxytoluene and butylated hydroxyanisole are used currently, but these substances may be inappropriate for chronic human consumption, as recent publications have mentioned their possible toxic properties for human health and the environment (Wu *et al.*, 2009). Natural phenolic antioxidants can scavenge reactive oxygen and nitrogen species thereby preventing the onset of oxidative diseases in the body. Many research works have shown that oilseeds are possible sources of phenolic compounds with antioxidant properties. Information on the influence of processing methods on the antinutrients of *Hura crepitans* seeds as well as its antioxidant potentials is scanty. Therefore, the aim of this study was to investigate the effect of cooking and fermentation on the antinutrients, total phenolic contents and antioxidant properties of *Hura crepitans* seeds with a view to increasing its utilization as food.

Materials and Methods

Sample collection and preparation

Sandbox (*Hura crepitans*) seeds dispersed by explosive mechanism were collected from various locations in Obafemi Awolowo University, Ile-Ife, Nigeria. The seeds were decorticated manually to reduce the bulk of materials to be processed, air dried and stored for further use. All chemicals used were of analytical grade and obtained from Sigma Chemicals, USA.

A modified method of Fowomola and Akindahunsi (2008) was used for the fermentation of *Hura crepitans* seed as follows. The decorticated dried seeds were divided into two portions. The first portion was cooked for 2 h at $100 \pm 2°C$ while the second portion remained uncooked. Parts of the cooked and uncooked portions were separately emptied into a calabash that has been uniformly lined (to about 5 layers) with clean plantain leaves, excess water was drained off and the cooked seeds were allowed to ferment at 30°C for 24, 48, 72 and 96 h while the uncooked seeds were fermented only for 96 h. This was followed by oven-drying at 70°C to terminate the fermentation process. Dried seeds obtained were milled and the resulting flour was subjected to cold extraction process using acetone (4°C) in a 1:5 w/v flour-to-solvent ratio with constant stirring on a magnetic stirrer. The slurry obtained was then filtered through a filter paper and the residue was re-extracted twice in a similar fashion. The defatted flour was desolventized by drying in a fume hood and the dried flour were finally ground into flour using Marlex Excella grinder (Marlex Appliances PVT., Daman). The samples were sieved through 200 μm sieve to obtain homogenous defatted flour samples that were kept in air tight containers and refrigerated at 4°C for further analyses.

Determination of tannins

The modified vanillin-hydrochloric acid (MV-HCl) method of Price *et al.* (1978) was used. *Hura crepitans* defatted flour was extracted separately with 10 ml of 1.0% (v/v) HCl – methanol. 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. Catechin was used as standard with various concentrations (0.0, 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml).

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

Where x = value obtained from standard catechin graph

Determination of oxalate and saponin

Oxalate and saponin contents of the samples were determined according to the method described by Nwinuka *et al.* (2005) and Mbagwu et al. (2011).

Extraction of antioxidant

Extraction of antioxidants was carried out on samples of defatted flour following the method of Yurttas *et al.* (2000) with minor modifications. Approximately 5 g of each of the sample were separately mixed with 200 ml of 80% methanol (methanol:water; 80:20 v/v) in a conical flask and the extraction was done on a magnetic stirrer for 8 h. The mixture was concentrated to dryness on a rotary evaporator and the extract was stored in a refrigerator until use. The total phenolic contents and antioxidant properties of the raw unfermented (RUS), raw fermented (RFS), cooked unfermented (CUF) and cooked fermented for 96 h (CFS96) sandbox seeds extract were determined as described below.

Total phenol content (TPC) of RUS, RFS, CUF and CFS96 extract

TPC was determined according to the method described by Gulcin *et al*. (2003) with some modifications. To a mixture of 0.1 ml of the flour extract and 0.9 ml of water was added 0.2 ml of Folin-Ciocalteu's phenol reagent and the resulting mixture was mixed thoroughly. After 5 minutes of standing, 1.0 ml of 7% (w/w) Na_2CO_3 solution was added and the solution was then distilled to 2.5 ml before incubated for 90 min at room temperature. The absorbance against a negative control containing 1ml of water in place of the sample was then taken at 750 nm. Gallic acid at 0.1 mg/ml was used as standard to prepare the calibration curve and the result was expressed as µg gallic acid equivalent (GAE) per gram of sample.

Table 1. Effects of cooking and fermentation on the antinutrient levels of *Hura crepitans* seed

Samples	Oxalate (mg/100 g)	Tannin (mg/100 g)	Saponin (mg/100 g)
RUS	8.35 ± 0.01^g	1.44 ± 0.01^e	0.18 ± 0.01^d
RFS	7.76 ± 0.01^f	1.34 ± 0.01^d	0.15 ± 0.01^c
CUS	7.48 ± 0.02^e	1.36 ± 0.01^d	0.16 ± 0.01^c
CFS24	7.42 ± 0.01^d	1.27 ± 0.01^c	0.14 ± 0.01^{bc}
CFS48	6.98 ± 0.01^c	1.23 ± 0.01^b	0.13 ± 0.01^{bc}
CFS72	6.84 ± 0.01^b	1.23 ± 0.01^b	0.12 ± 0.01^{ab}
CFS96	6.58 ± 0.01^a	1.18 ± 0.01^a	0.10 ± 0.01^a

RUS: Raw unfermented sandbox; RFS: Raw fermented sandbox; CUS: Cooked Unfermented sandbox; CFS24: Cooked Fermented sandbox (24 h); CFS48: Cooked Fermented sandbox (48 h); CFS72: Cooked Fermented sandbox (72 h); CFS96: Cooked Fermented sandbox (96 Hrs) Results are mean values ± S.D. of triplicate determination and means in a column sharing the same alphabet are statistically non-significant (p<0.05)

Determination of diphenyl-1-picryhydrazyl (DPPH) radical-scavenging activity assay of RUS, RFS, CUF and CFS96 extract

The radical scavenging ability of the extract was determined using the stable radical DPPH (2, 2-diphenyl-2-picrylhydrazyl hydrate) as described by (Pownall *et al*., 2010) with some modifications. To 1ml of different concentrations (5, 2.5, 1.25, 0.625, 0.3125 mg/ml) of the flour 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 1ml methanol in place of the extract.

The percentage of inhibition was calculated in the following way:

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

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. Sample concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotting inhibition percentage against extract concentration.

Determination of metal chelating ability assay of RUS, RFS, CUF and CFS96 extract

The metal-chelating assay was carried out according to the method of Singh and Rajini (2004) with some modifications. Solutions of 2 mM $FeCl_2 \cdot 4H_2O$ and 5 mM ferrozine were separately diluted 20 times. Briefly, an aliquot (1 ml) of different concentrations (0.5, 0.25, 0.125, 0.0625 and 0.03125 mg/ml) of flour extract was mixed with 1 ml of diluted $FeCl_2 \cdot 4H_2O$. After 5 min of

incubation, the reaction was initiated by the addition of 1 ml of diluted ferrozine. 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 by using the formula:

$$Metal \, chelating \, activity \, (\%) = \left(\frac{Acontrol - Asample}{Acontrol} \right) \times 100$$

where $A_{control}$ = absorbance of control sample and A_{sample} = absorbance of a tested sample.

Determination of ferric reducing antioxidant power (FRAP) of RUS, RFS, CUF and CFS96 extract

The FRAP of the sample extracts was determined according to method described by Benzie and Strain (1999). 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 $FeCl_3.6H_2O$ were mixed together in the ratio of 10:1:1 respectively, to give the working FRAP reagent. A 50 µl aliquot of the flour extract at 1 mg/ml and 50 µl of standard solutions of ascorbic acid (20, 40, 60, 80, 100 µg/ml) were separately added to 1 ml of FRAP reagent. The mixture was well mixed and absorbance measured at 593 nm against reagent blank (50 µl of distilled water and 1 ml of FRAP reagent) after allowing reaction to complete at exactly 10 minutes. The reducing power was expressed as µg

ascorbic acid equivalent (AAE) per gram of sample.

Statistical analyses

All analyses were conducted in triplicates and the mean data ± SD (standard deviation) were reported. Data were subjected to statistical analysis using analysis of variance (ANOVA). Differences between the treatment means were separated using Duncan's multiple range test.

Results and Discussion

Effects of cooking and fermentation on the antinutrient level of Hura crepitan seeds

Table 1 shows the effects of processing methods on the antinutrient levels of *Hura crepitans* flour samples. All the flour samples showed a reduction in oxalate (8.35 to 6.58 mg/100 g), tannins (1.44 to 1.18 mg/100 g) and saponins (0.18 to 0.10 mg/100 g) with the processing methods employed in this study. The results showed that the concentrations of each tested antinutrient decreased significantly ($p < 0.05$) with cooking compared to fermentation alone. However, the combination of cooking with fermentation brought about a more significant reduction in the levels of oxalate and tannin and the reduction increased with increase in the fermentation days. This could be due to the decomposition of tannin-protein complexes and leaching of free tannin released during cooking and fermentation. According to Enujiugha and Agbede (2000), insoluble complexes result from interaction of tannin with proteins, thereby interfering with their bioavailability and poor palatability is generally attributed to high tannin diets.

Enechi and Odonwodu (2003) reported that the toxic effects of oxalate, phytate and tannins could be avoided, provided the plant food is cooked before consumption. Ikemefuna *et al.* (1991) reported that soaking and fermentation as well as a combination of cooking and fermentation decreased the tannins content of sorghum (Guinesia) seeds. According to Soetan and Oyewole (2009), combination of cooking and fermentation of plant seeds synergistically improved nutrient quality and drastically reduced anti-nutritional factors to safe levels much greater than other processing methods.

The values of antinutrients obtained in this study were lower than the values (tannin: 18.61 to 5.8 mg/100 g; oxalates: 23.7 to 3.6 mg/100 g and saponins: 8.5 to 1.4 mg/100 g) reported by Fowomola and Akindahunsi (2008) that cooking coupled with increase in fermentation days caused a significant reduction in the anti-nutrients content of Hura crepitans seed. The results agreed with the reports
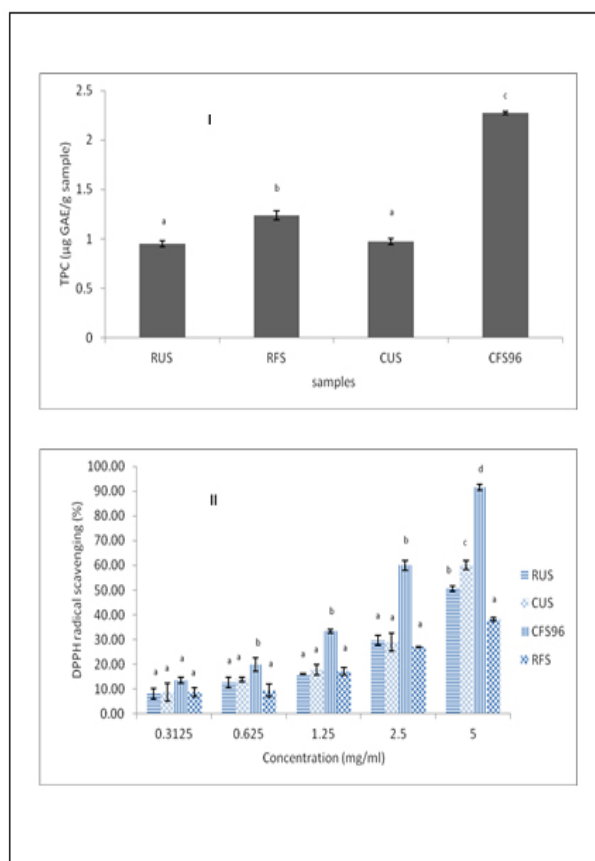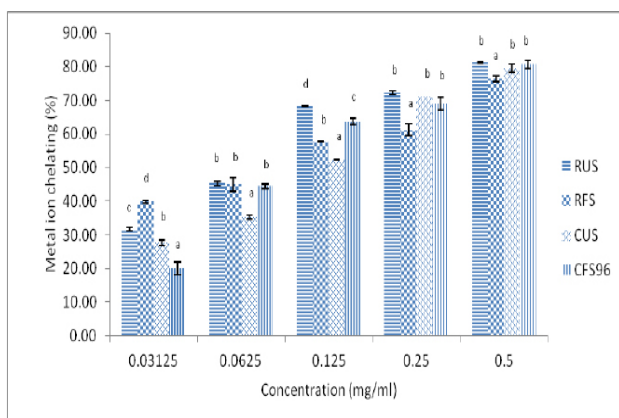

RUS: Raw unfermented sandbox; RFS: Raw fermented sandbox; CUS: Cooked unfermented sandbox; CFS96: Cooked Fermented sandbox (96 Hrs); Values sharing the same alphabet are statistically non-significant ($p < 0.05$)

Figure 1. Effects of cooking and fermentation on the (I) total phenol content (TPC) of *Hura crepitans* seed extracts; and (II) DPPH radical-scavenging activity of Hura crepitans seed extracts at different concentrations

of Ibukun and Anyasi (2012) where a reduction was observed in the oxalate, tannin and saponin levels of fermented sesame seeds (*Sesanum indicum*) (2.57 to 0.36, 0.019 to 0.008 and 2.68 to 1.01 mg/g extract respectively,); musk melon seeds (*Cucumis melo*) (2.1 to 0.27, 0.007 to 0.004 and 5.1 to 2.8 mg/g extract, respectively) and; white melon seeds (*Cucumeropsis mannii*) (1.35 to 0.14, 0.008 to 0.005 and 3.5 to 1.9 mg/g extract), respectively.

This reduction can be attributed to the activities of fermentative microorganisms involved (Fowomola and Akindahunsi, 2008) since a good number of microorganisms has been reported to be involved in the natural fermentation of *Hura crepitans* seeds (Fagbemi and Atum, 2001). Also, the activities of the indigenous microbes as well as processing could initiate the activities of some indigenous enzyme that degrade these antinutrients (Mubarak, 2005).

RUS: Raw unfermented sandbox; RFS: Raw fermented sandbox; CUS: Cooked unfermented sandbox; CFS96: Cooked Fermented sandbox (96 Hrs); Values sharing the same alphabet are statistically non-significant (p<0.05)

Figure 2. Effects of cooking and fermentation on the metal chelating (MC) ability assay of *Hura crepitans* seed extracts at different concentrations

Effects of cooking and fermentation on the antioxidant properties of RUS, RFS, CUF and CFS96 extract

Total phenol content (TPC)

The effects of fermentation and cooking on the total phenol contents of the flour samples are shown in Figure 1 (I). The samples showed that a TPC ranged from 0.95-2.27 µg GAE/g sample. Both fermentation and cooking increased the TPC in all the samples. However, the increase in TPC when the raw sample was cooked alone was not significant (p<0.05) when compared with when the seeds were cooked and fermented for 96 h. Sample CFS96 had the highest value of 2.27 µg GAE/g sample while the raw unfermented sample (RUS) had the lowest value 0.95 µg GAE/g sample. The study of Ghafar *et al*. (2011) has shown a direct relationship between antioxidant activity of citrus species and phenolic contents. The higher total phenol content of the fermented samples could be traced to the high vitamin C content of *Hura crepitan* seeds which was reported by Fowomola and Akindahunsi (2008) to have increased as fermentation proceeded. The hydrolysis of esterified and condensed phenolic compounds by the activities of the fermenting organisms could be responsible for the high TPC observed in the fermented sample.

DPPH radical-scavenging activity

Figure 1 (II) shows the results from the effects of fermentation and cooking on the DPPH radical-scavenging activity of *Hura crepitans* seed extracts at different concentrations. The DPPH radical scavenging activities of both the fermented and unfermented sample extracts increased progressively with concentration. Also, fermentation significantly

(p<0.05) increased the DPPH radical scavenging activities at all the concentrations used for the cooked samples. However, an insignificant decrease (p<0.05) in DPPH radical scavenging activities with fermentation was observed for the raw sample at all concentrations except at 5 mg/ml. Sample CFS96 had the highest DPPH radical scavenging activity (91.43%) at concentration of 5 mg/ ml while the RUS had the lowest DPPH value (7.98%) at concentration of 0.3125 mg/ml.

Table 2 indicates antioxidant potency based on IC_{50} values when compared with standards, a low value of IC_{50} indicates a higher antioxidant activity. Among the experimental samples, RFS had highest (p<0.05) IC_{50} value (6.56 mg/ml), hence the lowest DPPH radical scavenging activity while CFS96 had the lowest (p<0.05) IC_{50} value (2.32 mg/ml), hence the highest DPPH radical scavenging activity. However, the IC_{50} values of all the samples were significantly (p<0.05) higher than that of ascorbic acid (IC_{50}: 0.01 mg/ml) used as standard. Most of the processed samples showed moderate to higher levels of free radical inhibition activity than the raw samples at the various concentrations. The results obtained in this study agreed with the reports on the antioxidant activity of raw and differently processed underutilized tropical legume (*Canavalia ensiformis*) seeds Doss *et al*. (2011) and antioxidant activity of fermented and raw soybean extract (Samruan *et al*., 2012), where it was observed that the cooked and fermented samples were found to have higher antioxidant activity than unprocessed samples.

Metal chelating (MC) ability assay

The effects of cooking and fermentation on the ability of sandbox seed extracts to chelate and deactivate transition metals at different concentrations are shown in Figure 2. The metal chelating ability of the extract measures how effective the compounds in it can compete with ferrozine for ferrous ion. By forming a stable iron (II) chelate, an extract with high chelating power reduces the free ferrous ion concentration thus decreasing the extent of Fenton reaction which is implicated in many diseases (Gutteridge and Halliwell, 1990). The MC ability assay of all the sample extracts increased progressively with concentration. Cooking significantly decreased (p<0.05) the MC ability of the unfermented (RUS and CUS) samples at all the concentrations used. The fermented samples extracts followed the same trend only at concentrations of 0.03125 and 0.0625 mg/ml while at higher concentrations of 0.125, 0.25 and 0.5 mg/ml cooking significantly increased (p<0.05) the MC ability. On

Table 2. Antioxidant Potency of cooked and fermented
Hura crepitans seed extracts based on IC_{50} value

Samples	DPPH (mg/ml)	MC (mg/ml)
RUS	4.90 ± 0.01[d]	0.09 ± 0.01[b]
RFS	6.56 ± 0.03[e]	0.11 ± 0.01[c]
CUS	4.19 ± 0.02[c]	0.16 ± 0.02[e]
CFS96	2.32 ± 0.01[b]	0.14 ± 0.01[d]
AA. STD	0.01 ± 0.00[a]	------
EDTA STD	-------	0.07 ± 0.00[a]

RUS: Raw unfermented sandbox; RFS: Raw fermented sandbox; CUS: Cooked Unfermented sandbox; CFS96: Cooked Fermented sandbox (96 Hrs); AA. STD: ascorbic acid standard; EDTA STD: EDTA standard

Results are mean values ± S.D. of triplicate determination and means in a column sharing the same alphabet are statistically non-significant (p<0.05)
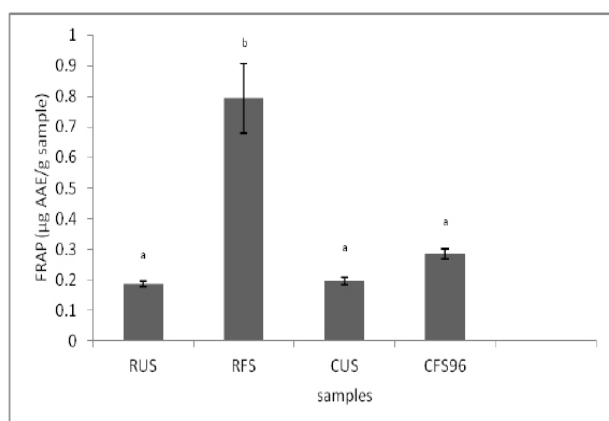
RUS: Raw unfermented sandbox; RFS: Raw fermented sandbox; CUS: Cooked unfermented sandbox; CFS96: Cooked Fermented sandbox (96 Hrs); Values sharing the same alphabet are statistically non-significant (p<0.05) Figure 3. Effects of cooking and fermentation on the ferric reducing antioxidant power (FRAP) of *Hura crepitans* seed extracts

the other hand, a significant decrease (p<0.05) in MC ability with fermentation was recorded except at concentrations of 0.0625, 0.125 and 0.5 mg/ml for only the cooked sample extracts. The RUS extract has the highest MC ability of 83.15% at 0.5 mg/ml concentration while the CFS96 sample extract has the lowest 20.02% at 0.03125 mg/ml concentration. Earlier works by Ibukun (2012) also presented poor chelating ability of fermented sesame seed (*Sesanum indicum*) as compared with the raw ones. From Table 2, RUS extract had significantly (p<0.05) higher metal chelating ability with IC_{50} value of 0.09 mg/ml than other samples while CUS showed the lowest

IC_{50} value of 0.16 mg/ml. The IC_{50} value of all the sample were significantly (p<0.05) lower than that of EDTA (IC_{50}: 0.07) used as standard.

Ferric reducing antioxidant power (FRAP)

The effects of cooking and fermentation on the ability of sandbox seed extracts to donate electron or hydrogen are shown in Figure 3. The FRAP of the samples increased with fermentation in all the samples, although the increase was significant (p<0.05) only in the raw sample (0.19-0.79 µg AAE/g sample). This agrees with Doss *et al*. (2011) who reported that processed tropical legume (*Canavalia ensiformis*) showed higher level of reducing power than those of raw seeds. Samples with higher reducing power have better abilities to donate electron and free radicals to form stable substances, thereby interrupting the free radical chain reactions (Juntachote and Berghofer, 2005). The results showed that fermentation of the raw seeds alone increased the reducing power of the extract but the ability to donate electrons or hydrogen decreased when cooking was combined with fermentation.

Conclusion

The present study has shown that combination of cooking and fermentation reduced the antinutrient levels of the samples and the effect increased with increase in fermentation days. Sample CFS96 exhibited the highest total phenolic contents and DPPH radical scavenging activity. However the raw samples, RUS and RFS, had the highest metal chelating activity and ferric reducing antioxidant power, respectively. Therefore, the extract of *Hura crepitans* flour could be employed as natural sources of antioxidants in the food industry to prevent lipid oxidation and maintain freshness during production and storage of food products.

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