

Effects of Fermentation on Nutritional and Microbial Qualities of Ash Neutralized Roselle (*Hibiscus sabdariffa* L.)

ABSTRACT

The study was conducted to investigate the effect of fermentation on the nutritional and microbial qualities of Roselle calyces from two States in Nigeria. Roselle calyx was fermented naturally (FN), fermented with coconut husk ash (FWCHA), Fermented with cocoa pod ash (FWCPA), Fermented with *Gmelina* tree ash (FWGTA) separately for 72 hours. The chemical and antinutrient compositions of raw and fermented calyx were determined using standard procedures. The microbial assessment of the samples was also determined. Moisture, ash, protein and carbohydrate contents increased significantly in fermented samples compared to raw calyx, however, the fat content reduced and crude fibre varied significantly likewise the antinutrients (Tannin, Hydrogen cyanide, Phytate) significantly reduced by the fermentation process ($\alpha=0.05$). A total of 8 organisms were isolated during fermentation, which included four (4) bacteria, one (1) yeast, and three (3) moulds. Microorganisms isolated during fermentation include *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa* (Bacteria) *Saccharomyces cerevisiae* (Yeast), *Mucor mucedo*, *Aspergillus niger*, *Aspergillus flavus* (Mold)

Keywords: [Roselle, fermentation, ash, proximate, mineral, antinutrient]

1. INTRODUCTION

Malnutrition is an unhealthy condition caused by not eating enough healthy food. Also, people are malnourished if unable to fully utilize the food they eat (undernutrition) or when they consume too many calories (overnutrition) [1].

Vegetables are agricultural products which are beneficial to the body; maintain good health and helps in the prevention of diseases [2]. Leafy vegetables are affordable and also possess high quality nutritional source which can aid in alleviating malnutrition rampant among the less wealthy population .

Roselle is an herbaceous upright plant growing up to 2 metres belonging to the family Malvaceae. Two varieties of Roselle has been recognized and also consumed, these are green calyx variety used to prepare soups, sauces and stews while the red calyx variety is commonly used for juice[3].

In West Africa, the green Roselle calyx variety is commonly steeped overnight in ash in water solution or parboiled before it is been used to prepare soup. Ash is the residue powder left after the combustion of wood. The major component of ash is calcium carbonate, about 25 or even 45 percent [4]. Less than 1 percent is phosphate, and less than 10 percent potash [5].

Comment [DP1]: State the microbial profiles before, during and at the of fermentation

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25 Environmental factors have an enormous effect on the growth and development of plants [6].
26 Previous studies have shown that plant morphology, most importantly, blade size, texture, thickness and
27 plant morphology are affected by biological environment [7]. Others include fertilizers, climatic factors, soil
28 nutrients, soil factors. Biological factors such as pests, soil microbes, planting density, weeds and
29 diseases [3] also play important roles.

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30 The traditional fermentation of foods serves as an improvement of diet with the change in the
31 aroma, flavour and texture in food[8]. The nutritional benefits of Roselle are however limited without
32 fermentation. The specific objective of the research is to determine the influence of climatic and
33 environmental factors on the chemical composition of Roselle calyx and the neutralizing efficacy of ash
34 on Roselle calyx.

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37 2. MATERIALS AND METHODS

39 2.1 SAMPLE COLLECTION AND PROCESSING

40
41 Kwara is a State in the Middle belt zone of Nigeria with the woodland and tall grass savanna
42 vegetation. The State Capital is Ilorin. The State is also known as the State of Harmony. Its coordinates
43 are 8° 30'N 5° 00'E. Ondo State, however, is in the South western region of Nigeria with the tropical rain
44 forest vegetation, it is also known as the Sunshine State. Akure is the State Capital. Its geographic
45 coordinates are 7° 10'N 5° 05' E.

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46 Green Roselle calyx was purchased from sellers at Oba market, Akure and from International
47 Market, Ilorin. Coconut husk was obtained from coconut sellers in Federal University of Technology,
48 Akure (FUTA) community [9]. Cocoa pod husk and *Gmelina* tree shavings were purchased from Cocoa
49 and *Gmelina* sellers in Uso Town, Ondo state and were all transported to the laboratory in sterile
50 polyethene bags.

51 Dry ashing technique was used to prepare ash from Coconut husk, cocoa pod and *Gmelina* tree
52 shavings according to the method described by Gaines. Coconut husk, cocoa pod and *Gmelina* tree
53 wood shaving were charred separately using an open flame. A 100g each of the charred sample was
54 weighed in separate porcelain crucibles labeled 1, 2, 3 respectively and placed inside the muffle furnace

55 at 500°C until all the carbon was oxidized. The ash residue obtained was then weighed and cooled in a
56 desiccator.

57 The Roselle calyx was sorted, cleaned and dried [10]. A 30g of ash was weighed and aseptically
58 poured into 2 litres of clean, sterile water in clean plastic containers with lid, followed by homogenization
59 and the addition of 150g of clean, sorted roselle calyx. The containers were labeled FN: Fermented
60 naturally (samples fermented with water alone), FWCHA: Fermented with coconut husk ash, FWCPA:
61 Fermented with cocoa pod ash, FWGTA: Fermented with *Gmelina* wood ash. The raw roselle calyx was
62 the control. The samples were allowed to ferment at $30 \pm 2^\circ\text{C}$ for 72 hours [3, 11].
63

64 2.2 Microbial analysis of the samples

65 Bacterial and fungal load were determined using standard methods as described by
66 Cheesbrough. Nutrient agar was used for bacteria while potato dextrose agar was used for fungal load. A
67 1g of the sample aliquot was measured using a sterile syringe into 9 mls of distilled clean, sterile water
68 followed by homogenization (10^{-1}), another 1ml was measured from 10^{-1} into 10 mls of sterile water and
69 then homogenized (10^{-2}). The serial dilution was done upto (10^{-5}) followed by pouring already prepared
70 molten agar in an aseptic environment. Microbial analysis was carried out at 24 hours interval throughout
71 the 72 hour fermentation process. The agar used was prepared according to the manufacturer's
72 specification. Bacteria plates were incubated at 37°C for 18 hours while fungi plates were incubated at
73 25°C for 72 hours. The number of colonies grown in each plate were was counted after incubation and
74 the isolated colonies were sub cultured to purify. The colonies were then characterized based on cultural,
75 morphological biochemical characteristics. Classified by microscopic examination of colony morphology
76 and biochemical tests after separation and growth on slants.

77 2.3 Proximate analysis

78 The proximate analysis of the samples was performed according to the Association of Official
79 Analytical Chemists (AOAC) procedures for crude fibre, ash, moisture, fat, protein using ($N \times 6.25$) and
80 Carbohydrate content was determined by difference.

81 2.4 Anti nutrient content determination

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82 Phytate was determined according to AOAC. Tannin content was determined according to the
83 method described by Makkar *et al*, Doss *et al*. Cyanide was determined by a modified AOAC, Onwuka
84 method.

85 2.5 Sensory evaluation

86 The Organoleptic properties of Roselle calyx fermented were evaluated and compared with raw
87 Roselle calyx. Organoleptic test was carried out by consumers of Roselle. The products were assessed
88 on a 5 point Hedonic scale by regular Roselle consumers as the Panelists [17]. Roselle (*Hibiscus*
89 *sabdariffa* L.) was prepared in five different ways: raw samples, samples fermented naturally, fermented
90 with cocoa pod ash, fermented with coconut husk ash and samples fermented with *Gmelina* tree ash
91 were cooked with melon soup and served. The attributes tested for were aroma, color, texture and
92 general acceptability.

93 2.6 Statistical analysis

94 Statistics computed include mean $\pm$ Standard deviation, analysis of Variance (ANOVA) and
95 Duncan New Multiple Range Test. All the data generated were statistically analyzed using mean $\pm$
96 standard deviation and analysis of variance statistical package for social sciences version 16.

97 3. RESULTS AND DISCUSSION

100 3.1 Microorganisms isolated during the fermentation of Roselle

101 A total of eight (8) microorganisms were isolated. They are *Bacillus subtilis*, *Pseudomonas*
102 *aeruginosa* and *Escherichia coli* as the only coliform, *Staphylococcus aureus*, *Saccharomyces cerevisiae*,
103 *Aspergillus flavus*, *Aspergillus niger*, *Mucor mucedo*. The changes in the microbial population during
104 fermentation revealed that the sample fermented with ash had a higher bacteria population while the
105 sample fermented without ash had a higher fungal population

106 3.2 Changes in the proximate composition during the fermentation of Roselle from Kwara and 107 Ondo States neutralized with different wood ash

108 Table 1: Proximate composition (%) of fermented Roselle from Kwara neutralized with
109 different ash

Comment [DP22]: How about the microbial loads, microbial profiles before fermentation and the bacteria responsible for the fermentation

Proximate	Ash content	Moisture content	Fat content	Crude fibre	Protein	Carbohydrate content
RAW	4.63±0.09 ^b	12.46±0.06 ^b	16.68±0.33 ^e	18.96±0.09 ^a	9.82±0.06 ^a	28.78±0.11 ^b
FN	4.10±0.02 ^a	21.13±0.13 ^e	8.40±0.08 ^b	22.01±0.09 ^d	11.18±0.02 ^c	41.85±0.10 ^e
FWCHA	6.76±0.12 ^c	13.35±0.02 ^c	9.15±0.14 ^c	24.76±0.08 ^e	10.17±0.04 ^b	35.81±0.03 ^c
FWCPA	7.42±0.02 ^d	19.75±0.07 ^d	14.86±0.04 ^d	19.95±0.04 ^b	10.14±0.01 ^b	27.88±0.05 ^a
FWGTA	7.76±0.02 ^e	12.15±0.08 ^a	8.08±0.04 ^a	20.42±0.06 ^c	11.51±0.03 ^d	39.87±0.14 ^d

110 Values represent means ± standard deviation of triplicate readings. Superscripts of the same letter in a
111 column are not significantly different at P≤0.05.

112 Legend:

113 FN: Fermented naturally; FWCHA: Fermented with coconut husk ash; FWCPA: Fermented with cocoa
114 pod ash; FWGTA: Fermented with *Gmelina* tree ash.

115 **Table 2: Proximate composition (%) of fermented Roselle samples from Ondo neutralized with**
116 **different ash**

Proximate	Ash content	Moisture content	Fat content	Crude fibre content	Protein	Carbohydrate content
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RAW	5.08±0.05 b	10.56±0.04 a	13.34±0.04 e	20.59±0.08 d	10.86±0.32 b	31.73±0.02 ^a
FN	3.60±0.05 a	11.12±0.01 b	6.55±0.04 ^b	22.82±0.02 e	13.68±0.03 e	42.05±0.04 ^b
FWCHA	5.82±0.01 b	17.20±0.10 c	6.69±0.00 ^c	16.54±0.04 a	9.58±0.02 ^a	50.70±0.01 ^e
FWCPA	7.12±0.01 c	11.28±0.02 c	6.96±0.05 ^d	17.70±0.17 c	12.91±0.01 d	43.84±0.03 ^c
FWGTA	7.06±0.05 c	13.06±0.05 d	5.75±0.04 ^a	17.05±0.04 b	11.34±0.03 c	45.56±0.05 ^d

117 Values represent means ± standard deviation of triplicate readings. Superscripts of the same letter in a
118 column are not significantly different at P≤0.05.

119 Legend:

120 FN: Fermented naturally; FWCHA: Fermented with coconut husk ash; FWCPA: Fermented with cocoa
121 pod ash; FWGTA: Fermented with *Gmelina* tree ash.

122 3.3 Changes in antinutrient content of fermented Roselle from Kwara and Ondo States neutralized 123 with different wood ash

124 **Table 3: Antinutrient content of fermented Roselle from Kwara neutralized with different ash**

Antinutrients	Phytates (mg/g)	Glycosides	Tannins (mg/g)
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	(mg/kg)		
RAW	14.42±0.20 ^e	82.49±0.10 ^e	1.43±0.06 ^d
FN	13.18±0.04 ^d	72.63±0.01 ^c	0.95±0.03 ^{bc}
FWCHA	7.83±0.08 ^a	44.25±0.02 ^a	0.78±0.07 ^a
FWCPA	8.45±0.06 ^b	80.59±0.08 ^d	1.00±0.04 ^c
FWGTA	9.48±0.06 ^c	60.82±0.17 ^b	0.88±0.05 ^b

125 Values represent means ± standard deviation of triplicate readings. Superscripts of the same letter in a
 126 column are not significantly different at P≤0.05

127 Legend:

128 FN: Fermented naturally; FWCHA: Fermented with coconut husk ash; FWCPA: Fermented with cocoa
 129 pod ash; FWGTA: Fermented with *Gmelina* tree ash

130 **Table 4: Antinutrient content of fermented Roselle from Ondo neutralized with different ash**

Samples	Phytates (mg/g)	Glycosides (mg/kg)	Tannins (mg/g)
RAW	12.97±0.08 ^e	107.87±0.09 ^e	1.20±0.03 ^d
FN	11.12±0.04 ^d	83.66±0.18 ^c	1.05±0.03 ^c

FWCHA	8.65±0.02 ^b	68.17±0.08 ^a	0.79±0.02 ^a
FWCPA	10.51±0.06 ^c	98.19±0.04 ^d	0.87±0.05 ^b
FWGTA	6.80±0.08 ^a	71.09±0.09 ^b	0.79±0.03 ^a

131 Values represent means ± standard deviation of triplicate readings. Superscripts of the same letter in a
 132 column are not significantly different at P≤0.05

133 Legend:

134 FN: Fermented naturally; FWCHA: Fermented with coconut husk ash; FWCPA: Fermented with cocoa
 135 pod ash; FWGTA: Fermented with *Gmelina* tree ash

136 **Table 5: Sensory evaluation profile of the Roselle samples from Kwara**

Samples	RAW	FN	FWCHA	FWCPA	FWGTA
AROMA	1.00±0.00 ^a	1.66±0.57 ^b	3.00±0.00 ^c	3.36±0.57 ^d	5.00±0.00 ^e
COLOUR	0.66±0.57 ^a	5.00±0.00 ^d	2.00±0.00 ^{bc}	1.66±0.57 ^b	2.66±0.57 ^c
TEXTURE	1.00±0.00 ^a	2.00±0.00 ^b	3.66±0.57 ^c	3.33±0.57 ^c	4.66±0.57 ^d
OVERALL ACCEPTABILITY	1.00±0.00 ^a	2.33±0.57 ^b	3.00±0.00 ^c	2.33±0.57 ^b	4.50±0.70 ^d

137 Values represent means ± standard deviation of triplicate readings

138 Superscripts of the same letter in a column are not significantly different at P≤0.05

139 Legend:

140 FN: Fermented naturally; FWCHA: Fermented with coconut husk ash; FWCPA: Fermented with cocoa
141 pod ash; FWGTA: Fermented with *Gmelina* tree ash.

142 **Table 6: Sensory evaluation profile of the Roselle samples from Ondo**

Samples	RAW	FN	FWCHA	FWCPA	FWGTA
AROMA	1.33±0.60 ^a	1.85±0.10 ^b	2.80±0.20 ^c	3.30±0.15 ^d	4.83±0.25 ^e
COLOUR	0.50±0.10 ^a	4.90±0.50 ^d	2.25±0.57 ^{bc}	1.50±0.20 ^b	2.50±0.00 ^c
TEXTURE	1.25±0.57 ^a	1.90±0.20 ^b	3.85±0.10 ^c	3.51±0.50 ^c	5.00±0.50 ^d
OVERALL ACCEPTABILITY	1.08±0.00 ^a	2.00±0.15 ^b	3.01±0.20 ^d	2.31±0.33 ^c	4.65±0.60 ^e

143 Values represent means ± standard deviation of triplicate readings

144 Superscripts of the same letter in a column are not significantly different at P≤0.05

145 Legend:

146 FN: Fermented naturally; FWCHA: Fermented with coconut husk ash; FWCPA: Fermented with cocoa
147 pod ash; FWGTA: Fermented with *Gmelina* tree ash.

148 The higher bacterial population recorded in the sample fermented with ash may be due to alkaline
149 nature of the ash which raised the initial pH that favoured their growth [18]. This was in agreement with
150 the result of [3,11].

151 *Saccharomyces cerevisiae* is a widely distributed spore former [19]. This may be due to
152 contamination from handling of the calyces in the process of fermentation since there are a lot of fungi
153 spores in the air. *Bacillus* sp has been reported to be the primary microorganism for the fermentation of
154 African locust bean to produce 'dawa dawa' [20] 'soubala' [21], some Asian fermented foods [22, 23].
155 Their presence in these fermentations may be due to their capability to start the fermentation of
156 nitrogenous as well as carbohydrate products [24]. In addition, *Bacillus* sp. is known to produce a
157 diversity of enzymes. Their metabolic activities can contribute to flavour and aroma generating reactions
158 [25].

159 *Staphylococcus aureus* occurrence agrees with the findings of [26] who reported the absence
160 of *Staphylococcus* in the 48 h fermentation period for 'dawadawa' and those of [27] and [28] who found
161 that *Staphylococcus* was present only at the early stages of the fermentation.

162 *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Mucor mucedo* were found
163 only at the onset of the fermentation and thereafter could not be isolated. The possible involvement of
164 these microorganisms in the fermentation is then ruled out. The microorganisms were presumed to be
165 probably contaminants obtained during the initial handling.

166 Of all the samples fermented, samples fermented naturally had the highest increase in protein
167 level (Table 2). The increase in protein content in the sample fermented naturally could be as a result of
168 an increase in the different species of microorganisms involved which may have secreted some
169 extracellular enzymes (proteins) [29]. The increase in the microbial biomass in the form of single cell
170 proteins may be another reason for the increase in the protein content [30].

171 The ash content of all the fermented samples (Table 1 and 2) shows significant decrease
172 ($P \leq 0.05$) in the sample fermented naturally. The high value of ash in the unfermented sample shows that
173 the calyx may have a reasonable quantity of mineral elements. Carbohydrate, moisture levels increased
174 while fat and crude fibre contents reduced significantly.

175 The results of the changes in the antinutrient of Roselle are as shown in Tables 3 and 4.
176 Fermentation of calyx brings about a significant decrease ($P \leq 0.05$) in the cyanide content of the calyx

Comment [DP23]: How would your findings agree with the findings of 26 and 27 Please rephrase

Comment [DP24]: *Klebsiella* is not listed in the abstract.

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when compared with the unfermented calyx. However, the sample fermented with wood ash recorded the highest decrease in cyanide content with fermentation. This however agrees with the report of [31] and [3] that cyanide content of the Roselle decreases with fermentation.

Fermentation brought about a significant decrease ($P \leq 0.05$) in the tannin content of the calyx samples fermented naturally and with ash, however, *Gmelina* tree ash fermented samples had the lowest decrease. The decrease in the tannin may be because of the processing [30] and [32] that samples were subjected to coupled with the activities of the microbial enzymes involved in the fermentation process.

The phosphorus in the phytic acid interferes with Calcium, Iron, Magnesium and Zinc absorption because of its ability to chelate divalent cationic minerals [33]. The results of the present study revealed that there was a significant decrease ($P \leq 0.05$) of the phytate content of fermented calyx samples. However, the phytate content of the sample fermented with *Gmelina* tree ash was significantly lower ($P \leq 0.05$). The phytate content of the unfermented calyx was lower than that of tropical vegetables (394.9-1692.3 mg/100g [34]. The decrease in the phytate content of the fermented calyx agrees with the report of [10] that phytate content decrease could be attributed to possible secretions of hydrolytic enzymes (phytase) by the microorganisms. This enzyme is capable of hydrolyzing phytate.

The result of sensory evaluation as shown in Table 5 and 6 revealed that fermentation improves taste, aroma, colour and texture of the calyx. Samples fermented with *Gmelina* tree ash had the best aroma with the raw sample having the least. In terms of colour, samples fermented naturally were the best with raw sample having the least. Samples fermented with *Gmelina* tree ash had the best texture with the raw samples having the least. For overall acceptability, samples fermented with *Gmelina* tree ash had the best with the raw samples having the least.

4. CONCLUSION

The study therefore reveals that fermentation as a precooking method and the use of ash especially *Gmelina* tree ash can be used to reduce the antinutrient, improve the nutrient in Roselle. Also, climatic and environmental factors play a key role in the chemical composition of the plant

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