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Nutrients, phytochemicals, fungal flora and aflatoxin in fresh and salted *Vernonia amygdalina* leaves

Fred O. J. OBOH*, Anita ALIU, Monday I. IDEMUDIA and Derek AHAMIOJE

Department of Basic Sciences, Benson Idahosa University, P.M.B.1100, Benin City, Edo State, Nigeria.

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In this study, the effect of salting on the pH, phytochemicals, fungal flora and nutrient composition of *Vernonia amygdalina* leaves was investigated. There was a decrease in pH from 5.88 for the fresh, to 5.80, 5.73, 5.24, and 5.02 for the light brined, light salted, heavy salted and light brine + vinegar treated leaves, respectively. Leaves treated with water alone had a pH of 6.63. Compared with the fresh leaves, there was a decrease in all the nutrients investigated, except for sodium and calcium which increased significantly. The fresh sample showed the highest concentration of total phenolic compounds (TPC). Compared with the fresh, the highest loss of TPC was for the light brine + vinegar preserved leaves (51.69%) and the lowest loss was for the water treated (27.28%). The heavy salted and light salted leaves lost about a third (36.05 and 33.42%, respectively) and the light brined 45.93% of their TPC. In terms of organoleptic properties, reduction in fungal count and genera, and loss of moisture, heavy salting appears to be the most effective preservation treatment. These, coupled with only moderate loss of nutrients and phytochemicals is recommended as an effective technique for the preservation of *V. amygdalina* leaves. Screening of the fresh and treated produce indicated the presence of aflatoxin.

Key words: *Vernonia amygdalina* leaves, salting, nutrients, phytochemicals, fungal flora, aflatoxin.

INTRODUCTION

The salting or brining of vegetables offers tremendous possibilities both for their commercial and home preservation. In the process, the salt exerts a selective action on the naturally occurring organisms to promote a desirable fermentation. Salt tolerant microorganisms use as their nutritive material the soluble constituents that diffuse out of the vegetable as a result of the action of the salt on vegetable tissue. These fermentative organisms bring about the production of various compounds, principally lactic acid but also acetic acid (both of which result in a reduction of pH), alcohols and considerable amounts of gas. The production of sufficient amount of acid makes the medium unsuitable for the growth of food spoilage bacteria. In addition, the acid and other microbial metabolites alter the flavour of the food. Substances and organisms in fermented foods can cause changes in the composition and/or activity of the gastrointestinal microbiota resulting in several health benefits (Perdigon et al., 1987; FAO/WHO, 2001; FAO, 2007; Farnworth, 2004; Farnworth et al., 2007; Granato et al., 2010).

Vernonia amygdalina, variously known as bitter leaf (English), oriwo (Edo), ewuro (Yoruba), shikawa (Hausa), and olubu (Igbo), is a tropical shrub, 1-3 m in height with petiole and leaf of about 6 mm in diameter, and elliptic in shape (Igile et al., 1995). The leaves are dark green in colour, with a characteristic odour and a bitter taste. The species is indigenous to tropical Africa where it is found wild or cultivated (Bosch et al., 2005). The leaves are eaten, after crushing and washing thoroughly to remove the bitterness (Mayhew and Penny, 1998). As with other high yielding leafy vegetables, post-harvest losses may

*Corresponding author: *Corresponding author. E mail: fredoboh67@yahoo.co.uk; Tel: +2348036677636.

occur due to inadequate preservation.

Previous authors have reported nutrient composition and antimicrobial activity (Oboh and Masodje, 2009) and blood lipid control activity of the methanolic and aqueous extracts of *V. amygdalina* leaves (Adaramoye et al., 2008; Oboh and Enobhayisobo, 2009), and the effects of blanching and drying, and salting on their organoleptic characteristics and nutrient composition (Osunde and Makama, 2007; Aliero and Abdullahi, 2009; Oboh and Madojemu, 2010), In this study, the effect of salting on the phytochemicals, fungal flora and nutrient composition of *V. amygdalina* leaves was investigated. The fresh and salted leaves were also screened for the presence of aflatoxin.

MATERIALS AND METHODS

To investigate the effect of salting on the nutrient composition, phytochemicals, mycological flora and organoleptic properties of *V. amygdalina* leaves, a study was conducted using the following treatments:

Heavy salting

V. amygdalina leaves were gently washed to remove dirt, and the water drained. Salt (37.5 g) and leaves (150 g) were mixed well in a plastic bucket. The mixture was covered with two layers of muslin cloth; a pressure plate and weight were placed on it. Brine made of salt (37.5 g) and water (150 ml) was added until the pressure plate was slightly submerged. The buckets were stored in a cool, dry and shaded place for two weeks (James and Kuipers, 2003). Ambient temperature was 26.5 to 27.0°C.

Light salting

Washed and drained *V. amygdalina* leaves (150 g) were mixed well with dry salt (3.75 g) in a plastic bucket, packing tightly. The mixture was covered with two layers of muslin cloth; a pressure plate and weight were placed on it. The bucket was stored in a cool, dry and shaded place for two weeks (James and Kuipers, 2003). Ambient temperature was 26.5 to 27.0°C.

Light brining

To washed and drained *V. amygdalina* leaves (150 g) light brine was added (3.75 g salt dissolved in 150 ml water). The brine was added to the vegetable in layers (that is, put a layer of vegetables, add brine, put another layer of vegetables add more, and so on) in a plastic bucket, and packed tightly. The mixture was covered with two layers of muslin cloth; a pressure plate and weight were placed on it. The bucket was stored in a cool, dry and shaded place for two weeks (James and Kuipers, 2003). Ambient temperature was 26.5 to27.0°C.

Light brine + vinegar

A solution of salt (7.5 g), white vinegar (7.5 ml) and water (150 ml) was added to rinsed and drained *V. amygdalina* leaves (150 g) in layers (that is, put a layer of vegetables, add brine and vinegar, put another layer of vegetables add more, and so on) in a plastic bucket and packed tightly. The mixture was covered with 2 layers of muslin cloth; a pressure plate and weight were placed on it. The bucket was stored in a cool, dry and shaded place for two weeks (James and Kuipers, 2003). Ambient temperature was 26.5 to 27.0°C.

Analytical procedure

Determination of nutrients

Vitamin C, β-carotene, carbohydrates, protein and moisture: Ascorbic acid was determined titrimetrically using the 2,6-dichlorophenolindophenol method according to Osunde and Musa Makama (2007). β-Carotene was determined spectrophotometrically according to the method of Nagata and Yamashita (1992). The sample (100 mg) was vigorously shaken in 10 ml of an acetone-hexane mixture (4:6) for 1 min and filtered through Whatman no. 4 filter paper. The absorbance was measured at 453, 505, and 663 nm. The content of β-carotene was calculated according to the following equations:

$$\beta\text{-carotene (mg/100 ml)} = 0.216\ (A_{663}) - 0.304\ (A_{505}) + 0.452\ (A_{453})$$

Where, A is absorbance. Total carbohydrate was determined spectrophotometrically at 620 nm using the anthrone method (Hedge and Hofreiter, 1962). Nitrogen was determined using the Kjedahl method and protein calculated as total nitrogen x 6.25. Moisture content was determined by drying 5 g wet sample to constant weight in a ventilated oven at 60°C (AOAC, 1984). Moisture content was calculated as follows:

$$\%\ \text{Moisture} = \frac{\text{Initial weight} - \text{Final weight}}{\text{Initial weight}} \times 100\%$$

Ether extract was determined by Soxhlet extraction with petroleum ether (40-60°C) and calculated as:

$$\%\ \text{Ether extract} = \frac{\text{Weight of sample before extraction} - \text{Weight of sample after extraction}}{\text{Weight of sample before extraction}} \times 100\%$$

Minerals: Minerals were determined according to Novosamsky (1983) and Okalebo (1985). Iron content was determined by atomic absorption spectrophotometry, after wet digestion with nitric acid-perchloric acid. Calcium was determined by ethylene diamine tetra acetic acid (EDTA) titration. Potassium and sodium were determined by flame photometry.

Phytochemical studies

Qualitative analysis was carried out according to Malec and Pomilio (2003) and Evans (1996).

Determination of total phenolic compounds (TPC)

TPC was determined spectrophotometrically according to Azizah et al. (2007). The standard curve was prepared as follows: To each gallic acid standard (50, 100, 150 upto 500 mg/l) and sample, 0.5 ml of 0.2 N Folin-Ciocalteau reagent was added. After 8 min, 1.5 ml

sodium carbonate (7.5% w/v) was added. The mixture was kept in the dark for 1 h and absorbance was measured at 765 nm. TPC content was read off the standard curve and expressed as mg of gallic acid equivalent (GAE) per litre of sample.

Microbiological analysis

Sterilization

The solid and liquid media were sterilized by autoclaving at 121°C for 15 min. Glassware was sterilized in the oven at 100°C for 1 h and allowed to cool down before use while the media were left to cool to 45°C before pouring into the Petri dishes

Enumeration and isolation of fungal isolates

Extract of fresh or preserved *V. amygdalina* leaves (1 ml) was aseptically transferred into 9 ml sterile distilled water in McCartney bottles and mixed thoroughly. Serial dilutions up to 10^{-2} and 10^{-3} were then carried out and 1 ml from dilutions 10^{-2} and 10^{-3} were transferred into the Petri dishes. Potato dextrose agar was poured into the Petri dishes and allowed to solidify. The plates were then incubated at ambient temperature (30±2°C) for three to five days and observed daily for growth of fungi. Discreet colonies were counted using a colony counter (Digital Colony Counter-Labtech, UK). Sub-culturing of the occurring fungi was made on sterile PDA plates to obtain pure isolates. The pure isolates were stored in PDA slants at 4°C for further laboratory studies.

Identification of Isolates

Pure cultures of the isolates obtained were used for identification. The identification of the isolates were based mainly on the structural features as observed from the growing colonies in plants (Sutton et al., 1998) and slide mounts seen under the microscope. The plate identification involved colour, presence of mycelia, spores and production of fruiting bodies. In the microscopic examination, a wet mount of each isolate was prepared on a microscopic slide, covered with a cover slip, stained with lactophenol cotton blue dye and viewed under the low and high power magnification to ascertain its features. These features were compared with those described in standard fungi manuals (Barnett and Hunter, 1998; Raper and Fennel, 1973).

Determination of percentage of fungal occurrence

The percentage frequency of occurrence of the fungal isolates was determined by dividing the occurrence of individual fungal isolates with the total occurrence. This was expressed as a percentage as follows:

$$\frac{X}{N} \times \frac{100}{1}$$

Where, X is the total number of each organism in the samples and N is the total number of all organisms in the samples.

Screening for aflatoxin

Screening for aflatoxin was done by the Contaminants Bureau FDA method (Heinrich, 1990; Richard et al., 1993) with modification, as follows:

Sample preparation

Fresh or salted *V. amygdalina* leaves (5 g) were ground to pass a no. 20 sieve, mixed thoroughly and placed in a 50 ml, glass-stoppered Erlenmeyer flask with 2.5 ml of H_20, 2.5 g of diatomaceous earth, and 25 ml of $CHCl_3$. The mixture was shaken for 30 min and filtered. The first 10 ml of the extract to emerge from the filter was collected and placed in the column.

Column preparation

Column chromatography was carried our using a 22 x 300-mm chromatographic tube packed with silica gel and anhydrous Na_2SO_4 in $CHCl_3$. The extract was added on top of the silica gel. The column was washed with 150 ml of hexane, followed by 150 ml of anhydrous ether, which were discarded. Aflatoxins were then eluted with 150 ml of solvent mixture MeOH-$CHCl_3$ (3:97v/v) and the entire fraction was collected and evaporated to dryness.

Thin layer chromatography (TLC)

Residue was dissolved in chloroform-acetonitrile (4:1v/v) and applied on a TLC plate, pre-coated with silica gel 60. Plates were developed for about 1 h using a chloroform-acetone-isopropanol (8:1:1v/v/v) solvent mixture in an equilibrated tank. Plates were removed from the tank, dried and examined under UV light.

RESULTS AND DISCUSSION

Table 1 shows the result of an organoleptic evaluation of the fresh and treated *V. amygdalina* leaves. The fresh leaves were a bright dark green in colour, had a distinctive green leaf smell and were firm, with slightly rough surface. Light brined leaves had a dull dark green colour with a slightly offensive smell. They were deformed and their surface was rougher than that of the fresh leaves. Light salted leaves were similar to the light brined in structure but were closer to the fresh leaves in texture.

Light brine + vinegar treated leaves had an almost smooth texture and their structure was better maintained than that of the light brined and light salted leaves. Leaves subjected to heavy salt treatment had properties similar to those of the original leaves but had a salty taste. Leaves treated with water alone had a dull dark green colour and a very offensive smell. They had a deformed structure and a rough texture. Based on these observations, heavy-salted leaves were the closest to the fresh leaves in organoleptic properties.

The pH values of fresh and fermented *V. amygdalina* leaves are given in Table 2. There was a decrease in pH from 5.88 for the fresh, to 5.80, 5.73, 5.24, and 5.02 for the light brined, light salted, heavy salted and light brine + vinegar treated leaves, respectively. These values indicate that salting resulted in fermentation, with production of acid and decrease in pH, the better preserved heavy-salted and light brine vinegar treated leaves having the lowest values. Leaves treated with water alone had a pH of 6.63.

Table 1. Organoleptic characteristics of fresh and fermented (*Vernonia amygdalina*).

Property	Fresh	Light brine	Light salt	Light brine+ vinegar	Heavy salt	No salt
Colour	Bright dark green	Dull dark green	Dull dark green	Dull light green	Bright dark green	Dull dark green
Odour	Fresh leafy smell	A slight but not offensive smell	A slight but not offensive smell	A slight but not offensive smell	Fresh leafy smell retained	Very offensive smell
Appearance and texture	Firm with slightly rough surface	Slightly deformed structure; surface slightly rougher than that of the fresh leaves.	Slightly deformed structure; similar to the fresh leaves in texture.	Retained more of the original structure than the light salted and light brined. Leaves had an almost smooth texture	Similar to that of the fresh leaves.	Deformed structure rough surface

Table 2. pH values for fresh and fermented *Vernonia amygdalina* leaves.

Treatment	pH
Fresh	5.88
Light brine	5.80
Light salt	5.73
Light brine + vinegar	5.02
Heavy salting	5.24
No salt	6.63

Table 3 shows the nutrient content of fresh and fermented leaves. Compared with the fresh leaves, there was a decrease in the content of all the nutrients investigated, except for sodium and calcium which increased significantly. This is in agreement with previous findings (Oboh and Madojemu, 2010). The water treated leaves had less of each nutrient than the fresh or fermented leaves, except for their protein content, which was not significantly different from that of the heavy-salted and light brine and vinegar treated leaves. Loss of nutrients during the salting of vegetables has been observed previously (Jones and Etchells, 1944) and might be due to leaching into the aqueous medium in which the leaves were fermented. The sodium content was much higher in the salted samples than in the fresh sample because of the addition of salt to the fermentation medium. The higher calcium levels in the salted leaves (relative to the fresh) were due, probably to the presence of calcium impurity in the salt (Jones and Etchells, 1944) and/or the presence of this mineral in the water used in the experiments.

Compared with fresh sample, the moisture content of the fermented leaves showed a significant decrease due to the osmotic effect of the salt. The heavy-salted leaves had the lowest moisture content (less than half of that of the fresh leaves). This, coupled with their lower pH gave an environment less favourable than the fresh leaves, for the proliferation of spoilage microorganisms, resulting in excellent preservation as indicated by the organoleptic properties of the leaves subjected to this treatment.

The results of tests for phytochemicals and the total phenolic compounds content of the fresh and preserved leaves are presented in Table 4 and 5. These compounds when present in the diet could provide biological and pharmacological benefits. Saponins and glycosides were found in all the leaves (fresh, salted and unsalted) and tannins were present in all except the unsalted. Steroids were detected in the fresh, light salted and water treated (unsalted) leaves. Flavonoids and alkaloids were not detected in any of the samples. The fresh sample gave the highest value of total phenolic compounds (TPC). The highest loss of TPC was for the light brine + vinegar preserved leaves (51.69%). The lowest loss was for the badly preserved water treated leaves (27.28%). Of the salted leaves, the heavy-salted and light-salted lost about a third of their TPC (36.05 and 33.42% respectively). Compared with the fresh, light brining resulted in the loss of 45.93% of the total phenolic compounds. Thus, the best preserved leaves in terms of organoleptic properties (that is, the heavy-salted) lost only about a third of their TPC.

Table 6 shows the fungal counts of fresh and fermented leaves. In all cases, salting resulted in a decrease in fungal count. The fresh leaves had a higher fungal count (120×10^2 cfu/ml) than the salted samples (49, 47, 43 and 8×10^2cfu/ml respectively for light brined, light salted, light brine + vinegar treated and heavy salted leaves, respectively). Unsalted leaves had the highest count (292×10^2cfu/ml, about a two and half-fold increase, compared with the fresh leaves). Heavy salting was the most effective

Table 3. Nutrients of fresh and fermented *Vernonia amygdalina* leaves.

Bitter leaf	Fresh	Light brine	Light salt (dry)	Light brine ± vinegar	Heavy salt	No salt
Fe (mg/100 g)	16.50±0.71^{b}	11.80±0.14^{b}	9.80±0.00^{a}	11.20±0.00^{c}	10.15±0.07^{c}	9.65±0.07^{a,c}
Ca (mg/100 g)	235.26±0.01^{c}	257.82±0.01^{c}	245.29±0.01^{c}	242.79±0.01^{c}	240.28±0.01^{c}	220.25±0.07^{a,b}
K (mg/100 g)	23.57±0.01^{c}	24.48±0.01^{c}	27.15±0.07^{c}	25.39±0.01^{c}	20.82±0.01^{a,c}	9.03±0.01^{a,c}
Na (mg/100 g)	1.56±0.01^{c}	165.48±0.01^{a}	161.39±0.01^{a}	204.80±0.14^{a,b}	289.77±0.01^{a}	1.48±0.01^{a}
Protein (%)	4.59±0.01^{b}	3.13±0.00^{a}	2.63±0.01^{a,c}	1.98±0.01^{a,c}	1.65±0.01^{a,c}	2.20±0.01^{a,c}
Carbohydrate (mg/100 g)	0.054±0.039^{b}	0.001±0.001^{a,c}	0.001±0.001^{a,c}	0.022±0.002^{a,c}	0.029±0.002^{a,c}	-
Lipid (g/100 g)	0.85±0.07^{c}	0.55±0.07^{a,b}	0.35±0.07^{a,b}	0.51±0.01^{a,c}	0.62±0.03^{a,c}	0.31±0.01^{a,c}
Moisture (%)	74.60±4.26^{c}	46.00±4.37^{a,c}	48.40±3.50^{a,b}	43.80±7.45^{a,b}	36.90±1.94^{a,b}	48.70±3.52^{a,b}
β-carotene (mg/100 g)	0.44±0.67^{b}	0.35±0.59^{a,c}	0.37±0.58^{a,c}	0.31±0.52^{a,b}	0.28±0.46^{a,b}	0.02±0.02^{a,b}
Vit. C (mg/100 g)	1049.03±2.87^{b}	821.29±0.01^{a,c}	625.52±0.01^{a,c}	680.84±0.03^{a,c}	582.94±0.06^{a,b}	285.12±0.01^{a,c}

Values are recorded as mean ± standard deviation of three independent samples. t-Test: ^{a}, Values differ significantly compared with the fresh sample mean $P < 0.05$; ANOVA: ^{b}, mean values differ significantly from other means within the same group $P<0.05$; ^{c}, values are not significantly different $P < 0.05$ within the same group.

Table 4. Phytochemicals of fresh and salted *Vernonia amygdalina* leaves.

Sample	Saponin	Tannin	Flavonoid	Alkaloid	Steroid	Glycoside
Fresh	+	+	-	-	+	+
Light brine	+	+	-	-	-	+
Light salt	+	+	-	-	+	+
Light brine + vinegar	+	+	-	-	-	+
Heavy salt	+	+	-	-	-	+
No salt	+	-	-	-	+	+

+, Present; -, absent. Values are recorded as mean ± standard deviation of two independent samples. t-Test: ^{a}, values differ significantly compared with the fresh sample mean $P < 0.05$; ANOVA: ^{b}, mean values differ significantly from other means within the same group $P < 0.05$; ^{c}, values are not significantly different $P < 0.05$ within the same group.

for the reduction of fungal load (93.3% reduction) and was therefore the most effective preservation treatment.

Table 7 shows the occurrence of fungal species and genera in fresh and salted *V. amygdalina* leaves. *A. nige*r occurred in the fresh, light brined and the water treated (no salt) leaves. *A. flavus* was associated with all the samples (fresh and treated) and *Penicillium* with the light salted and water treated. *Fusarium* was associated with only the light salt and vinegar treated leaves.

Mycotoxins are small (MW ~ 700), toxic chemical products formed as secondary metabolites by a few fungal species that readily colonise crops and contaminate them with toxins in the field, between harvest and drying, and during storage. The major fungal genera producing mycotoxins are *Aspergillus, Fusarium* and *Penicillium.* The most common mycotoxins are aflatoxins, ochatoxin A, fumonisins, deoxyni-valenol, T-2 toxin and zearalenone (Turner et al., 2009 and Zheng et al., 2006).

A green colour characteristic of aflatoxins B_1 and B_2 (Surekha et al., 2011) was observed when

Table 5. Total phenolic compounds (TPC) of fresh and salted leaves.

Sample	Total phenolic compounds (mg/100g)	Loss relative to the fresh (%)*
Fresh	0.799 ± 0.001^{b}	-
Light brine	$0.432 \pm 0.001^{a,c}$	45.93
Light salt	$0.532 \pm 0.001^{a,c}$	33.42
Light brine + vinegar	$0.386 \pm 0.002^{a,c}$	51.69
Heavy salt	$0.511 \pm 0.001^{a,c}$	36.05
No salt	$0.581 \pm 0.001^{a,c}$	27.28

*Percentage loss relative to the fresh = 0.799 - TPC/ 0.799 x 100.

Table 6. Fungal counts of fresh and fermented *V. amygdalina* leaves.

Sample	10^2 CFU/Ml	loss relative to the fresh (%)
Fresh	120	-
Light brine	49	59.16
Light salt	47	60.83
Light brine + vinegar	43	64.17
Heavy salt	8	93.33
No salt	292	-

Table 7. Occurrence of fungal species and genera, and aflatoxin in fresh and preserved leaves.

Leaves	*Aspergillus niger*	*Aspergillus flavus*	*Penicillium* spp.	*Fusarium* spp.	Aflatoxin
Fresh	+	+	-	-	+
Light brine treated	+	+	-	-	+
Light salt treated	-	+	+	-	+
Light brine + vinegar treated	-	+	-	+	+
Heavy salt treated	-	+	-	-	+
Water (no salt) treated	+	+	+	-	+

+, Present; -, absent.

Table 8. Frequency of occurrence of fungi isolates.

Fungal isolate	Percentage frequency of occurrence
Aspergillus flavus	79.12
Aspergillus niger	74.21
Penicillium sp.	48.26
Fusarium sp.	19.36

leaf extracts were cleaned up, separated by thin layer chromatography (TLC), and viewed under ultra violet light. Aflatoxins are highly toxic and carcinogenic secondary metabolites of *Aspergillus flavus, Aspergillus parasiticus* and *Aspergillus nomius. A. flavius* produces only aflatoxin B_1 and B_2, while the other two species produce both aflatoxins B and G (Baydar, 2007). In this study, only *A. flavus* was found to be associated with the leaves. Some species of *Fusarium* produce the mycotoxins zearalenone and fumonisin B_1, which are possibly carcinogenic in humans. One species of *Penicillium, Penicillium verrucosum* produces ocratoxin, which is suspected to be a human carcinogen (GASGA, 1997).

Results (Table 8) show that the percentage frequency of occurrence of fungi associated with fresh, salted and unsalted leaves was highest for *A. flavus* followed by *A.*

niger, and Penicillium. Fusarium had the lowest occurrence. Unlike the other treatments which gave leaves with two or more organisms, only *A. flavus* was found in the heavy salted leaves, which in addition, had the lowest fungal count.

Conclusion

In terms of organoleptic properties, reduction in fungal count and genera, and moisture reduction, heavy salting appears to be the most effective preservation treatment. These, coupled with the only moderate loss of nutrients and phytochemicals recommend it as an effective technique for the preservation of *V. amygdalina* leaves. A rigorous study is required to identify and quantify the mycotoxins present in the preparation.

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