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FRACTIONATION AND RATIONAL UTILISATION OF *Vernonia amygdalina* LEAVES

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ABSTRACT
The aim of this study was to develop a waste-free process for the utilisation of *Vernonia amygdalina* leaves by fractionation. The leaves were pulped and pressed to yield the leaf juice (53%) and press cake (fibrous fraction, 47%). The juice was heated to coagulate its protein and filtered to yield the deproteinised juice (46.4%). The residue was pressed, washed and pressed again to give the leaf protein concentrate (LPC, yield, 3.8%). The fibrous fraction had a composition similar to that of the fresh leaves, except for its lower ash and higher carbohydrate concentration. Compared with the fresh leaves, LPC had much higher protein (about four times as much) and lipid (one and a half times), but lower carbohydrate concentrations than the fresh leaves. Both fractions had lower β-carotene content (fibrous fraction, 33.30mg/g; LPC, 82.28 mg/g) than the fresh leaves (97.82 mg/g). Total phenolic content of the LPC (13.63mg GAE/g) was twice that of that of the fresh leaves (6.4 mg GAE/g), which was similar to that of the fibrous fraction (5.82 mg GAE/g). Leaf protein and deproteinised juice contained flavonoids, glycosides, saponins, terpenoids and tannins, and were rich in phenolic compounds (TPC of 713.59 and 812.82 mg GAE/l respectively for leaf juice and deproteinised juice). Possibilities for the utilisation of the fractions are discussed.

Keywords: *Vernonia amygdalina* leaves, Fractionation, Analysis of fractions

INTRODUCTION
Vernonia amygdalina variously known as bitter leaf (English), oriwo (Edo), ewuro (Yoruba), shikawa (Hausa), and olubu (Igbo), is a shrub indigenous to tropical Africa, found wild or cultivated all over sub- Saharan Africa (Bosch *et al.*, 2005; Oboh and Masodje, 2009). The leaves are used for food and medicine in Nigeria and other West African countries (Mayhew and Penny, 1988; Mensah *et al.,* 2008). The leaves are consumed as a vegetable. On a dry basis, they are rich in minerals, protein, lipids, fibre, carbohydrate and vitamins (Oboh and Madojemu, 2010); the proteins are rich in essential amino acids (Alabi *et al.*, 2005; Asaolu *et al.*, 2010). All parts of the plant are pharmacologically useful. Both the roots and leaves are used in ethno-medicine to treat fever, hiccups, kidney disease and stomach discomfort, among others (Abosi and Raseroka, 2003; Yeap *et al.,*

2010). Numerous experimental studies have been carried out on extracts from this plant revealing a wide range of bioactivities: - antibacterial, antifungal, antimalarial, anti-helminthic, anticancer, anti-mutagenic, hypoglycaemic, hypolipidemic, antioxidant and anti-inflammatory activities amongst others (Izevbigie, 2004; Nwanjo, 2005; Iwalewa *et al.*, 2005; Oladimoye, 2007; Oboh and Masodge, 2009; Oboh and Enobhayisobo, 2009; Khalafalla *et al*, 2009; Mboto *et al*, 2009; Yeap *et al.,* 2010).

When used for food, the leaves are washed in water to remove most of the bitterness. Mostly, the water is thrown away and the residue is used as an ingredient. When the fresh leaves are used as medicine, the usual practice is to throw away the residue and drink the water extract. When used as herbal tea, the dried and crushed leaves are extracted with hot water and the residue is discarded. Thus, clearly, the available methods for the utilisation of this valuable resource are very wasteful and a waste-free rational utilisation is needed.

Leaf fractionation is a mechanical process of separating fresh leaves into fibrous fraction, deproteinised juice (whey) and protein concentrate. The fractionation of green herbage has been based on the utilisation of green forages (e.g. grasses and legumes) as a source of high yielding food protein, using fractionating technologies to separate protein enriched juice from the cellulosic material and so ultimately provide a useful protein source for monogastric animal production and for use as food for humans (Stephen, 2009). The conventional technological fractionation process essentially involves pulping and pressing of the raw material (leaves) to express both green juice and press cake (pressed crop residue). The juice extract is then heat-coagulated and the protein curd recovered as leaf protein concentrate (LPC). This basic process has been refined and used since the 1940's and has progressed from conventional animal dietary protein usage and by-product feeding, to pre-treatment for further alternative biomass processing applications and multi-purpose fractionation uses, including fibre pulp processing, chemical and solvent production, human nutrition, bioremediation and biofuels (Pirie, 1942, 1971; Stephen, 2009).

The aim of this study was to develop a waste-free process for the utilisation of *V. amygdalina* leaves by fractionation. The leaves were fractionated to obtain the fibrous fraction and the leaf juice. Protein concentrate and deproteinised juice were then obtained from the leaf juice. Quantitative and qualitative analyses of the various fractions were undertaken. The results and their implications for the rational utilisation of the leaves are discussed.

MATERIALS AND METHODS

Plant Material

Leaf samples were harvested from cultivated stands at Eyaen, Benin-City, Nigeria, in the month of March 2011.

Microorganisms

Microorganisms used in the study were obtained from the Microbiology Unit, Department of Basic Sciences, Benson Idahosa University, Benin City, Nigeria.

Table 1: Microbial strains used to test for antibacterial activity

Microbial group	Microorganism	Cultivation conditions
Gram positive bacteria	*Bacillus cereus*	NA, 37°C
	Staphylococcus aureus	NA, 37°C
Gram negative bacteria	*Escherichia coli*	NA, 37°C
	Pseudomonas aeruginosa	NA, 37°C

NA = Nutrient Agar

Fig. 1: The hydraulic press used in the study

Leaf Fractionation

The fractionation scheme is shown in Fig. 2. Fresh leaves (10 kg) were rinsed with distilled water and pulped with a mortar and pestle followed by pressing in a hydraulic press (Fig. 1) to give the leaf juice and a fibrous fraction (press cake). The leaf juice was heated in batches to 80-100°C for about 10mins to coagulate and pasteurize the leaf protein. The protein coagulum was separated from the deproteinised juice by filtering through a cheese cloth followed by pressing with a hydraulic press (Fig. 1). The LPC was then washed with water and re-pressed. The fibrous fraction, leaf concentrate, leaf juice and deproteinised juice were stored in the deep freezer prior to analysis.

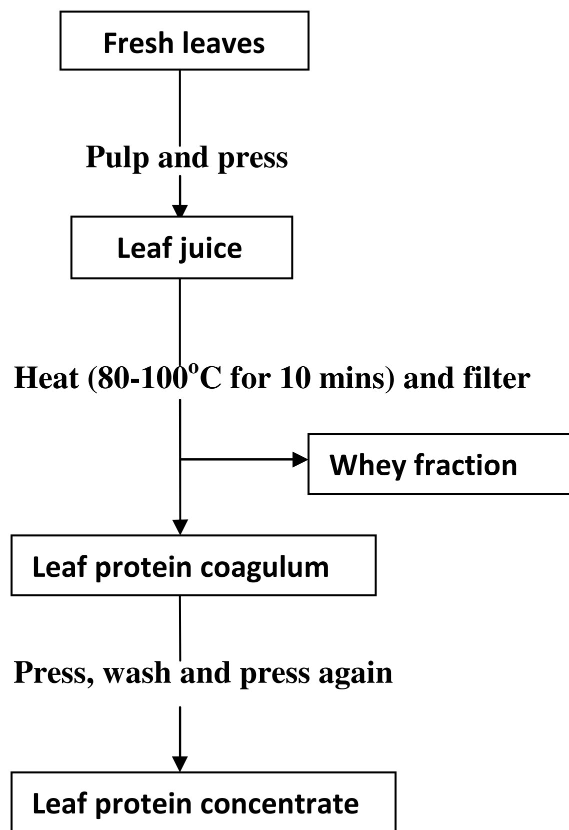

Fig. 2: Flow chart of leaf fractionation (Fellows, 1987)

Analytical Procedure

Phytochemical Screening

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

Test for Alkaloids

A small portion of each sample was stirred separately with few drops of dilute hydrochloric acid and filtered. The filtrate was tested with the following reagents for the presence of alkaloids.

Mayer's reagent - Cream precipitate
Hager's reagent - Yellow precipitate
Wagner's reagent - Reddish brown precipitate

The non-appearance of the corresponding colours after treatment with the regents indicated the absence of alkaloids.

Test for Flavonoids

A few drops of 25% ammonia solution were added to the sample in a test tube. A yellow colouration indicated the presence of flavonoids.

Test for Glycosides

Sample (5 ml) was mixed with 2 ml of glacial acetic acid containing 1 drop of ferric chloride. Concentrated H_2SO_4 (1 ml) was carefully added along the side of the test tube so that it was underneath the mixture. The appearance of a brown ring indicated the presence of glycosides.

Test for Saponins

To 0.5 g of sample was added 5 ml of distilled water in a test tube. The solution was shaken vigorously resulting in the formation of a stable persistent froth. An emulsion was formed when the froth was mixed with 3 drops of olive oil and shaken vigorously, indicating the presence of saponins.

Test for Terpenoids

Sample (2.5 ml) is mixed with 1ml of chloroform in a test tube. Concentrated H_2SO_4 (3 ml) was carefully added to the mixture to form a layer. An interface with a reddish brown colouration indicated the presence of terpenoids.

Test for Tannins

About 0.5 ml of sample was added to 10 ml of distilled water and filtered. Ammonia (5 ml of a 10% solution) was added to the filtrate and the mixture was shaken. Appearance of a blue-black, green or blue-green colouration showed the presence of tannins.

Quantitative Determination of Bioactive Compounds

Determination of Total Phenolic Content (TPC)

TPC in each sample was determined by a slight modification of the procedure described by Azizah *et al.* (2007). Samples and gallic acid standards; 50, 100, 250 and 500 mg/l (0.1 ml, in triplicate) were mixed with 0.5 ml of 0.2 N Folin-Ciocalteau reagent and after 8mins, 1.5ml of 7.5% sodium carbonate was added. The mixture was kept in the dark for 1 hr, and the absorbance measured at 765 nm.

β-Carotene and Lycopene Determination

β-carotene and lycopene were determined by slight modifications of the method of Nagata and Yamashita (1992). Sample (100 mg) was weighed and mixed by vortex with 10 ml of an acetone–hexane mixture (4:6 v/v) for 1 min and centrifuged to separate the layers. The upper layer containing the extracted carotenoids was carefully removed into a clean already labelled test tube and the lower layer discarded. The absorbance was measured at wavelengths 453, 505, and 663 nm. The β-carotene and lycopene contents were calculated according to the following equations:

β-carotene (mg/100 ml) = 0.216 (A_{663}) - 0.304 (A_{505}) + 0.452 (A_{453})

Lycopene (mg/100 ml) = 0.0458 (A_{663}) + 0.372 (A_{505}) - 0.0806 (A_{453})

Where A = absorbance.

Determination of Antibacterial Activity

Four strains of bacteria were tested, two gram positive bacteria, *Bacillus cereus* and *Staphylococcus aureus* and two gram negative bacteria; *Escherichia coli* and *Pseudomonas aeruginosa.* Antibacterial activity was determined by the disc diffusion method described by Mackeen *et al*. (2000). A swab of the test microorganism (approximately 1 - 2 x 10^8 CFU/ml based on McFarland turbidity standard 0.5) was spread onto Petri dishes containing nutrient agar (3 mm deep). Sterile filter paper discs (6 mm in diameter) completely soaked in the samples were placed on the culture dishes. Water served as positive control, while standard gentamicin (10 µg) discs were used as positive control. The dishes were inverted and incubated at 37°C for 24 hours. At the end of the incubation, the distinct zones of inhibition surrounding the discs were measured. Antimicrobial activities were expressed as inhibition diameter zones in

millimetres (mm) as follows: - (negative) = 0 mm; + (weak) = 1-4 mm; ++ (moderate) = 5-10 mm; +++ (strong) = 10-15 mm and ++++ (very strong) ≥ 16 mm. The determination was carried out in duplicate and the average zones of inhibition were calculated.

Proximate Analysis of Fresh Leaves, Fibrous fraction and Leaf Protein Concentrate

Moisture, crude protein, ether extract, ash and crude fibre were determined according to Official Methods (AOAC, 1999).

Carbohydrate

Total carbohydrate was estimated using the phenol sulphuric acid method. The sample (2 g) was crushed in a mortar and hydrolysed by addition of 0.2 N HCL and heating in a water bath. The hydrolysate was allowed to cool and then neutralised with solid sodium carbonate. Phenol solution (5%) and 96% sulphuric acid (5ml) were added and the mixture was mixed. After 10 min the mixture was heated at 30°C for 20min. Absorbance was read at 490 nm. Total carbohydrate content was calculated using a glucose standard plot (Krishnaveni *et al.*, 1984).

Statistical Analysis

Experimental replicates within individual experiments were averaged and expressed as mean ± SD. Comparisons between means were determined by unpaired student's t test with 2 tailed P values reported, employing Microsoft Excel 2003 and Statistical Package for Social Sciences (SPSS) version 15.0. Each experiment was replicated three times with comparable results. Results with $P < 0.05$ were regarded to be statistically significant.

RESULTS

Yields and Composition of Fractions

The yields of these fractions are given in Table 2.

Table 2: Leaf fractionation

Component	Fresh wt. (kg)		Dry wt. (kg)
Fresh leaf	10.00		1.98
Yield of fractions			
	Wet Wt (kg)	% by wet wt.	% by dry wt.
Fibrous fraction	4.70	47	86.38
Fresh leaf juice	5.30	53	-
Deproteinised juice	4.64	46.4	-
Leaf Protein Concentrate	0.38	3.8	6.17

Pulping and hydraulic pressing of fresh leaves yielded a fibrous fraction (47%) and fresh leaf juice (53%). Heating of the fresh leaf juice yielded the leaf protein concentrate (3.8% of fresh leaves) and deproteinised juice (46.4% of the fresh leaves). Loss due to evaporation of water was 0.28%. Based on dry weight, the yield of the fibrous fraction was 86.38% while that of the leaf protein concentrate was 6.17%, with soluble solids in the deproteinised juice making up the balance.

Table 3 shows the proximate composition, β- carotene, and total phenolic content (TPC) of the fresh *V. amygdalina* leaves, and its fibrous and leaf protein fractions.

Table 3: Proximate composition, β-carotene and total phenolic content of fresh leaves, fibrous fraction and leaf protein concentrate

	Fresh leaves	Fibrous fraction	Leaf protein concentrate
Moisture (%)	80.24 ± 0.75	63.61 ± 0.38	67.83 ± 0.72
Ash (% DM)	12.19 ± 0.18^{a}	2.92 ± 0.17^{b}	2.89 ± 0.19^{b}
Crude protein (% DM)	11.95 ± 0.09^{a}	10.31 ± 0.32^{b}	40.12 ± 0.80^{c}
Crude fibre (% DM)	10.33 ± 0.04^{a}	13.10 ± 0.12^{b}	0 ± 0^{c}
Ether extract (% DM)	9.79 ± 0.09^{a}	8.27 ± 0.25^{b}	15.55 ± 0.30^{c}
Carbohydrate (% DM)	55.73 ± 0.17^{a}	64.91 ± 0.23^{b}	41.83 ± 0.85^{c}
β-Carotene (mg/g DM)	97.82 ± 0.37^{a}	33.33 ± 0.21^{b}	82.28 ± 0.20^{c}
TPC (mg GAE/g)	6.40	5.82	13.63

Values are expressed as means ± SD (n = 3)
^{abc} Means with different superscripts on same row differ significantly (P < 0.05).

The moisture content of the fresh leaves, fibrous fraction and protein fraction were 80.24, 63.61 and 67.83% respectively, thus the materials had water as their dominant component. Ash content (dry basis) was 12.19% for the fresh leaf, 2.92% for the fibrous fraction and 2.89% for the protein fraction. This indicates that on fractionation, the mineral matter in the leaf ended up largely in the whey. The crude protein concentration of the leaf protein concentrate (40.12%) was about four times as high as that of the fresh leaves (11.05%) or the fibrous fraction (10.21%). The crude fibre content of the fresh leaves (10.33%) was slightly lower than that of the fibrous fraction (13.10%). The leaf protein concentrate had no crude fibre. Carbohydrate constituted slightly over half of fresh leaves, about two- thirds of the fibrous fraction (64.91%) and less than half (41.83%) of the leaf protein concentrate. Thus, on a dry basis, it formed the major component of the fresh leaves and the fibrous fraction, and a dominant component (the other being protein) of leaf protein concentrate.

The fat content (ether extract) of the fibrous fraction (8.27%) was only slightly less than that of the fresh leaves (9.79%); both were much less than the fat content of the leaf protein concentrate (15.55%). The materials had high β-carotene (provitamin A) and total phenolic content. The fresh leaves had the highest β-carotene content (97.82 mg/g); the fibrous fraction contained 33.33 mg/g and leaf protein concentrate 82.28 mg/g. Total phenolic content of fresh leaves, fibrous fraction and leaf protein concentrate was 6.4 mg GAE/g, 5.82 mg GAE/g, and 13.63 mg GAE/g respectively.

A consideration of the composition of the *V. amygdalina* leaves and their fractions suggests that in spite of its lower content of minerals (ash) and β-carotene (relative to the fresh leaves),

the fibrous fraction could substitute for the fresh leaves as food ingredient. It appears to be the equivalent of the washed bitter leaf that is used as a food ingredient. When the fresh leaves are washed, mineral matter and soluble phytochemicals (some of which give the bitter taste), are leached into the wash water. Thus the fibrous fraction, with its lower mineral content and phenolic compounds (as measured by its TPC) is eminently suitable for use as washed bitter leaves. Its residual phytochemicals indicate that it could still confer the slightly bitter taste and medicinal benefits associated with the food preparations in which it as an ingredient, without the antinutritional effects that are usually associated with high content of certain phytochemicals in foods. This fraction can be preserved by blanching and drying or by salting and fermentation (the latter under certain conditions may produce a material suitable for use as a condiment), thereby adding value to the fraction (Oboh and Madojemu, 2010). It can be packaged and sold, to be used without further processing.

V. amygdalina leaves are suitable for inclusion in diets for monogastric and ruminant animals. They are eaten as forage by goats and are added as dried meal to poultry feed (Owen and Amakiri, 2011; Kabirizi and Ejobi, 2005). The fibrous fraction, except for its lower mineral content, is similar in composition to the fresh leaves and could be fed wet or dried as an ingredient of animal ration, or as silage. Ensiling may be beneficial by reducing the content of antinutrients and enriching the material through the action of microorganism (FAO, 2000). Also, being rich in carbohydrate (carbohydrate content was 65%), the fibrous fraction could be a useful material for conversion to bioethanol. Here again, the alcohol-free stillage (mash) could be dried for high-value animal feed (Rutz and Janssen, 2008).

Though the leaf protein concentrate is of poor yield (3.8% wet basis, 6.17% dry basis), a consideration of its high carbohydrate, protein and β-carotene content, coupled with its considerable fat content appears to be desirable from a nutritional viewpoint. The absence of crude fibre is good, especially if it is intended for use as an additive in feed for monogastric animals. But it has a considerable content of phenolic compounds, which could adversely affect utilisation of its protein. It holds promise also, for use as a nutritional and medicinal supplement for man and livestock, to provide vitamin A and phenolic compounds, with their associated antioxidant, antimicrobial, antitumour, hypoglycaemic and blood lipid regulatory activities. Thus this fraction appears interesting for use as a substitute for antibiotics and other drugs in livestock feed, with a view to reducing cost of production and minimising their use or eliminating them altogether due to health and safety concerns about drug residues in livestock products. Further studies are therefore required in order to find out if this fraction is suitable for such applications.

The phytochemicals in leaf juice and deproteinised juice, as well as their total phenolic content, β-carotene and lycopene are shown in Table 4.

Table 4: Phytochemicals, β-carotene, lycopene and total phenolic content (TPC) of leaf juice and deproteinised juice

	Leaf juice	Deproteinised juice
Alkaloids	-	-
Flavonoids	+	+
Glycosides	+	+
Saponins	+	+
Terpenoids	+	+
Tannins	+	+
Total phenolic compounds (mg of GAE/litre)	713.59 ± 5.01^{a}	812.82 ± 4.51^{b}
β-Carotene (mg/l)	4.5 ± 0.20^{b}	0.42 ± 0.02^{a}
Lycopene (mg/l)	0.73 ± 0.05^{b}	0.03 ± 0.01^{a}

+ = Present; - = Absent

Values are expressed as mean ± SD (n = 3)

^{ab} Means with different superscripts on same column differ significantly (P < 0.05)

Both contained flavonoids, glycosides, saponins, terpenoids and tannins. Alkaloids were absent. They had considerable total phenolic content; deproteinised juice contained more TPC (812.821mgGAE /l) than leaf juice (713.59 mg GAE /l). Phenolic compounds have been intensively investigated and have been reported to posses many useful properties. They act as antioxidants, preventing the oxidation of low density lipoprotein, platelet aggregation and damage to red blood cells. They act as metal chelators, anti-mutagens, anticarcinogens and antimicrobial agents (Cheynier *et al.,* 2005). The juices had low carotenoid content; β-carotene constituted 4.5 and 0.42 mg/l, and lycopene 0.73 and 0.03 mg/l respectively of fresh juice and deproteinised juice.

The antibacterial activity of the fresh juice and deproteinised juice against *B. cereus, S. aureus, E. coli and P. aeruginosa* are shown in Table 5.

Table 5: Antibacterial activities of fresh juice and deproteinised juice (DPJ)

	Zone of inhibition (mm)			
Zone of inhibition	Gram positive bacteria		Gram negative bacteria	
Sample	*B. cereus*	*S. aureus*	*E. coli*	*P. aeruginosa*
Fresh juice	- (<1)	- (<1)	- (<1)	+ (1)
DPJ	+ (1)	- (<1)	+ (1)	+ (2)

* Antimicrobial activities were expressed as inhibition diameter zones in millimetres (mm) : - (negative) = < 1 mm; + (weak) = 1-4mm.

* () = Average zone of inhibition in mm.

Both had weak antibacterial activity. The fresh juice inhibited growth only of *P. aeruginosa* (zone of inhibition of 1 mm). The deproteinised juice inhibited

the growth of *B. cereus, E. coli* (each with zone of inhibition of 1mm), and of *P. aeruginosa* (2mm). This indicates that the separation of protein from juice gave a liquid, with a higher TPC content and antimicrobial potency against these organisms than the juice itself. Further increase in TPC by the removal of water, should therefore increase the antimicrobial activity of the juice. Thus, material having high antimicrobial activity can be prepared from the deproteinised juice by spray drying, evaporation or other suitable method(s) for the removal of water. Such concentrated material, or extracts produced from it by the use of organic solvents or by supercritical CO_2, could also possess (in addition to antimicrobial activity) the other benefits claimed for *V. amygdalina* extracts and may be suitable for formulation into medicines. The spray dried material could be used as a substitute for hops in beer (Ajebesona and Aina, 2004). These applications (medicinal and as hops substitute) hold great potential for deproteinised *V. amygdalina* juice utilisation.

The fibrous fraction and the leaf protein concentrate each had a lower content of minerals than the fresh leaves; soluble minerals were therefore concentrated in the deproteinised juice. Thus the deproteinised juice may be drunk as a mineral-rich tonic, with the additional health benefits listed previously.

CONCLUSION

Vernonia amygdalina leaves have been separated into three fractions – a fibrous fraction, a protein concentrate and a deproteinised juice. A consideration of their composition indicates that the fibrous fraction would be suitable for use as an ingredient ('washed bitter leaf') in various food preparations, as a component of livestock feed and as raw material for conversion to bioethanol. The leaf protein concentrate was rich in protein, lipid and carbohydrate. This, coupled with its considerable content of phenolic compounds and β- carotene indicates that it could be used as a nutritional supplement for humans and livestock. In the latter it could substitute for antibiotics and other drugs, thereby reducing cost of production and eliminating their undesirable effect on consumers of livestock products. The deproteinised juice could be drunk as a mineral and phenolic compounds- rich tonic, or concentrated for use in medicinal preparations and as a substitute for hops in beer.

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