Ezeonu et al., Afr J Tradit Complement Altern Med. (2016) 13(6):11-17
10.21010/ajtcam. v13i6.3

IMMUNO-MODULATORY PROPERTIES OF PREBIOTICS EXTRACTED FROM *vernonia amygdalina*

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Abstract

Background*: Vernonia amygdalina*, commonly called bitter-leaf, is widely consumed in many parts of Africa, and Nigeria, in particular. The leaf extract has been reported to have antimicrobial, anti-plasmodial, anti-helminthic, as well as prebiotic properties, but its immuno-modulatory effects have not been well-studied, neither have the prebiotics been identified. This study evaluated the immuno-modulatory properties of the aqueous leaf extract and identified the prebiotic components.
Methods: The immuno-modulatory potential was evaluated by monitoring the effects of oral administration of the extract on immunological, haematological and lipid profiles of *Rattus norvegicus*, while the prebiotic components were identified by thin layer chromatography (TLC), following liquid-liquid fractionation of the extract.
Results: Consumption of the extract caused significant increases in $CD4^+$-, white blood cell-, total lymphocyte- and high density lipid (HDL) counts; decreases in low density lipid (LDL) and triglycerides and no significant effect on haemoglobin (Hb) and packed cell volume (PCV) in the blood of test animals. The water-soluble fraction of the extract contained most of the phyto-constituents of the extract and Thin Layer Chromatographic analysis of the fraction revealed the presence of fructo-oligosaccharide and galacto-oligosaccharide prebiotics.
Conclusion: The results from this study have shown that the aqueous leaf extract of *V. amygdalina* has positive immune-modulatory and haematologic effects and contains some important prebiotic compounds.

Keywords: *Vernonia amygdalina*, prebiotic, fructo-oligosaccharide, galacto-oligosaccharide, haematology

Introduction

Vernonia amygdalina, a member of the *Asteraceae* family, is a widely used local vegetable in Nigeria, Uganda and other African countries. It grows in a range of ecological zones in Africa and the Arabian Peninsula. The leaf is commonly called "bitter leaf" in English. It is a shrub of 2-5 m tall with petiolate leaves of about 6.0 mm wide. It is up to 20 cm long and its bark is rough (Ojiako and Nwanjo, 2006; Ijeh and Ejike, 2011). The bitter taste of the leaf has been attributed to the presence of anti-nutritive principles like saponins, alkaloids, tannins and glycosides (Ijeh and Ejike, 2011). There have been several reports on its antimicrobial, antiplasmodial, antitumor, antioxidant and antihelminthic properties (Farombi and Owoeye, 2011; Udochukwu et al., 2015). Aqueous leaf extracts of *V. amygdalina* have also been previously reported to have prebiotic effects (Ezeonu et al., 2012).

Prebiotics are non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of beneficial bacteria in the colon and thus improve host health (Gibson and Roberfroid, 1995). The roles of prebiotics in modulation of bowel function, inhibition of pathogenic bacteria and reduction of colon cancer have been widely studied and reported (Gibson and Roberfroid, 1995; Hamilton-Miller, 2004; Lomax and Calder, 2009; Chakraborti, 2011; Licht *et al*., 2011). Prebiotics have also been reported to have positive effects on haematological and biochemical parameters, as well as lipid metabolism in both human and animal models (Lomax and Calder, 2009; Ooi and Liong, 2010; Jackson and Lovegrove, 2012; Scavuzzi et al, 2014).

The most widely studied prebiotics are inulin and non-digestible oligosaccharides such as oligofructose (Watzl et al., 2005; Leenen and Dieleman, 2007; Guarner, 2007; Lomax and Calder, 2009; Patel and Goyal, 2012). However, some studies suggest that there may be other candidate-prebiotics such as xylitol, sorbitol, mannitol and lactulose (Chakraborti, 2011). This study aimed to evaluate the immuno-modulatory properties of *V. amygdalina* extracts and identify the prebiotic components in the extract.

Methodology

Collection of plant materials and preparation of aqueous plant leaf extracts

The leaves of *V. amygdalina* used for this study were obtained from a local market in Nsukka and authenticated by a botanist from the Department of Botany, University of Nigeria, Nsukka (Voucher number: U.N.H. {University of Nigeria Herbarium}/001).

Extraction procedure

The leaves were washed in clean water and dried. The dried leaves were pulverized into a homogenous powder using mortar and pestle. One hundred grams (100 g) of the powder was soaked in 1 L of water for 24 h with intermittent stirring. The aqueous extract was filtered through a 2 mm mesh filter and the filtrate was stored at 4˚C in clean sterile bottles.

Liquid-liquid fractionation of crude extract

Fractionation of the *V. amygdalina* extract was carried out by sequential extraction with chloroform, ethyl-acetate and N-butanol, according to the methods of Ajali (2004). Briefly, the crude extract obtained as described above was concentrated to dryness by air-drying. The concentrated extract was re-extracted with chloroform and filtered through a Whatman no. 1 filter paper. The filtrate (chloroform-soluble fraction) was evaporated to obtain the extract. The residue was air-dried and extracted with methanol for 6 hr, washed five times with methanol and then filtered. The methanol extract was dried and the residue washed three times with ethyl-acetate. The ethyl-acetate fraction was separated and the residue re-extracted with N-butanol. All solvents were from JHD, China.

Phytochemical analysis of crude extract

The crude extract was analyzed phytochemically in the Department of Pharmacognosy, University of Nigeria, Nsukka, using standard methods outlined by Harborne (1984) and later modified by Trease and Evans (1996). The extracts were tested for carbohydrates, reducing sugars, steroids and terpenoids, phenols and tannins, alkaloids, saponins, flavonoids, oils and glycosides.

Thin Layer Chromatography (TLC)

The various fractions were analyzed using Thin Layer Chromatography method described by Harborne (1984) and later modified by Trease and Evans (1996). TLC plates of 5 cm height and of various convenient widths were cut from large sheets. The plates were handled carefully in order not to disturb the coating of adsorbent or get them dirty. A distance of 0.5 cm was measured from the bottom of the plate. Then, using a pencil, a line was drawn across the plate at the 0.5 cm mark, to mark the origin. Under the line, the names of the samples to be spotted were labelled lightly. Samples were spotted using a 10 µl microcapillary. The spots were spaced out appropriately to prevent mixing. The prepared TLC plate was then placed in the developing beaker and covered with a watch glass. The set-up was left undisturbed to develop until the solvent was about half a centimeter below the top of the plate. The plate was then removed from the beaker and the solvent front immediately marked with a pencil. The plate was allowed to air-dry. Coloured spots were circled lightly with a pencil and the plates were placed on a prepared iodine chamber to enhance visibility of the bands. Sample sugars (standards) were run alongside the extracts. The Retardation factor (R_f) values of the standards and extracts were measured and compared to determine the probable sugars present in the extracts.

Collection of animals

Experimental animals (*Rattus norvegicus*) of either sex, weighing between 150-200 g, were purchased from a commercial supplier. The rats were housed in well ventilated cages in the animal house of the Faculty of Biological sciences, University of Nigeria, Nsukka. The animals were allowed free access to feed (growers guinea feed) and clean water according to the guidelines of the National Institute of Health (NIH) publications for laboratory animal care and use (NIH, 1985). The animals were acclimatized to the housing and feeding conditions for a period of ten days prior to testing.

Administration of aqueous extract to animals

The animals were carefully grouped according to similar body weights. There were a total of six groups with six animals per group. One group was set as a control while the other five groups were administered different concentrations of the aqueous leaf extracts. The control groups were administered distilled water while other groups were administered oral doses of 50, 100, 200, 400 and 800 mg/kg of the aqueous extracts reconstituted in distilled water, respectively, twice daily for three weeks. The animals were monitored for another seven days before they were sacrificed.

Haematological assays

Blood samples were obtained by the orbital bleeding method. A microhaematocrit capillary tube was inserted at the medial canthus to gain access to the orbital venous plexus of the rats. Twenty microlitre (20 µl) aliquots of blood were used to determine Haemoglobin level, Packed Cell Volume, White Blood Cell, $CD4^+$ cells population and Lymphocyte counts, respectively, according to the methods described by Cheesbrough (2006).

Biochemical assays

The animals were allowed to fast for about 16 h. Then, blood samples were drawn as described above. The tubes containing the blood samples were kept at 4^oC for the sera to separate. Aliquots (200 µl) were used for the determination of serum Total Cholesterol (TC), Triglyceride (TG), High Density Lipoprotein Cholesterol (HDL-C) and Low Density Lipoprotein Cholesterol (LDL-C), respectively, according to the methods outlined by Cheesbrough (2006).

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Statistical Analysis

The results obtained in this research work were expressed as mean ± standard deviation. The differences between mean values were assessed for significance by Analysis of variance (ANOVA) at $P < 0.05$ level of significance.

Results and Discussion

Four fractions were recovered from the leaf extract with chloroform, ethyl-acetate and N-butanol (2 fractions, a lower and upper). The residue, which did not dissolve in any solvent but water, was called the water residue and taken as a fifth fraction. The fractions were designated E1 to E5. From 35 g of crude extract, the yields of the five fractions were as follows: chloroform fraction (E1), 0.1 g (0.29%); water residue (E2), 18 g (51.43%); ethyl acetate fraction (E3), 0.5 g (1.43%); N-butanol lower faction (E4), 13.8 g (39.43%) and N-butanol upper fraction (E5), 1.0 g (2.86%). The total weight recovered was 33.4 g (95.4%) and the weight loss was calculated to be 1.6 g (4.6%). Phytochemical analysis revealed that the crude extract contained carbohydrates, reducing sugars, glycosides, saponin, steroid, tannin and oil. These components were distributed among the different fractions as shown in Table 1. The water residue (E2) was the most abundant fraction and also contained most of the phyto-constituents, including carbohydrates, reducing sugars, steroids, saponins and tannins. A previous study had shown this fraction to be the most active, in terms of stimulation of growth of beneficial bacteria (Ukwah and Ezeonu, 2009).

Table 1: Phytochemical composition of the crude *Vernonia amygdalina* aqueous leaf extract and five fractions (E1 to E5) produced by liquid-liquid fractionation of the crude extract

	Fractions					
Constituents	**Crude extract**	**E1**	**E2**	**E3**	**E4**	**E5**
Carbohydrates	+++	+	++	+	+	+
Reducing sugars	++	-	++	NT	-	-
Glycosides	++	-	++	NT	-	-
Saponins	+++	-	+++	-	-	-
Flavonoids	-	-	-	-	-	-
Resin	-	-	-	-	-	-
Steroids	++	-	+	NT	+	NT
Terpenoids	-	-	-	-	-	-
Alkaloids	-	-	-	-	-	-
Tannins	++	-	+	-	+	+
Oil	++	+	-	+	++	+

Key: - = absent; + = trace quantity; ++ = moderate quantity; +++ = high quantity; NT = not tested

The presence of saponin is of particular interest in this study. Saponins are complex glycosides made up of sugar (glycone) and non-sugar (aglycone) groups. The glycone can consist of a single group of sugars (monosaccharides) or several groups of sugars (oligosaccharides) (LASCU, 2008). The foaming characteristic usually observed with saponins is caused by the combination of the non-polar aglycone and the water soluble side chain (glycone) and on contact with water, the sugar group dissolves releasing the non-sugar group (Ajali, 2004; Shi et al, 2004; LASCU, 2008). Saponins have been reported to have biological activities including anti-inflammatory, anticancer, immune stimulating, antimicrobial and anti-plasmodial properties (Ajali, 2004; Ray, 2007). In addition, saponins are believed to be non-systemic and able to escape digestion in the upper gut to arrive in the colon (Ajali, 2004). This latter property is an important property of all prebiotics.

The effect of the extract on $CD4^+$ (cluster of differentiation 4) cell counts, haematological and lipid profiles was evaluated using the water residue. In the control (untreated) animals, there were no significant differences in counts throughout the experimental period. However, in the test groups, there was a general increase in $CD4^+$ counts with administration of the extract. The effect was dose-dependent, with counts increasing with increase in dose of extract up to 200 mg/kg body weight, after which the $CD4^+$ counts decreased again. The $CD4^+$ count at 800 mg/kg was lower than the count recorded at 50 mg/kg but higher than control.

The differences were significant ($p<0.05$). Similarly, consumption of the extract caused increases in total white blood cell (WBC) and total lymphocyte counts (Table 2).

cells that organize the immune system's response to infections. They are the core of adaptive immunity, the system that tailors the body's immune response to specific pathogens. They are called helper cells because one of their main roles is to send signals to other types of immune cells, including $CD8^+$ killer cells, which destroy the infectious particle. If $CD4^+$ cells become depleted, for example in untreated HIV infection, or following immune suppression prior to a transplant, the body is left vulnerable to a wide range of infections that it would otherwise have been able to fight (Ansari-Lari et al., 1996). The increase in $CD4^+$ cell counts observed in test animals administered with oral doses of the *V. amygdalina* extract, in this study, strongly suggests a positive immuno-modulatory effect of the extract. The extract did not produce any significant effects on haemoglobin and PCV in this study.

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Table 2: Effects of Different Oral Doses of *V. amygdalina* Aqueous Leaf Extract on Some Blood Parameters

Parameters	Baseline Values (Values at zero time)	Treatment Groups/ Dose	Values after 21 days Treatment	7 Days after Completion of Treatment
$CD4^+$ cell counts (cells/ml)	52.1 ± 0.07	A (Control)	52.3±0.05	55.0±0.06
		B (50 mg/kg)	356.0±0.12*	227.0±0.11*
		C (100 mg/kg)	509.3±0.05*	301.0±0.06*
		D (200 mg/kg)	1147.7±0.11*	701.0±0.12*
		E (400 mg/kg)	486.0±0.00*	109.0±0.05*
		F (800mg/kg)	239.7±0.00*	113.0±0.11*
Haemoglobin (g/dL)	13.6 ± 0.02	A (Control)	14.0±0.12	15.3±0.00
		B (50 mg/kg)	13.3±0.05	13.9±0.06
		C (100 mg/kg)	13.7±0.11	14.3±0.12
		D (200 mg/kg)	14.0±0.11	14.7±0.00
		E (400 mg/kg)	14.0±0.12	15.5±0.11
		F (800mg/kg)	14.7±0.00	17.0±0.12
Packed Cell Volume (%)	39.8 ± 0.06	A (Control)	41.0±0.00	34.0±0.06
		B (50 mg/kg)	40.3±0.05	37.0±0.06
		C (100 mg/kg)	39.7±0.05	30.0±0.00
		D (200 mg/kg)	40.3±0.05	39.0±0.00
		E (400 mg/kg)	41.7±0.00	39.0±0.00
		F (800mg/kg)	43.0±0.05	40.0±0.06
Total WBC Counts ($x10^9$/L)	6.9 ± 0. 04	A (Control)	6.7±0.00	6.8±0.00
		B (50 mg/kg)	14.7±0.00*	8.3±0.06*
		C (100 mg/kg)	16.1±0.12*	9.6±0.12*
		D (200 mg/kg)	15.0±0.00*	10.2±0.00*
		E (400 mg/kg)	21.0±0.00*	17.8±0.05*
		F (800mg/kg)	17.5±0.06*	9.0±0.00*
Lymphocytes ($x10^9$/L)	6.8 ± 4.56	A (Control)	7.08±4.16	5.92±4.08
		B (50 mg/kg)	73.00±8.32	48.00±0.00*
		C (100 mg/kg)	71.33±5.03	60.00±1.02*
		D (200 mg/kg)	64.00±12.16	70.00±0.03*
		E (400 mg/kg)	75.33±11.54	62.00±0.00*
		F (800mg/kg)	69.33±8.08	54.00±0.06*

*: significant ($p<0.05$)

Evaluation of the lipidaemic effect of the *V. amygdalina* extract showed a general increase in serum cholesterol in the blood of the test animals. However, separate measurements of high density lipoprotein cholesterol (HDL-C) and low density lipoprotein cholesterol (LDL-C) showed that while there were significant increases in HDL-C levels of the test animals, there were decreases in LDL-C. Similarly, there were significant decreases in triglycerides. The effects were also dose-dependent (Table 3). Lipid profile is useful in assessing the risk of cardiovascular disease and is usually altered in the serum in various disease states in humans (Betteridge, 1994). LDL-C, sometimes called "bad" cholesterol transports cholesterol mainly to the arterial wall, thus leading to formation of arteriosleretic plaque, which is a major cause of cardiovascular disease (Latunde-Dada, 1990). In contrast, HDL-C, called "good" cholesterol binds to arterial cholesterol and transports it to the liver for metabolism. Therefore, any substances that can increase the HDL-C level, but decrease the LDL-C level will play an important role in the reduction of cardiovascular diseases (Yokozawa et al., 2006). The results in this study are therefore significant.

The best known prebiotics are Inulin, fructooligosacharides and galactooligosaccharides, even though the existence of other candidate prebiotics has been suggested (Sharma et al, 2012). In this study, attempts to identify prebiotic components of the *V. amygdalina* extract by TLC, showed the presence of compounds with R_f values of 0.20, 0.28, 0.16 and 0.23, similar to those of D-Fructose, D-Xylose, D-Glucose , D-Arabinose, D-Mannose and D-Galactose (Table 4). These results indicate that the extract contains fructo-oligosaccharides and galacto-oligosaccharides, among others, which are known prebiotics.

Table 3: Effects of Oral Administration of *V. amygdalina* Aqueous Leaf Extract on Lipid Profile

Parameters	Baseline Values (Values at zero time; mg/dL)	Treatment Groups/ Dose	Values after 21 days Treatment (mg/dL)	7 Days after Completion of Treatment (mg/dL)
Total Cholesterol	38.0 ± 0.15	A (Control)	60.3±0.00	65.4±0.00
		B (50 mg/kg)	54.7±0.06*	49.0±0.00*
		C (100 mg/kg)	62.7±0.00*	54.0±0.00*
		D (200 mg/kg)	59.7±0.00*	67.0±0.05*
		E (400 mg/kg)	52.0±0.05*	63.0±0.00*
		F (800mg/kg)	65.7±0.00*	50.0±0.12*
High Density Lipids (HDL)	15.1 ± 0.06	A (Control)	16.0±0.00	19.7±0.04
		B (50 mg/kg)	33.6±0.09*	28.0±0.06*
		C (100 mg/kg)	37.6±0.03*	31.1±0.12*
		D (200 mg/kg)	40.3±0.06*	35.3±0.11*
		E (400 mg/kg)	42.3±0.01*	39.4±0.06*
		F (800mg/kg)	27.0±0.09*	23.7±0.00*
Low Density Lipids (LDL)	48.2 ± 4.24	A (Control)	52.3±0.05	52.9±0.06
		B (50 mg/kg)	50.0±0.52*	54.0±0.00*
		C (100 mg/kg)	31.3±0.03*	35.0±0.05*
		D (200 mg/kg)	32.1±0.00*	37.0±0.06*
		E (400 mg/kg)	31.3±0.05*	36.0±0.00*
		F (800mg/kg)	29.6±0.05*	32.0±0.07*
Triglycerides	43.5 ± 3.56	A (Control)	53.6±0.13	57.0±0.00
		B (50 mg/kg)	40.6±0.10*	43.0±0.06*
		C (100 mg/kg)	37.3±0.03*	39.0±0.00*
		D (200 mg/kg)	30.6±1.24*	36.0±0.00*
		E (400 mg/kg)	27.6±0.15*	29.0±0.12*
		F (800mg/kg)	19.3±0.05*	23.0±0.11*

*: significant ($p<0.05$)

Cholesterol is synthesized from long chain fatty acids, which are attached to the glycerol side chain of triglycerides. Thus, increase in triglyceride level often increases synthesis of cholesterol from the liver, and therefore increases the risk of cardiovascular disease (Richards et al., 1989). Reduction in level of triglycerides of test animals by consumption of *V. amygdalina* extracts, in this study, agrees with reports of other investigators that *V. amygdalina* has a serum- lipid-modulation property and can reduce artherscleorsis in animal models (Ijeh and Ejike, 2011; Abdulmalik et al., 2016). They are also in agreement with studies which have reported that prebiotics can cause favourable redistributions of lipids among the various lipoproteins (Busserolles et al., 2003; Lomax and Calder, 2009; Ooi and Liong, 2010; Jackson and Lovegrove, 2012; Sharma et al., 2012; Salahuddin et al., 2013; Scavuzzi et al, 2014).

The best results were observed in this study using the dose of 200 mg/kg. This is in agreement with the report of Abdulmalik et al. (2016).

White blood cells are the cells that are involved in defending the body against both infectious disease and foreign invaders. The increased WBC count in treated groups suggests improved immune-response. Increase in total WBC count could occur by different mechanisms, including increased production of white blood cells, absence of re-distribution of white blood cells from peripheral blood into the tissues or reduced destruction of white blood cells (Bruce et al., 2002; Handin et al., 2003; El-Demerdash, 2004). In this study, the exact mechanism responsible for the increased WBC counts could not be confirmed. However, an increase in number of lymphocytes was observed and T-cell activation is usually an indication of a boost in the immune system. It is usually measured as a way to assess the health of patients with HIV/AIDS and in other disorders (Kwong et al., 1998; Zamani et al., 2010). $CD4^+$ cells, a subset of T-cells, are of particular importance in assessing the immune status of a patient. $CD4^+$ cells are white blood.

Table 4: Thin Layer Chromatographic Identification of Oligosaccharides in Aqueous Extract of *V. amygdalina* Leaves

Standards/ Fractions	Samples Tested	R_f Values (cm)	Probable Oligosaccharides Identified
Standards			
	D-Fructose	0.23	-
	D-Mannose	0.20	-
	D-Xylose	0.28	-
	D-Galactose	0.16	-
Extract Fractions			
	Chloroform	0.20	Mannosyl-oligosaccharide
	Water Residue		
		0.15	Galacto-oligosaccharide
		0.18	Galacto-oligosaccharide
	N-butanol (Upper Layer)		
		0.28	Xylo-oligosaccharide
		0.23	Fructo-oligosaccharide
	N-butanol (Lower Layer)	0.21	Arabino-oligosaccharide

Conclusion

The results from this study have shown that the aqueous leaf extract of *V. amygdalina* has positive immune-modulatory and haematologic effects and contains some important prebiotic compounds.

Acknowledgements

The authors sincerely appreciate Mr. Emma Mbaoji, of the Department of Pure and Industrial Chemistry, University of Nigeria, Nsukka and Mr. Kenneth Ugwu of Classic Laboratories, Nsukka, for their technical assistance. The authors also acknowledge the staff of the Taxonomy Unit, Department of Plant Science and Biotechnology and the Department of Pharmacognosy, University of Nigeria, Nsukka for all their technical assistance. This study was conducted with support from Bioresources Development Group Ltd., Nigeria.

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