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Research Article

Effect of Freeze-dried *Vernonia amygdalina* Del. Leaves on Glycaemia, Oxidative Stress Biomarkers and Selected Metals in Type 2 Diabetics with or without Foot/Leg Ulcer

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Abstract
Vernonia amygdalina (VA) extracts have been reported to possess antioxidant and hypoglycaemic properties in animal studies with paucity of data in human study. To bridge this gap, this study investigated the effect of 8 weeks supplementation of freeze-dried VA leaves on glycaemic indexes and oxidative stress biomarkers in Type 2 diabetics with or without foot/leg ulcer and compared with non-diabetics with or without chronic foot/leg ulcer. One hundred and twenty participants were recruited and grouped into four. Each group was randomly divided into 3 subgroups: subgroup-1 (250 mg VA); subgroup-2 (500 mg VA) and subgroup-3 (No supplementation, NS). Ten millilitres of fasting blood samples were collected for determination of fasting plasma glucose (FPG), glycated haemoglobin A1c (HbA1c), total plasma peroxides (TPP), total antioxidant status (TAS), oxidative stress index (OSI), antioxidant micronutrients. Also, the wounds were clinically assessed and rated (WA) using modified ABDEFS. Statistical significance was considered at $p<0.05$. In the subgroups supplemented with 250 and 500 mg VA for 8 weeks, decreases in FPG, HbA1c, TPP, OSI and improvement in WA ($p<0.05$) as well as increases in TAS and antioxidant micronutrients ($p<0.05$) were found in all the groups. However, in NS subgroups, increases in FPG, HbA1c, and worsening of wounds ($p>0.05$), TPP, OSI ($p<0.05$) with significant decreases in TAS and antioxidant micronutrients were observed. Supplementation with *Vernonia amygdalina* could be used as a potential adjunct for treatment of diabetes mellitus and diabetic foot ulcer, but with caution when used as prophylactic in non-diabetics to prevent occurrence of hypoglycaemia..

Key Words: *Vernonia amygdalina*, Antidiabetic and antioxidative properties, Oxidative stress biomarkers, Diabetic foot ulcer, chronic ulcer

INTRODUCTION

Diabetes mellitus (DM), a group of metabolic disorders characterized by hyperglycaemia, is an endemic disease that demands an urgent attention. Globally, DM prevalence is on exponential increase (Saeedi *et al*., 2019). In 2019, about 463 million (9.3%) people were reported with DM, this is projected to increase to 578 million (10.2%) by 2030 and 700 million (10.9%) by 2045 (Saeedi *et al*., 2019). The sub-Saharan Africa has been reported to experience the greatest increase in DM (International Diabetes Federation, IDF, 2017), with Nigeria recording more than a hundred percent increase in the prevalence (from 2.2% in 1997 to around 6% in 2015) (Adeloye *et al*., 2017). As DM increases globally, it is accompanied by an increase in its attendant complications, among which are diabetic foot ulcers (DFUs), and necessitating lower extremity amputation (Atosona and Larbie, 2019). Worldwide, the prevalence of DFUs varies by continent from 3% in Oceania to 13% in North America giving a global average of 6.3% (Zhang *et al*., 2017). Nigeria has been

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reported to bear a greater burden with prevalence ranging from 11% to 32% (Ogbera *et al.*, 2006; Anumah *et al*., 2017). Therefore, the lifetime risk of a person with DM developing foot ulcers is 25% (IDF, 2015) with 40-60% risk of lower extremity amputation (Bakker and Schaper, 2012). In Nigeria, DFU has been reported to be the second leading cause of diabetes-related deaths accounting for 24% of all diabetes mortalities (Ogbera *et al.*, 2006) with attendant high treatment cost. Thus, the question of how best to manage and treat DFU with minimal cost that can be affordable by patients then arises.

The World Health Organization (WHO) has since supported the use of herbal remedies for treatment of DM (Bailey and Day, 1989). Premised on this, interest in herbal medicines continues to play an important role in diabetic therapy, particularly in developing countries where most people have limited resources and do not have adequate access to modern treatment (Ali *et al.*, 2006). As a result of the increasing importance of traditional medicine in various healthcare systems around the world, the World Health Assembly resolution on Traditional Medicine adopted in 2009 requested that WHO Traditional Medicine Strategy '2002-2005' be updated (WHO, 2013). Several medicinal plants, among which is *Vernonia amygdalina* (VA), have been scientifically reported in animal and *in vivo* studies to lower glycaemia (Akah *et al*., 2009; Toyang and Verpoorte, 2013; Ezuruike and Prieto, 2014; Mohammed *et al*., 2014) and improve antioxidant status (Nwanjo, 2005; Iwalokun *et al*., 2006; Farombi and Owoeye, 2011).

Vernonia amygdalina Del., also known as bitter leaf because of its bitter taste, is a vegetable used in preparing traditional soup in Nigeria (Farombi and Owoeye, 2011; Alara *et al*., 2019). It belongs to the Asteraceae family and widely spread across tropical Africa including Nigeria, Cameroon, Gabon, Democratic Republic of Congo and South Africa as well as Asia (Afolayan and Sunmonu, 2010; Farombi and Owoeye, 2011; Agbogidi and Akpomorine, 2013; Alara *et al*., 2019). *Vernonia amygdalina* leaves have been reported to be exceptionally rich in fats, proteins, fibres, carbohydrates, vitamins and mineral elements such as iron, phosphorus, calcium, potassium, zinc, copper, folic acids and ascorbic acid (Eyong *et al*., 2011; Agbogidi and Akpomorine, 2013; Alara et al., 2018). Furthermore, studies have shown that extract of VA leaves possess several secondary metabolites, which include flavonoids, polyphenols, saponins, tannins, terpenoids and glycosides (Agbogidi and Akpomorine, 2013; Alara *et al*., 2018). Consequently, due to these nutrients and secondary metabolites, it has been reported to have a number of potential uses and numerous medicinal values. The medicinal uses, which include anti-diabetic, anti-oxidative, anti-cancer, anti-microbial, anti-allergic, anti-bacterial, anti-malarial, anti-fungi, anti-inflammatory, anti-helminthic, anti-pyretic, analgesic, hepatoprotective, and hypolipidemic properties have been well documented in animal studies (Yeap *et al*., 2010; Farombi and Owoeye, 2011; Ngatu *et al*., 2012; Ezuruike and Prieto, 2014). Similarly, its aqueous extracts have been used by many herbalists and naturopathic doctors as treatment for diabetes and several other ailments (Kupchan *et al*., 1969).

Diabetes mellitus and its complication over the years have been managed conventionally through non-pharmacological (diet and exercise) and/or pharmacological means such as insulin and oral hypoglycaemic agents (Michael *et al*., 2010). This conventional management protocol is expensive, thereby resulting in lack of or low compliance by the patients (Adeneye and Agbaje, 2008; Okoli *et al*., 2008). Similarly, it may be associated with adverse effects including lactic acidosis, diarrhoea, and liver diseases among others (Adeneye and Agbaje, 2008; Okoli *et al*., 2008; Mane *et al*., 2012; Ezuruike and Prieto, 2014). Thus, there is need for scientific search to explore for relatively less toxic and less expensive natural resources from medicinal plants with effective therapeutic values to either be used as substitute or act in synergy to other hypoglycaemic drugs (Okoduwa *et al*., 2017). The medicinal uses of VA extracts have been well documented in animal studies; however, there is paucity of data on the use of freeze-dried samples of VA in clinical studies. The freeze-dried powder sample is necessary because high moisture content in any organic compound or material can cause microbial contamination and growth and this may have negative impact on the long term storage of the aqueous form of the medicinal plants (Ruberto and Baratha, 2000; Dah-Nouvlessounon *et al*., 2015). To bridge this gap, therefore, this study aimed at investigating the effect of 8 weeks supplementation of freeze-dried *Vernonia amygdalina* leaves on glycaemic indexes and oxidative stress biomarkers in Type 2 diabetics with or without foot/leg ulcer and compared with non-diabetics with or without chronic foot/leg ulcer.

MATERIALS AND METHODS

Ethical Consideration: The study protocol was reviewed and approved by the joint University of Ibadan/University College Hospital Institutional Research Ethics Committee (approval number: UI/EC/15/0399). All participants were briefed about the study and informed consent was obtained from each participant before they were enrolled into the study.

Data collection: Basic characteristics of the participants were obtained through the use of a validated structured self-administered questionnaire covering socio-demographic and anthropometric characteristics, social activities/life styles, exercise history, as well as medical history.

Study Design, Study Population, Description and Intervention: This interventional case-control study with 8 weeks of *Vernonia amygdalina* supplementation was conducted at the Metabolic Research Ward, Medical-Out-Patient and Surgical Out-Patient of the University College Hospital, UCH, Ibadan. One hundred and twenty male and female participants between 40 and 80 years were recruited into this study from January 2016 to September 2018. The sample size was determined using the case-control formula: [$n = r+1(p^*)(1-p^*)[(Z_\beta + Z_{\alpha/2})^2/(p_1 - p_2)^2$] as described by Charan and Biswas (2013). Participants were consecutively recruited and divided into 4 main groups namely: 30 Type-2 diabetes mellitus with foot/leg ulcer (Group 1), 30 Type-2 diabetes mellitus without ulcer (Group 2), 30 Non-diabetics with chronic foot/leg ulcer (Group 3) and 30 apparently healthy non-diabetics without ulcer (Group 4). Each of the main group was further divided into 3 subgroups: subgroup-1 [received 250 mg/kg body weight of encapsulated VA (250 mg VA)]; subgroup-2 [received 500 mg/kg body weight of encapsulated VA (500 mg VA)] and subgroup-3 [received no supplementation (NS)], using consecutive numbering system:

the first three numbers represent 250 mg GK, 500 mg GK and NS, respectively and so on.

The general population were classified into five categories based on body mass index (BMI), which is a ratio between weights and the squared height (Kg/m^2) of a participant. The BMI was classified as: underweight (BMI<18.5 kg/m^2), normal weight (BMI 18.5-24.9 kg/m^2), overweight/class I obesity (BMI 25.0-29.9 kg/m^2), obese/class II obesity (BMI 30.0-39.9 kg/m^2) and extremely obese/class III obesity (BMI > 40 kg/m^2) (WHO, 2018). Similarly, participants' age were stratified as follows: 40-49 years; 50-59 years; 60-69 years; 70-80 years.

The diabetics with foot/leg ulcers were clinically graded using 'Wagner's Grades 1 and 2 ulcer' grading system (Wagner, 1987). All the participants in Groups 1 and 2 were previously diagnosed according to the criteria of American Diabetes Association (2004; 2020) and continued on the medications prescribed by the attending physician, which include the use of single doses of metformin, glibenclamide or insulin only or combined doses of metformin with glibenclamide or metformin with insulin. In addition to these medications, they received the herbal treatment (250 mg VA or 500 mg VA) as a supplement. However, participants of Groups 1 and 2 in the NS subgroups were only on the medications prescribed by the attending physician with no supplementation. They were not given placebo because of their diabetic conditions. Similarly, all participants in this study were asked to abstain from eating 'bitter leaf soup' and also stop any multivitamins and mineral supplements during the 8 week intervention with VA supplements.

Preparation and encapsulation of the freeze-dried *Vernonia amygdalina* powder: *Vernonia amygdalina* leaves were freeze-dried using a pressure freeze-drying machine at temperature of -65ºC and -40ºC (machine and sample temperature respectively) to a constant weight. The dried leaves were then blended into powder using a blender, sieved with laboratory sieve with a mesh size of 0.5 mm and the sieved powdered samples were stored in air-tight containers at -20ºC until required. Two hundred and fifty (250) mg of freeze-dried powder of VA leaves were measured into size 2 capsules using a manual mini homemade capsule filler CN–20 CL (CapsulCN International Co., Ltd, Zhejiang, China). The participants in the 250 mg VA sub-group were administered a capsule per day, while participants in the 500 mg VA sub-group were administered 2 capsules per day (1 capsule in the morning and 1 capsule at night). The dosage used was based on the extrapolated age and weight from the acute toxicity test carried out on rat model (Bolajoko *et al*., 2019).

Inclusion Criteria:

1. Male and female (non-pregnant/non-lactating) participants between 40 and 80 years.
2. Participants with Type-2 diabetes mellitus with or without foot/leg ulcer of at least 6 weeks.
3. Type-2 diabetic participants with Wagner's Grade 1 and 2 diabetic ulcers.
4. Non-diabetic participants with or without chronic foot/leg ulcers (pressure/venous ulcers) of at least 6 weeks
5. Non-diabetics with FPG < 5.6 mmol/L (<100 mg/dL) (ADA, 2004; 2020)
6. Participants who sign the written informed consent form.

Exclusion Criteria:

1. Participants below 40 years and above 80 years
2. Pregnant/lactating females
3. Participants with Wagner's Diabetic Ulcers Grade 3 and above
4. Diabetics with other complications such as retinopathy, nephropathy, autonomic neuropathy, and other co-morbidities
5. Non-diabetic participants with post-trauma ulcer, haematological ulcer and vasculitis
6. Non-diabetic participants with fasting plasma glucose (FPG) in pre-diabetes level (5.6 - 6.9 mmol/L or 100 - 125 mg/dL) (ADA, 2004; 2020).
7. Participants who do not sign the written informed consent

Physical Examination Procedure

Clinical Assessment of Wound (WA): In this study, the modified ABDEFS tool of evaluating chronic ulcers by Oluwatosin *et al.*, (1998) was adopted and used in clinical assessment of the wound healing progress.

Aetiology:
1) Local, e.g. trauma, infection
2) Controlled systemic disease
3) Uncontrolled systemic disease
4) Malignancy

Size:
1) Less than or equal to 2.5 cm in one dimension
2) Greater than 2.5 cm in one dimension

Depth
1) Superficial partial thickness
2) Deep dermal
3) Full thickness

Re-epithelisation
1) >75% of the wound
2) 50 – 75 of the wound
3) 25 – 49 of the wound
4) <25% of the wound

Granulation tissue
1) >75% of the surface area
2) 50 – 75 of the surface area
3) 25 – 49 of the surface area
4) <25% of the surface area

Slough
1) <25% of the surface area
2) 25 – 49 of the surface area
3) 50 – 75 of the surface area
4) >75% of the surface area

Necrotic tissue
1) <25% of the surface area
2) 25 – 49 of the surface area
3) 50 – 75 of the surface area
4) >75% of the surface area

Odour
1) No odour
2) Faint odour at close range
3) Moderate odour in the room
4) Strong odour in the room

Exudates quantity
1) No exudates
2) Scanty exudates
3) Moderate exudates
4) Large exudates

Edge:
1) Flat, shelving, punched out
2) Undermined, raised

Points corresponding to appropriate description were allocated to each feature on the ulcer. The minimum possible score of 10 corresponds to the best healing ulcers while the maximum score of 35 corresponds to the worst healing ulcers. This wound assessing tool is easy to measure and no special training is required.

Follow-up Details: All participants were followed up for 8 weeks.

1. An agreed day, date, and time was fixed and participants were requested to undergo an overnight fast of about 10 to 12 hours until when blood samples were collected at 8.00 am the following morning.
2. Participants with wound were assessed by the attending physician and these were documented.
3. Participants supplemented with either 250 mg VA or 500 mg VA were given the encapsulated VA that was enough for use for two weeks. Each participant was asked to come back to the hospital fortnightly with the unused encapsulated herbs until the 8 weeks were completed.
4. Participants in the NS subgroup were also asked to come back fortnightly for wound assessment.

5. At each visit to the hospital, all participants were asked to list the multivitamins and mineral supplements, type of vegetables they ate during the last 2 weeks. This was done to measure the level of compliance with the instructions given at the very beginning of the study.
6. At 4 and 8 weeks of treatment, blood samples were collected from each participant respectively for the second and last determination of biochemical parameters. The attending physician to the participants again assessed the wounds and these were recorded.

Collection and storage of Blood: Ten millilitres of blood samples were collected after a 10 hour overnight fast from each participant at 0 (baseline), 4 and 8 weeks of supplementation with 250 mg VA, 500 mg VA or NS. The blood samples were collected into either fluoride oxalate, EDTA or heparin tubes. About 2 mL of EDTA whole blood samples were aliquoted for metal analysis. Samples were centrifuged at 3000 rpm for 10 minutes to separate the plasma. All samples (plasma and whole blood) were stored in small aliquots at -80ºC until the day of analysis. Levels of glycaemic index [FPG, glycated haemoglobin A1c (HbA1c)], and oxidative stress biomarkers [total plasma peroxides (TPP), total antioxidant status (TAS), vitamin A, vitamin C, vitamin E, manganese (Mn), zinc (Zn), and selenium (Se)] were determined.

Determination of Fasting Plasma Glucose: Glucose from fluoride oxalate plasma was determined using a commercial kit from DIALAB® (Austria). The technique, described by the manufacturer, was based on Trinder's reaction (Barham and Trinder, 1972). In this method, glucose was oxidized to gluconic acid and hydrogen peroxide in the presence of glucose oxidase. The formed hydrogen peroxide then reacts, in the presence of peroxidase, with phenol and 4-aminoantipyrine to form a quinonimine dye. The intensity of the pink colour formed was proportional to the glucose concentration and was measured spectrophotometrically at wavelength of 500 nm using Spectro SC spectrophotometer (Labomed Inc., USA).

Determination of Glycated Haemoglobin A1c: The levels of HbA1c were determined using Clover A1c test cartridge on the Clover A1c analyser (Infopia Co., Ltd, Korea). The method was based on automated boronate affinity assay. This Clover A1c test cartridge is composed of a cartridge and a reagent pack with blood sample collecting area. The reagent packs were pre-filled with wash solutions and reaction solutions, which contain agents that lyse erythrocytes and bind haemoglobin specifically. Similarly, the reagent packs were pre-filled with boronate resins that bind cis-diols of glycated haemoglobin. In this method, 4 µL of EDTA whole blood were collected at the sample collecting area of the reagent packs, then the reagent packs were inverted into the cartridges, where the blood samples were instantly lysed releasing the haemoglobin and the boronate resin binding the glycated haemoglobin. Then each cartridge was inserted into the Clover A1c Analyser. The blood sample mixture was automatically rotated, placing the blood sample in the measuring zone. The total haemoglobin were photometrically measured by the diffused reflectance of the optical sensor composed of both a Light Emitting Diode (LED) and a Photo Diode (PD). Then, the assembled cartridges were rotated so that the washing solutions wash out non-glycated haemoglobin from the blood samples, enabling photometrical measurement of glycated haemoglobin. The ratio of glycated haemoglobin with respect to total haemoglobin in the blood samples were calculated using the formula "HbA1c% = A x [(HbA1c x 100)/Total Haemoglobin] + B". Where, 'HbA1c' and 'Total haemoglobin' were values obtained from the Clover A1c analyser and 'A' and 'B' were the slope and intercept factor respectively, to correct the value for the calibration standard of National Glycohemoglobin Standardization Program (NGSP).

Determination of Total Plasma Peroxide: Total plasma peroxide (TPP) concentrations were determined using FOX-2 assay according to the method described by Miyazawa (1989) with minor modifications by Harma *et al*., (2003). This method was based on the oxidation of ferrous ion to ferric ion by various types of peroxides contained within the plasma samples to produce a coloured ferric-xylenol orange complex, which were measured spectrophotometrically at 560 nm (Spectro SC spectrophotometer, Labomed Inc., USA).

Determination of Total Antioxidant Status*:* Plasma levels of TAS were determined by a Fenton-type reaction described by Koracevic *et al.* (2001). In this method, standardization solution of Fe-EDTA complexes react with hydrogen peroxides leading to the formation of hydroxyl radicals ($HO^{\cdot}$). These reactive oxygen species degrade benzoate resulting in the release of thiobarbituric acid reactive substance (TBARS). The antioxidants from the added samples of human blood or standards result in suppression of the production of TBARS and were measured spectrophotometrically at 532 nm using spectro SC spectrophotometer (Labomed Inc., USA). The rates of inhibition of colour were proportional to the concentrations of antioxidant status present in the samples.

Calculation of Oxidative Stress Index: Oxidative stress index is an indicator of the degree of oxidative stress. It was calculated from the values of total plasma peroxide and total antioxidant status using the formula (TPP/TAS x 100) (Harma *et al*., 2003)

Determination of Vitamins A, C and E by High Performance Liquid Chromatography (HPLC): Vitamin A, vitamin C, and vitamin E were determined by HPLC system (Waters 616/6261c). Precipitating reagents were added to precipitate the higher molecular components, which were removed by centrifugation. The supernatants were then injected into the HPLC system. The separation via HPLC follows an isocratic method at 30°C using a reversed phase column. Vitamin A were determined at 325 nm as described by Talwar *et al*. (1998), vitamin C were determined at 270 nm using the method of Sanderson and Schorah (1987) and vitamin E were determined at 290 nm (Talwar *et al.*, 1998).

Determination of Manganese (Mn), Zinc (Zn), and Selenium (Se) by Atomic Absorption spectrophotometry (AAS): Manganese, Zn and Se were determined using AAS technique. In this technique, the atoms of the element aspirated into the AAS vaporize and absorb light of the same wavelength as that emitted by the element when in the excited state. The procedures include thawing of frozen plasma samples and diluting with 0.1N hydrochloric acid (1:20) to release bound trace metals in order to enhance accurate measurement. The digested samples were aspirated directly into the AAS for analyses.

Table 1:
Basic characteristics of all participants

	Variables	Group 1 Frequency (%)	Group 2 Frequency (%)	Group 3 Frequency (%)	Group 4 Frequency (%)	Chi-square	p-value
Age (years)	40-49	2 (6.7)	6 (20)	12 (40)	16 (53.3)	30.433*	0.000
	50-59	18 (60)	9 (30)	14 (46.7)	9 (30)		
	60-69	9 (30)	10 (33.3)	4 (13.3)	4 (13.3)		
	70-80	1 (3.3)	5 (16.7)	0	1 (3.3)		
Gender	Male	17 (56.7)	18 (60)	14 (46.7)	12 (40)	3.034	0.386
	Female	13 (43.3)	12 (40)	16 (53.3)	18 (60)		
BMI	Underweight	0	0	0	2 (6.7)	20.497*	0.015
	Normal weight	16 (53.3)	10 (33.3)	19 (63.3)	21 (70)		
	Overweight	10 (33.3)	15 (50)	11 (36.7)	5 (16.7)		
	Obese	4 (13.3)	5 (16.7)	0	2 (6.7)		
Marital Status	Married	30 (100)	27 (90)	29 (96.7)	29 (96.7)	11.165	0.265
	Divorced	0	0	0	1 (3.3)		
	Widower	0	2 (6.7)	0	0		
	Widow	0	1 (3.3)	1 (3.3)	0		
Education Status	No Formal Education	3 (10)	0	1 (3.3)	0	42.642*	0.000
	Primary School	7 (23.3)	3 (10)	6 (20)	0		
	Secondary School	13 (43.3)	19 (63.3)	12 (40)	5 (16.7)		
	Graduate	6 (20)	6 (20)	8 (26.7)	13 (43.3)		
	Postgraduates	1 (3.3)	2 (6.7)	3 (10)	12 (40)		
Smoking Status	Ever	11 (36.7)	4 (13.3)	6 (20)	6 (20)	5.114	0.164
	Never	19 (63.3)	26 (86.7)	24 (80)	24 (80)		
Alcohol Consumption	Ever	1 (3.3)	9 (30)	3 (10)	10 (33.3)	12.640*	0.005
	Never	29 (96.7)	21 (70)	27 (90)	20 (66.7)		
Exercise History	Yes	0	16 (53.3)	0	17 (56.7)	45.601*	0.000
	No	30 (100)	14 (46.7)	30 (100)	13 (43.3)		
Hypertension Status	No	13 (43.3)	12 (40)	22 (73.3)	30 (100)	31.133*	0.000
	Yes	17 (56.7)	18 (60)	8 (26.7)	0		
Hypertension Medication	Amlodipine plus Lisofil	1 (3.3)	1 (3.3)	0	-	32.075*	0.043
	Lisinopril only	1 (3.3)	4 (13.3)	0	-		
	Nifedipine plus Lisinopril	2 (6.7)	2 (6.7)	2 (6.7)	-		
	Lisofil only	1 (3.3)	1 (3.3)	0	-		
	Amlodipine only	5 (16.7)	2 (6.7)	1 (3.3)	-		
	Ramipril plus Nifedipine	0	1 (3.3)	0	-		
	Nifedipine only	2 (6.7)	5 (16.7)	0	-		
	Dopatab	0	2 (6.7)	0	-		
	Ramipril	5 (16.7)	0	2 (6.7)	-		
	Amlodipine plus Lisinopril	0	0	3 (10)	-		
Hypertension Duration (Years)	< 10	21 (70)	23 (76.7)	30 (100)	-	20.538*	0.002
	11-20	6 (20)	6 (20)	0	-		
	21-30	3 (10)	1 (3.3)	0	-		
T2DM Duration (Years)	< 10	13 (43.3)	20 (66.7)	-	-	42.816*	0.000
	11-20	17 (56.7)	9 (30)	-	-		
	21-30	0	1 (3.3)	-	-		
	>30						
Diabetes Medication	Metformin only	8 (26.7)	21 (70)	-	-	13.392*	0.010
	Metformin plus Glibenclamide	4 (13.3)	2 (6.7)	-	-		
	Metformin plus Insulin	11 (36.7)	2 (6.7)	-	-		
	Insulin only	4 (13.3)	2 (6.7)	-	-		
	Glibenclamide only	3 (10)	3 (10)	-	-		
Wound duration	6-8 weeks	7 (23.3)	-	2 (6.7)	-	3.269	0.195
	9-12 weeks	18 (60)	-	22 (73.3)	-		
	13-16 weeks	5 (16.7)	-	6 (20)	-		
Wound Dressing	Compression bandage with nano crystallised silver	10 (33.3)	-	13 (43.3)	-	136.383*	0.000
	Normal saline with dressing powder, iodine	14 (46.7)	-	17 (56.7)	-		
	Normal saline and honey	6 (20)	-	0	-		

Working standard solutions were prepared in part per million (ppm) and used for the standardization of the corresponding elements. Manganese, Zn and Se in EDTA whole blood samples were determined by the technique respectively

described by Casey *et al*. (1987), Kaneko *et al*., 1997), and Pleban *et al*., (1982) using a 210/211 VGP atomic absorption spectrophotometer (Buck Scientific, USA).

Data Analysis Method: Categorical variables were reported as numbers and percentages and non-normally distributed variables as median with interquartile range (IQR). Data were analysed using a Statistical Software Package for Social Sciences (SPSS) version 20 (IBM SPSS, Armonk, NY, USA). Chi-square tests were used for categorical variables and Wilcoxon signed rank tests were used for non-normally distributed continuous variables. Partial correlations among all the variables were carried out controlling for disease and supplementation. A value of $p<0.05$ was set for the statistical significance.

RESULTS

The mean age of the participants were 51.97 ± 8.71 (Group 4), 52.00 ± 6.96 (Group 3), 58.73 ± 6.14 (Group 1) and 59.23 ± 9.96 (Group 2). The basic characteristics of all parameters in this study revealed that diabetes mellitus and diabetic foot ulcers with duration of 20 years or less occurred frequently in married male participants between ages 50 and 69 years. These participants were either overweight or within the normal weight with at least secondary school education. Most of these participants were hypertensives with no smoking and alcohol history. The hypertension duration of less than ten years were mostly managed with single dose therapy, while DM were managed with either single dose of metformin only or with combined therapy of metformin with insulin. Most of the participants had foot/leg ulcers of 9 to 12 weeks and were dressed with normal saline, dressing powder and iodine (Table 1).

Figure 1 revealed the study population distribution: 30 participants each in 4 groups with 3 subgroups (250 mg VA, 500 mg VA and NS). Each subgroup comprised of 10 participants. A total of 12 participants (3 participants from Group 1; 6 participants from Group 2; and 3 participants from Group 4) were lost to follow-up.

No significant differences were observed in BMI in all the Groups and subgroups except in Group 2 (500 mg VA supplementation), Group 3 (NS) and Group 4 (500 mg VA supplementation) where significant decreases were found. In all supplemented groups, FPG and HbA1c were significantly decreased at 8 weeks, except in Group 4 (250 mg VA supplementation) where no significant decreases were observed in FPG. However, FPG values were similar from baseline to 8 weeks in the NS subgroups across all groups except in Group 2 where significant increases were observed. Similarly, in the NS subgroups, HbA1c values were similar across all groups (Table 2).

At 8 weeks of supplementation with 250 mg VA or 500 mg VA, significant decreases in TPP and OSI with significant increases in TAS, vitamin A, vitamin C and vitamin E were observed in all the Groups. However in the NS subgroup, significant increases were found in TPP and OSI with decreases in TAS, vitamin A, vitamin C and vitamin E of Groups 1 to 3, while in Group 4, significant decreases in TAS, vitamin A and vitamin E with similar values in TPP, OSI and vitamin C ($p>0.05$) were observed (Tables 3 and 4).

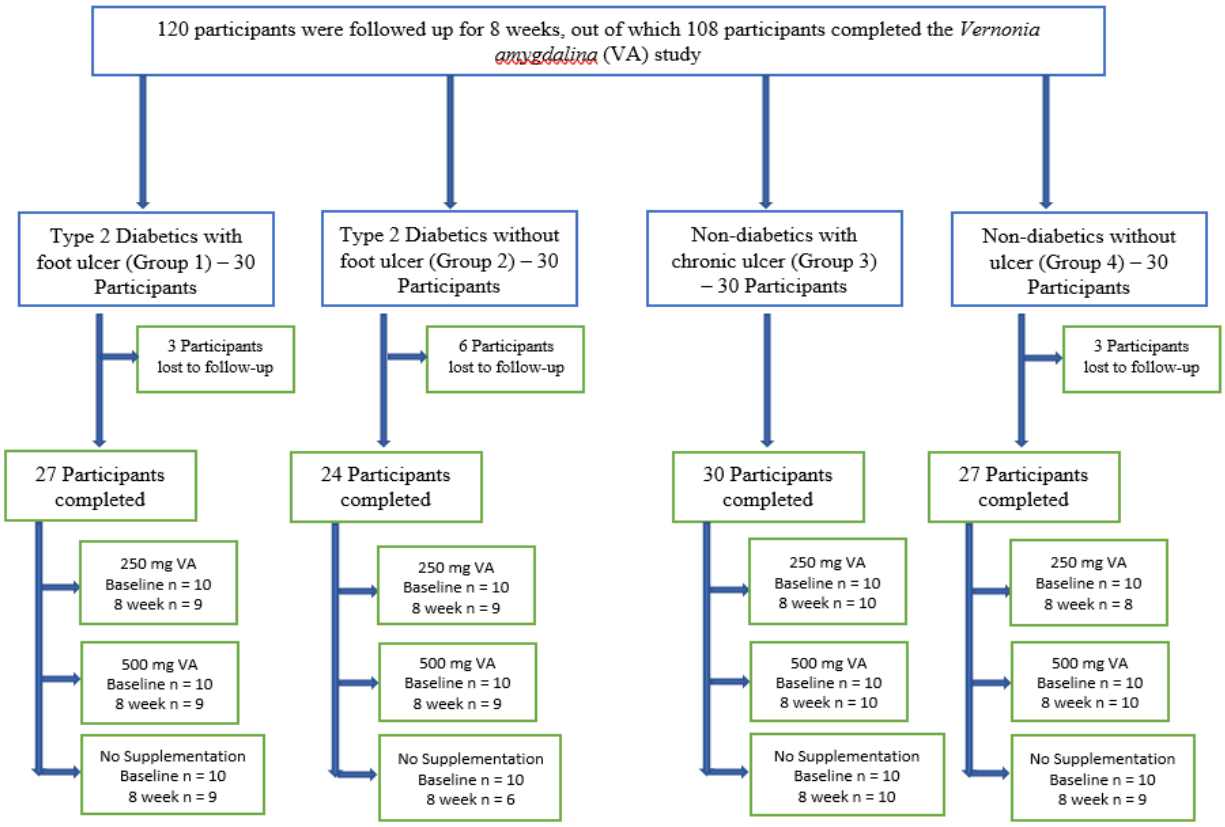

Figure 1:
Study Population Distribution

Table 2:
8 weeks supplementation comparison of BMI, glycaemic indexes and wound assessment in all Groups supplemented with V. amygdalina – 250 mg VA, 500 mg VA and no supplementation (NS) subgroups

	250 mg VA				500 mg VA				NS			
Parameters	**Baseline Med(IQR)**	**4 weeks Med(IQR)**	**8 weeks Med(IQR)**	**p-value**	**Baseline Med(IQR)**	**4 weeks Med(IQR)**	**8 weeks Med(IQR)**	**p-value**	**Baseline Med(IQR)**	**4 weeks Med(IQR)**	**8 weeks Med(IQR)**	**p-value**
Group 1	**(n=10)**	**(n=9)**	**(n=9)**		**(n=10)**	**(n=10)**	**(n=9)**		**(n=10)**	**(n=10)**	**(n=9)**	
BMI (Kg/m²)	24.8(24.5, 27.3)	25.2(23.9, 27.0)	25.6(23.6, 27.3)	0.916	24.9(24.5, 31.7)	25.5(23.9, 32.1)	25.0(23.1, 31.4)	0.051	24.7(23.3, 28.9)	24.0(22.8, 24.7)	23.6(23.1, 25.1)	0.372
FPG(mmol/dL)	6.5(6.0, 7.1)	6.5(5.6, 6.7)	6.2(5.5, 6.6)	0.008	7.0(6.4, 7.2)	6.4(6.0, 6.8)	6.3(5.8, 6.6)	0.008	7.1 (6.8, 7.3)	7.1(6.7, 7.2)	7.2(6.7, 7.2)	0.171
HbA1c (%)	6.2(5.9, 8.0)	6.1(5.8, 7.8)	6.1(5.7, 7.7)	0.011	6.4(6.1, 8.4)	6.2(5.9, 8.1)	6.1(5.7, 8.7)	0.011	6.9(5.9,10.5)	6.9(5.9, 10.4)	6.8(5.9, 10.4)	0.856
WA	20.0(19.0, 24.0)	19.0(17.0, 22.0)	16.0(15.0, 19.0)	0.007	22.0(20.5, 23.0)	19.0(18.5, 20.0)	17.0(15.0, 18.0)	0.007	22.5(21.5, 25.3)	24.0(22.5, 26.0)	25.0(23.5, 26.5)	0.010
Group 2	**(n=10)**	**(n=9)**	**(n=9)**		**(n=10)**	**(n=9)**	**(n=9)**		**(n=10)**	**(n=8)**	**(n=6)**	
BMI (Kg/m²)	28.6(25.5, 31.1)	28.0(24.8, 30.8)	27.2(24.8, 30.8)	0.150	25.9(23.3, 28.1)	24.8(22.3, 27.6)	24.4(21.7, 26.1)	0.012	25.0(23.0, 28.4)	25.7(23.8, 29.0)	24.3(23.1, 30.2)	0.028
FPG(mmol/dL)	6.4(5.5, 6.7)	5.9(5.3, 6.4)	5.6(5.0, 6.1)	0.038	6.0(5.4, 6.2)	5.4(5.1, 6.5)	5.2(5.0, 5.6)	0.008	5.7(5.2, 6.8)	6.0(5.6, 6.2)	6.2(6.1, 6.4)	0.046
HbA1c (%)	5.7(5.0, 6.8)	5.5(4.8, 6.8)	5.3(4.8, 6.9)	0.028	5.9(5.1, 7.4)	5.5(4.7, 7.3)	5.5(4.6, 7.1)	0.007	5.5(4.9, 6.0)	5.6(5.2, 5.9)	5.9(5.3, 6.1)	0.027
WA	-	-	-	-	-	-	-	-	-	-	-	-
Group 3	**(n=10)**	**(n=10)**	**(n=10)**		**(n=10)**	**(n=10)**	**(n=10)**		**(n=10)**	**(n=10)**	**(n=10)**	
BMI (Kg/m²)	23.5(21.6, 26.2)	23.9(22.2, 25.8)	23.1(23.0, 25.0)	0.878	24.5(23.6, 26.1)	24.6(23.3, 25.8)	23.8(23.1, 25.9)	0.314	24.7 (23.9, 25.4)	24.3(23.7, 24.6)	23.4(23.1, 23.8)	0.017
FPG(mmol/dL)	4.7(4.5, 5.4)	4.6(4.4, 5.4)	4.5(4.3, 4.9)	0.005	4.5(4.4, 5.0)	4.4(4.2, 5.1)	4.2(4.0, 5.1)	0.284	4.6(4.2, 6.1)	5.0(4.7, 5.5)	5.1(4.6, 5.5)	0.878
HbA1c (%)	4.8(4.6, 5.2)	4.7(4.3, 5.0)	4.5(4.2, 5.0)	0.007	4.9(4.6, 5.1)	4.6(4.2, 5.0)	4.4(4.0, 4.8)	0.005	5.2(4.8, 5.6)	5.4(5.1, 5.5)	5.5(5.2, 5.7)	0.058
WA	18.5(17.8, 22.8)	16.5(15.0, 20.5)	14.5(11.8, 17.8)	0.005	17.0(15.0, 19.5)	14.5(13.8, 16.5)	13.0(12.0, 15.0)	0.005	17.5(17.0, 20.0)	18.5(17.0, 19.3)	18.5(17.5, 20.0)	0.762
Group 4	**(n=10)**	**(n=9)**	**(n=8)**		**(n=10)**	**(n=10)**	**(n=10)**		**(n=10)**	**(n=10)**	**(n=9)**	
BMI (Kg/m²)	23.5 (20.7, 27.8)	23.5(19.5, 26.0)	23.3(21.7, 24.9)	0.674	23.5 (22.6, 24.9)	23.3(21.8, 24.8)	22.7(22.0, 24.4)	0.038	23.4 (22.6, 24.5)	23.4(22.1, 24.2)	23.3(21.9, 24.4)	0.144
FPG(mmol/dL)	5.4(5.0, 5.8)	5.2(4.5, 5.5)	4.8(4.0, 5.1)	0.069	5.2(4.8, 6.0)	4.6(4.1, 4.9)	4.6(4.1, 4.8)	0.007	4.9(4.1, 5.1)	5.0(4.7, 5.3)	5.3(4.5, 5.5)	0.086
HbA1c (%)	4.8(4.5, 5.1)	4.5(4.4, 4.8)	4.4(4.2, 4.7)	0.018	5.0(4.7, 5.4)	4.7(4.2, 5.1)	4.6(4.0, 4.7)	0.007	4.5(4.2, 4.9)	4.6(4.0, 4.9)	4.6(4.0, 5.0)	0.777
WA	-	-	-	-	-	-	-	-	-	-	-	-

Significant at $p<0.05$;
n=sample size;
p-value=Baseline compared with 8 weeks;
Med(IQR)=median(interquartile range);
BMI=Body mass index;
FPG=Fasting plasma glucose; HbA1c=Glycated hemoglobin A1c;
WA=wound assessment tool

Table 3:
8 weeks supplementation comparison of oxidative stress biomarkers in all Groups supplemented with V. amygdalina – 250 mg VA, 500 mg VA and no supplementation (NS) subgroups

	250 mg VA				500 mg VA				NS			
Parameters	**Baseline Med(IQR)**	**4 weeks Med(IQR)**	**8 weeks Med(IQR)**	**P-value**	**Baseline Med(IQR)**	**4 weeks Med(IQR)**	**8 weeks Med(IQR)**	**P-value**	**Baseline Med(IQR)**	**4 weeks Med(IQR)**	**8 weeks Med(IQR)**	**P-value**
Group 1	**(n=10)**	**(n=9)**	**(n=9)**		**(n=10)**	**(n=10)**	**(n=9)**		**(n=10)**	**(n=10)**	**(n=9)**	
TPP (µmol/L)	87.3(45.1, 94.7)	79.7(41.0, 90.9)	77.2(39.2, 89.3)	0.007	61.2(21.3, 87.9)	57.4(19.8, 85.0)	51.3(16.8, 81.0)	0.012	82.0(78.7, 90.9)	85.8(81.2, 96.4)	87.8(81.7, 97.5)	0.037
TAS (µmol/L)	2700.0(2050.0, 4150.0)	2950.0(2375.0, 5050.0)	3100.0(2550.0, 5250.0)	0.007	2850.0(1575.0, 3600.0)	3000.0(1675.0, 3800.0)	3200.0(1750.0, 4100.0)	0.006	1650.0(1125.0, 9800.0)	1550.0(1200.0, 9500.0)	1500.0(950.0, 9100.0)	0.020
OSI (%)	1.6 (0.9, 3.6)	1.5(1.1, 3.5)	1.4(1.0, 3.0)	0.008	1.5(1.1, 3.5)	1.4(0.7, 3.2)	1.2(0.6, 3.0)	0.008	4.5(0.9, 6.2)	4.8(1.8, 6.5)	5.4(2.6, 8.0)	0.008
Group 2	**(n=10)**	**(n=9)**	**(n=9)**		**(n=10)**	**(n=9)**	**(n=9)**		**(n=10)**	**(n=8)**	**(n=6)**	
TPP (µmol/L)	40.1(25.1, 57.4)	39.1(22.3, 60.4)	37.6(20.6, 59.1)	0.012	43.4(33.0, 67.1)	37.6(28.7, 66.0)	36.0(27.2, 64.2)	0.008	78.4(48.6, 84.6)	82.7(79.4, 89.5)	84.8(70.6, 92.8)	0.043
TAS (µmol/L)	3600.0(2375.0, 4075.0)	3900.0(2400.0, 4250.0)	4100.0(2500.0, 4550.0)	0.021	3200.0(2125.0, 4475.0)	3300.0(2150.0, 6050.0)	3500.0(2400.0, 6150.0)	0.007	3500.0 (2300.0, 5525.0)	3350.0(2300.0, 4700.0)	3150.0(1725.0, 3650.0)	0.027
OSI (%)	1.5(0.9, 3.3)	1.2(0.8, 3.0)	1.0(0.8, 2.5)	0.008	1.4(0.7, 3.2)	1.1(0.6, 1.9)	1.0(0.5, 1.7)	0.008	2.3(1.2, 2.9)	2.6(1.3, 3.7)	2.9(1.5, 5.0)	0.028
Group 3	**(n=10)**	**(n=10)**	**(n=10)**		**(n=10)**	**(n=10)**	**(n=10)**		**(n=10)**	**(n=10)**	**(n=10)**	
TPP (µmol/L)	50.6(45.2, 54.8)	50.5(39.6, 53.3)	48.2(36.8, 51.8)	0.007	59.4(49.7, 83.2)	56.9(47.8, 78.7)	56.6(45.0, 75.5)	0.005	51.8(41.0, 68.5)	53.3(48.0, 68.0)	55.1(48.7, 69.0)	0.016
TAS (µmol/L)	4600.0(3700.0, 5100.0)	4800.0(3925.0, 5275.0)	5000.0(4250.0, 5550.0)	0.004	5600.0(4725.0, 8200.0)	5800.0(4925.0, 9075.0)	6100.0(5275.0, 9300.0)	0.005	6200.0 (4575.0, 7462.5)	6125.0(4525.0, 7525.0)	5950.0(4300.0, 7800.0)	0.260
OSI (%)	1.0(0.7, 1.3)	0.9(0.5, 1.2)	0.8(0.5, 1.1)	0.005	1.0(0.2, 1.1)	0.9(0.2, 1.0)	0.8(0.2, 0.9)	0.005	1.1(0.6, 4.3)	1.5(0.7, 4.4)	1.8(1.0, 5.1)	0.007
Group 4	**(n=10)**	**(n=9)**	**(n=8)**		**(n=10)**	**(n=10)**	**(n=10)**		**(n=10)**	**(n=10)**	**(n=9)**	
TPP (µmol/L)	60.7(45.2, 88.6)	56.9(31.0, 78.4)	53.3(19.3, 71.6)	0.012	46.2 (27.5, 68.4)	40.4(20.7, 60.5)	37.6(19.2, 57.2)	0.005	47.0(23.9, 85.4)	50.5(23.5, 87.9)	51.3(28.2, 90.6)	0.050
TAS (µmol/L)	5450.0(3400.0, 13750.0)	6600.0(3500.0, 14300.0)	6750.0(3612.5, 16050.0)	0.012	14000.0(13200.0, 15875.0)	14100.0(12700.0, 16050.0)	14350.0(13000.0, 16275.0)	0.073	14525.0(12225.0, 23475.0)	14350.0(11975.5, 23400.0)	14300.0(12800.0, 25250.0)	0.008
OSI (%)	1.3(0.7, 1.9)	1.0(0.5, 1.6)	0.8(0.4, 1.4)	0.017	0.3(0.2, 0.9)	0.2(0.1, 0.8)	0.2(0.1, 0.7)	0.005	0.3(0.1, 0.6)	0.3(0.2, 0.6)	0.4(0.2, 0.6)	0.086

Significant at $p<0.05$;
n=sample size;
p-value=Baseline compared with 8 weeks;
Med(IQR)=median(interquartile range);
TPP: Total plasma peroxides;
TAS: Total antioxidant status;
OSI: Oxidative stress index

Table 4:
8 weeks supplementation comparison of antioxidant vitamins in all Groups supplemented with V. amygdalina – 250 mg VA, 500 mg VA and no supplementation (NS) subgroups

	250 mg VA				500 mg VA				NS			
Parameters	**Baseline Med(IQR)**	**4 weeks Med(IQR)**	**8 weeks Med(IQR)**	**P-value**	**Baseline Med(IQR)**	**4 weeks Med(IQR)**	**8 weeks Med(IQR)**	**P-value**	**Baseline Med(IQR)**	**4 weeks Med(IQR)**	**8 weeks Med(IQR)**	**P-value**
Group 1	**(n=10)**	**(n=9)**	**(n=9)**		**(n=10)**	**(n=10)**	**(n=9)**		**(n=10)**	**(n=10)**	**(n=9)**	
Vit. A (µmol/L)	3.0(2.6, 3.3)	3.2(2.7, 3.4)	3.2(2.8, 3.5)	0.008	2.9(2.6, 3.1)	3.0(2.7, 3.2)	3.1(2.7, 3.3)	0.008	2.7(2.7, 3.2)	2.6(2.5, 3.1)	2.4(2.3, 2.8)	0.007
Vit. C (µmol/L)	15.7(11.7, 19.3)	18.8(12.9, 20.7)	20.3(14.4, 21.5)	0.008	16.8(13.6, 19.0)	18.1(15.1, 20.5)	18.9(17.2, 22.2)	0.008	13.3(12.8, 18.7)	12.8(11.7, 17.5)	11.3(10.4, 16.6)	0.108
Vit.E*10^6 (µmol/L)	11.5(10.4, 12.7)	11.7(10.9, 12.8)	11.9(10.9, 12.8)	0.007	11.2(9.8, 12.4)	11.5(10.2, 12.4)	11.7(10.8, 12.4)	0.007	9.1(8.1, 10.9)	8.9(8.1, 10.2)	8.3(7.1, 9.7)	0.008
Group 2	**(n=10)**	**(n=9)**	**(n=9)**		**(n=10)**	**(n=9)**	**(n=9)**		**(n=10)**	**(n=8)**	**(n=6)**	
Vit. A (µmol/L)	3.1(3.0, 3.2)	3.2(3.1, 3.4)	3.3(3.2, 3.5)	0.008	3.2(3.0, 3.3)	3.3(3.1, 3.7)	3.4(3.3, 3.7)	0.008	3.1(2.9, 3.2)	2.8(2.7, 3.1)	2.7(2.6, 3.0)	0.028
Vit. C (µmol/L)	17.3(16.3, 19.0)	18.9(17.6, 20.0)	20.6(19.0, 21.1)	0.008	19.3(17.5, 20.1)	21.8(18.3, 22.4)	23.2(19.9, 23.9)	0.008	20.1(19.2, 20.8)	18.6(17.2, 20.1)	16.8(16.5, 19.0)	0.028
Vit.E*10^6 (µmol/L)	12.3(11.8, 13.0)	12.6(12.1, 13.0)	12.6(12.2, 13.4)	0.007	12.3(11.9, 13.5)	13.1(12.8, 13.8)	13.5(12.8, 14.0)	0.007	13.0(12.6, 13.9)	12.7(12.2, 13.3)	12.4(11.9, 12.7)	0.028
Group 3	**(n=10)**	**(n=10)**	**(n=10)**		**(n=10)**	**(n=10)**	**(n=10)**		**(n=10)**	**(n=10)**	**(n=10)**	
Vit. A (µmol/L)	3.8(3.7, 4.1)	3.9(3.8, 4.3)	4.2(3.9, 4.5)	0.005	3.9(3.6, 4.1)	4.2(3.8, 4.4)	4.3(3.8, 4.9)	0.005	3.6(3.4, 4.1)	3.4(3.2, 3.9)	3.3(3.2, 3.8)	0.005
Vit. C (µmol/L)	30.8(26.4, 38.9)	32.2(27.9, 40.1)	33.6(29.4, 41.9)	0.005	29.0(27.1, 52.0)	30.1(28.7, 54.5)	32.3(30.4, 56.1)	0.005	31.3(28.4, 56.1)	29.7(27.0, 55.5)	27.8(25.7, 56.2)	0.012
Vit.E*10^6 (µmol/L)	14.6(13.7, 16.2)	14.8(14.1, 16.2)	14.9(14.2, 16.3)	0.282	15.5(15.0, 18.0)	15.9(15.0, 18.3)	16.3(15.1, 18.3)	0.005	16.2(13.3, 17.6)	15.9(13.3, 17.6)	15.8(12.8, 17.4)	0.005
Group 4	**(n=10)**	**(n=9)**	**(n=8)**		**(n=10)**	**(n=10)**	**(n=10)**		**(n=10)**	**(n=10)**	**(n=9)**	
Vit. A (µmol/L)	4.3(3.9, 4.6)	4.3(4.0, 4.6)	4.5(4.1, 4.6)	0.012	4.1(4.0, 4.5)	4.4(4.1, 4.6)	4.5(4.2, 4.9)	0.005	4.3(4.0, 4.5)	4.1(3.8, 4.4)	4.1(3.7, 4.3)	0.008
Vit. C (µmol/L)	56.4(52.5, 59.3)	58.2(55.2, 62.3)	60.1(59.1, 63.8)	0.012	55.3(53.6, 57.5)	57.5(55.8, 60.3)	59.0(57.1, 62.7)	0.005	57.3(56.2, 59.5)	56.8(54.6, 58.8)	56.2(54.2, 58.2)	0.767
Vit.E*10^6 (µmol/L)	19.2(18.6 27.9)	19.2(17.7, 20.1)	20.0(18.7, 22.7)	0.012	18.8(18.0, 26.3)	19.6(18.2, 26.3)	20.2(18.4, 26.3)	0.005	17.9(16.9, 19.0)	17.6(16.9, 18.8)	16.9(16.9, 18.8)	0.028

Significant at $p<0.05$;
n=sample size;
p-value=Baseline compared with 8 weeks;
Med(IQR)=median(interquartile range);
Vit. A: vitamin A;
Vit. C: vitamin C;
Vit. E: vitamin E

Table 5:
8 weeks supplementation comparison of antioxidant metals in all Groups supplemented with V. amygdalina – 250 mg VA, 500 mg VA and no supplementation (NS) subgroups

	250 mg VA				500 mg VA				NS			
Parameters	**Baseline Med(IQR)**	**4 weeks Med(IQR)**	**8 weeks Med(IQR)**	**P-value**	**Baseline Med(IQR)**	**4 weeks Med(IQR)**	**8 weeks Med(IQR)**	**P-value**	**Baseline Med(IQR)**	**4 weeks Med(IQR)**	**8 weeks Med(IQR)**	**P-value**
Group 1	**(n=10)**	**(n=9)**	**(n=9)**		**(n=10)**	**(n=10)**	**(n=9)**		**(n=10)**	**(n=10)**	**(n=9)**	
Mn (nmol/L)	131.0(106.4, 150.5)	138.1(104.9, 154.9)	139.2(106.5, 156.2)	0.008	131.5(110.1, 158.1)	132.9(111.2, 159.9)	139.2(118.1, 161.5)	0.008	117.9(112.3, 120.5)	116.7(110.9, 120.4)	115.9(111.2, 120.8)	0.015
Zn (µmol/L)	12.5(11.1, 15.0)	13.2(11.6, 15.6)	13.6(11.9, 16.3)	0.008	12.4(10.4, 15.0)	13.1(10.7, 16.2)	13.6(11.6, 16.8)	0.008	10.7(8.9, 11.1)	10.3(8.0, 10.6)	9.9(7.7, 10.7)	0.011
Se (µmol/L)	0.018(0.014, 0.027)	0.019(0.016, 0.029)	0.021(0.017, 0.031)	0.008	0.019(0.015, 0.022)	0.020(0.016, 0.024)	0.026(0.016, 0.030)	0.008	0.018(0.016, 0.023)	0.016(0.013, 0.019)	0.014(0.012, 0.019)	0.008
Group 2	**(n=10)**	**(n=9)**	**(n=9)**		**(n=10)**	**(n=9)**	**(n=9)**		**(n=10)**	**(n=8)**	**(n=6)**	
Mn (nmol/L)	142.7(140.2, 156.2)	144.8(141.9, 159.6)	145.8(142.8, 160.8)	0.008	138.6(131.9, 150.4)	142.3(133.8, 154.2)	143.4(135.2, 155.2)	0.008	163.3(158.1, 179.2)	159.0(152.6, 174.2)	155.3(145.2, 162.1)	0.028
Zn (µmol/L)	15.1(14.6, 15.4)	15.6(15.0, 16.1)	16.2(15.6, 16.7)	0.008	15.5(15.1, 15.7)	16.2(15.6, 16.9)	17.0(16.3, 17.8)	0.008	15.4(15.2, 15.4)	14.9(14.7, 15.0)	14.6(14.3, 14.6)	0.028
Se (µmol/L)	0.025(0.023, 0.028)	0.027(0.025, 0.031)	0.029(0.026, 0.031)	0.008	0.027(0.024, 0.030)	0.029(0.028, 0.032)	0.030(0.029, 0.033)	0.007	0.029(0.027, 0.035)	0.028(0.024, 0.033)	0.026(0.022, 0.031)	0.027
Group 3	**(n=10)**	**(n=10)**	**(n=10)**		**(n=10)**	**(n=10)**	**(n=10)**		**(n=10)**	**(n=10)**	**(n=10)**	
Mn (nmol/L)	181.8(147.7, 203.3)	182.7(149.1, 205.1)	184.3(150.4, 206.5)	0.005	188.4(179.3, 229.5)	189.6(180.0, 231.0)	190.7(181.3, 232.3)	0.005	187.2(164.1, 205.0)	186.6(162.3, 203.3)	185.9(161.8, 203.0)	0.005
Zn (µmol/L)	13.0(11.7, 14.4)	13.3(12.3, 14.9)	13.7(12.6, 15.4)	0.005	13.6(12.4, 15.4)	14.2(12.8, 16.3)	14.5(13.2, 17.0)	0.005	13.3(11.0, 14.7)	13.1(10.5, 14.3)	12.4(9.9, 14.2)	0.007
Se (µmol/L)	0.030(0.029, 0.035)	0.032(0.030, 0.043)	0.035(0.030, 0.046)	0.005	0.031(0.026, 0.043)	0.032(0.029, 0.046)	0.034(0.030, 0.047)	0.005	0.035(0.028, 0.041)	0.031(0.027, 0.038)	0.030(0.026, 0.037)	0.005
Group 4	**(n=10)**	**(n=9)**	**(n=8)**		**(n=10)**	**(n=10)**	**(n=10)**		**(n=10)**	**(n=10)**	**(n=9)**	
Mn (nmol/L)	232.9(216.2, 313.6)	239.1(220.8, 316.9)	240.5(230.1, 313.1)	0.012	260.0(217.7, 309.3)	261.9(218.9, 313.2)	263.1(220.0, 314.5)	0.005	243.6(210.6, 277.2)	232.2(209.6, 275.1)	238.9(202.3, 258.7)	0.008
Zn (µmol/L)	16.1(15.3, 16.3)	16.9(15.8, 17.1)	17.2(16.4, 17.9)	0.012	16.3(15.3, 17.7)	17.5(16.3, 19.1)	18.0(17.0, 19.9)	0.005	15.9(14.9, 19.4)	15.7(14.8, 19.1)	15.4(14.5, 19.9)	0.021
Se (µmol/L)	0.044(0.040, 0.052)	0.047(0.042, 0.062)	0.047(0.044 0.072)	0.012	0.043(0.039, 0.045)	0.045(0.043, 0.048)	0.046(0.044, 0.053)	0.005	0.041(0.039, 0.047)	0.039(0.038, 0.047)	0.038(0.037, 0.046)	0.011

Significant at $p<0.05$;
n=sample size;
p-value=Baseline compared with 8 weeks;
Med(IQR)=median(interquartile range); Mn: Manganese; Zn: Zinc; Se: Selenium

In Table 5, significant increases were found in antioxidant micronutrients (Mn, Zn, Se) in all the Groups supplemented with 250 mg VA or 500 mg VA. However in the NS subgroups, significant decreases were found in these antioxidant micronutrients across the groups.

At baseline and 8 weeks of supplementation with VA; FPG correlated positively with BMI, HbA1c, WA, TPP, OSI and inversely with TAS, vitamin A, vitamin C, vitamin E, Mn, Zn and Se ($p<0.05$). Similar trend of relationships was observed when TPP and OSI were correlated with these parameters, except for BMI, where inverse associations were observed. However, TAS revealed inverse associations with BMI, HbA1c, WA, TPP, OSI and positive relationships with vitamin A, vitamin C, vitamin E, Mn, Zn and Se ($p<0.05$). Similarly, vitamin A, vitamin C and vitamin E followed similar trend as TAS (Tables 6 and 7).

DISCUSSION

The WHA resolution on traditional medicine, which was adopted in 2009 and updated by WHO in 2013 to support Member States for the decade (2014–2023) were to (a) "Harness the potential contribution of traditional medicine to health, wellness and people-centred health-care"; and (b) "Promote the safe and effective use of traditional medicine by regulating, researching and integrating traditional medicine products, practitioners and practice into health systems where appropriate" (WHO, 2013). In line with these updated goals and the medicinal properties reported in various experimental studies on VA (Yeap et al., 2010; Farombi and Owoeye, 2011; Ngatu et al., 2012; Ezuruike and Prieto, 2014), this study evaluated the use of freeze-dried VA leaves for the management of Type 2 diabetics with or without foot ulcers.

In this study, supplementation with either 250 mg or 500 mg VA does not seem to have any effect on BMI of all the participants as similar BMI were observed from baseline to the 8 weeks of supplementation. The significant decreases found in FPG and HbA1c of Type 2 diabetics with or without foot/leg ulcer in this study further buttresses the various experimental studies that VA possesses antidiabetic properties (Akah et al., 2009; Asante et al., 2016; Okoduwa et al., 2016, 2019). Similarly, using VA as a preventive supplement against development of hyperglycaemia in non-diabetics with or without chronic ulcer may be beneficial as depicted by the findings in FPG and HbA1c of the participants. However, this should be done with caution, so as to avoid hypoglycaemic in these non-diabetic people.

Table 6:
Partial correlation between parameters at baseline

Parameters	r-values	p-values	Parameters	r-values	p-values	Parameters	r-values	p-values
FPG-BMI	0.315*	0.015	TPP-BMI	-0.292*	0.025	Vit A-BMI	-0.276*	0.034
FPG-HbA1c	0.714*	0.000	TPP-HbA1c	0.126	0.343	Vit A-HbA1c	-0.559*	0.000
FPG-WA	0.489*	0.000	TPP-WA	0.127	0.338	Vit A-WA	-0.437*	0.001
FPG-TPP	0.283*	0.030	TPP-TAS	-0.047	0.725	Vit A-Vit C	0.750*	0.000
FPG-TAS	-0.447*	0.000	TPP-OSI	0.340*	0.008	Vit A-Vit E	0.663*	0.000
FPG-OSI	0.352*	0.006	TPP-Vit A	-0.322*	0.013	Vit A-Mn	0.743*	0.000
FPG-Vit A	-0.662*	0.000	TPP-Vit C	-0.277*	0.033	Vit A-Zn	0.296*	0.023
FPG-Vit C	-0.544*	0.000	TPP-Vit E	-0.369*	0.004	Vit A-Se	0.727*	0.000
FPG-Vit E	-0.439*	0.000	TPP-Mn	-0.247	0.060			
FPG-Mn	-0.519*	0.000	TPP-Zn	-0.378*	0.003			
FPG-Zn	-0.216	0.100	TPP-Se	-0.338	0.009	Vit C-BMI	-0.319*	0.014
FPG-Se	-0.485*	0.000				Vit C-HbA1c	-0.532*	0.000
						Vit C-WA	-0.412*	0.001
						Vit C-Vit E	0.773*	0.000
TAS-BMI	0.010	0.942	OSI-BMI	-0.299*	0.021	Vit C-Mn	0.741*	0.000
TAS-HbA1c	-0.082	0.535	OSI-HbA1c	-0.072	0.588	Vit C-Zn	0.520*	0.000
TAS-WA	-0.163	0.217	OSI-WA	0.018	0.895	Vit C-Se	0.815*	0.000
TAS-OSI	-0.565*	0.000	OSI-Vit A	-0.402	0.002			
TAS-Vit A	0.379*	0.003	OSI-Vit C	-0.299*	0.022			
TAS-Vit C	0.387*	0.002	OSI-Vit E	-0.308*	0.018	Vit E-BMI	-0.174	0.187
TAS-Vit E	0.223	0.090	OSI-Mn	-0.294*	0.024	Vit E-HbA1c	-0.429*	0.001
TAS-Mn	0.306*	0.018	OSI-Zn	-0.428*	0.001	Vit E-WA	-0.406*	0.001
TAS-Zn	0.179	0.176	OSI-Se	-0.393*	0.002	Vit E-Mn	0.617*	0.000
TAS-Se	0.270*	0.039				Vit E-Zn	0.460*	0.000
						Vit E-Se	0.647*	0.000

**Significant at p<0.05;*
BMI=Body mass index;
FPG=Fasting plasma glucose;
HbA1c=Glycated hemoglobin A1c;
WA=wound assessment tool;
TPP: Total plasma peroxides;
TAS: Total antioxidant status;
OSI: Oxidative stress index;
Vit. A: vitamin A;
Vit. C: vitamin C;
Vit. E: vitamin E; Mn: Manganese; Zn: Zinc; Se: Selenium

Table 7:
Partial correlation between parameters at 8 weeks of VA supplementation

Parameters	r-values	p-values	Parameters	r-values	p-values	Parameters	r-values	p-values
FPG-BMI	0.177	0.193	TPP-BMI	-0.359*	0.007	Vit A-BMI	-0.164	0.227
FPG-HbA1c	0.657*	0.000	TPP-HbA1c	0.115	0.399	Vit A-HbA1c	-0.554*	0.000
FPG-WA	0.398*	0.002	TPP-WA	0.192	0.157	Vit A-WA	-0.326*	0.014
FPG-TPP	0.459*	0.000	TPP-TAS	-0.126	0.356	Vit A-Vit C	0.699*	0.000
FPG-TAS	-0.334*	0.012	TPP-OSI	0.304*	0.023	Vit A-Vit E	0.656*	0.000
FPG-OSI	0.297*	0.026	TPP-Vit A	-0.300*	0.025	Vit A-Mn	0.692*	0.000
FPG-Vit A	-0.755*	0.000	TPP-Vit C	-0.242	0.072	Vit A-Zn	0.303*	0.023
FPG-Vit C	-0.665*	0.000	TPP-Vit E	-0.318*	0.017	Vit A-Se	0.543*	0.000
FPG-Vit E	-0.552*	0.000	TPP-Mn	-0.202	0.136			
FPG-Mn	-0.661*	0.000	TPP-Zn	-0.374*	0.005			
FPG-Zn	-0.323*	0.015	TPP-Se	-0.244	0.070	Vit C-BMI	-0.223	0.099
FPG-Se	-0.512*	0.000				Vit C-HbA1c	-0.506*	0.000
						Vit C-WA	-0.287*	0.032
						Vit C-Vit E	0.779*	0.000
TAS-BMI	-0.142	0.296	OSI-BMI	-0.136	0.319	Vit C-Mn	0.731*	0.000
TAS-HbA1c	-0.068	0.617	OSI-HbA1c	-0.137	0.312	Vit C-Zn	0.481*	0.000
TAS-WA	-0.157	0.247	OSI-WA	0.115	0.400	Vit C-Se	0.736*	0.000
TAS-OSI	-0.451*	0.000	OSI-Vit A	-0.312*	0.019			
TAS-Vit A	0.325*	0.015	OSI-Vit C	-0.220	0.104			
TAS-Vit C	0.362*	0.006	OSI-Vit E	-0.244	0.070	Vit E-BMI	-0.112	0.412
TAS-Vit E	0.235	0.082	OSI-Mn	-0.225	0.095	Vit E-HbA1c	-0.454*	0.000
TAS-Mn	0.279*	0.037	OSI-Zn	-0.441*	0.001	Vit E-WA	-0.306*	0.022
TAS-Zn	0.121	0.373	OSI-Se	-0.265*	0.048	Vit E-Mn	0.603*	0.000
TAS-Se	0.149	0.274				Vit E-Zn	0.365*	0.006
						Vit E-Se	0.547*	0.000

*Significant at $p<0.05$; BMI=Body mass index; FPG=Fasting plasma glucose; HbA1c=Glycated hemoglobin A1c; WA=wound assessment tool; TPP: Total plasma peroxides; TAS: Total antioxidant status; OSI: Oxidative stress index; Vit. A: vitamin A; Vit. C: vitamin C; Vit. E: vitamin E; Mn: Manganese; Zn: Zinc; Se: Selenium

The NS subgroups had increases in FPG across all the groups. This observed increase in FPG of the diabetics with or without foot/leg ulcer may be due to lack of control of their medication, which may require review by the attending Physicians. Secondly, it may be due to partial or non-compliance with dietary regulation as depicted by the results of HbA1c. Whereas, the observed increase in FPG of the non-diabetic participants with or without foot/leg ulcers may be due to diet. Therefore, it would be recommended that as ageing occur, each individual should be careful of his/her diet, especially diet containing high glycaemic index foods should be reduced, with lot of fruits and vegetables incorporated. Similarly, at least 30 minutes of active exercise twice a week is recommended. Correlation of FPG with HbA1c revealed positive and strong association between these parameters. This suggested that decrease in the levels of FPG concurrently lead to the decrease in levels of HbA1c and therefore indicative of good glycaemic control in these participants.

Similarly in this study, significant decreases in TPP and OSI with improvement in TAS were observed in all the groups supplemented with either 250 or 500 mg VA. These findings agree with the previous studies by Iwalokun et al., (2006), Adaramoye et al., (2008), Farombi and Owoeye (2011), Ekaluo et al., (2015), Omede et al., (2018). These researchers reported the antioxidative properties of VA in animal studies. However in the NS subgroups, significantly elevated levels of oxidative stress biomarkers were observed across all groups. This was depicted by increases in TPP and OSI with concomitant depletion of TAS. These findings were further strengthened by the correlation analysis, where TAS correlated inversely with FPG, HbA1c, TPP and OSI. Thus, indicating that proper glycaemic control resulted in reduction of oxidative stress and concurrent improvement of circulating antioxidants and vice versa.

In addition, this study revealed significant increases in the antioxidant micronutrients including vitamin A, vitamin C and vitamin E, Mn, Zn and Se in all the groups supplemented with 250 mg and 500 mg VA. In the NS subgroups however, there were decreases in these antioxidant micronutrients. This may further explain the reason why the biomarkers of oxidative stress were elevated with depleted antioxidants in the participants of this subgroups. These data on the antioxidant micronutrients were further corroborated with the correlation results; decreases in FPG, HbA1c, TPP or OSI levels resulted in increases in levels of these micronutrients. The findings of this study can be supported by previous studies, where VA were reported to be rich in vitamins and minerals (Oboh, 2006; Eyong et al., 2011; Aregheore, 2012; Agbogidi and Akpomorine, 2013; Shewo and Girma, 2017: Alara et al., 2018).

Diabetes mellitus and oxidative stress have been identified to interfere with wound healing (Guo and DiPietro, 2010). Therefore, proper glycaemic control and reduction of oxidative stress in diabetics with foot/leg ulcer is essential to attain wound healing. In this study, wound healing in Type 2 diabetics with foot/leg ulcer and non-diabetics with chronic foot/leg ulcer supplemented with 250 or 500 mg VA were observed to improve significantly. Whereas in the NS subgroups, no improvement in healing of the wound was observed as shown by the modified wound assessment tool. The observed improvement in foot/leg ulcers may be due to the decrease in glycation of cellular proteins involved in wound healing and higher antioxidative capacity observed in

diabetic foot ulcer and non-diabetic with chronic ulcer patients supplemented with VA. This finding can be buttressed further with the correlation results, where FPG positively and TAS inversely correlated with the wound assessment tool (WA). This finding suggested that decrease in levels of FPG resulted in improvement of TAS and corresponding decrease in the wound assessment tool, thus, facilitating wound healing process.

The main limitation of this study was the use of small sample size. We, therefore, recommend a further clinical study with *V. amygdalina* using a larger sample size in order to validate the antidiabetic and antioxidative properties of this herb in human.

In conclusion, the findings of this study revealed that supplementation with 250 mg/kg or especially, 500 mg/kg body weight of freeze-dried powder of *V. amygdalina* leaves could attenuate hyperglycaemia, oxidative stress and improve antioxidant defence system in type 2 diabetics with or without foot/leg ulcer as well as in non-diabetics with or without chronic ulcers. Furthermore, supplementation of V. amygdalina improved wound healing in type 2 diabetics with foot/leg ulcer as well as in non-diabetics with chronic ulcers. This study further buttresses the animal studies, where V. amygdalina were reported to possess both the antidiabetic and antioxidative properties. Therefore, *V. amygdalina*, as either therapeutic or preventive measure, could be used as a potential source of natural antioxidant and antidiabetic agents. Most house-holds in Nigeria consume V. amygdalina (bitter leaf) as vegetable soup, and sometimes drink its extracts for prophylactic purposes; however, premised on the hypoglycaemic effect observed in this study, it will therefore be recommended that caution should be taken when using this herb for prophylactic purposes in non-diabetics to prevent occurrence of hypoglycaemia.

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