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Original Research Article

Phytochemical Components of Butanol Fraction of *Vernonia amygdalina* Leaf Extract Using GC-MS Analysis

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Abstract

Earlier studies have shown the butanol fraction of *Vernonia amygdalina* to be ypoglycaemic, hepatoprotective, and to ameliorate diabetes induced decreases in the level of male hormone and testicular damage. The aim of this study was to evaluate the phytochemical composition of the butanol fraction of *V.amygdalina* leaf extract using Gas Chromatography-Mass spectroscopy (GC-MS) with a view to unravelling the phytochemical constituents responsible for these antidiabetic activities. The GC-MS analysis of the butanol fraction revealed the presence of 18 major important bioactive compounds namely: 3, 5-bis 1, 1 dimethylethyl Phenol, Tetradecanoic acid, 1, 2-epoxyhexadecane (Oxirane), Methylhexadecanoate (Palmitic acid), Hexadecanoic acid (Eicosanoic acid), 9, 12-octadecadienoic acid (Linoleic acid), 3, 7-dimethyldodecan-1-ol (Phytol), 6-octadecenoic acid (Oleic acid), octadecanoic acid (Stearic acid), Cholest-5, 3-ol, 5-acetate (Cholestane), 1,2-Benzenedicarboxylic acid (Di-n-octyl phthalate), Hexadecanoic acid butyl ester, 16-Hexadecanoyl hydrazide, Decane, 1-fluoro- Decyl fluoride 1-(Fluorodecane),9,12-Octadecadien-1-ol, 9,12,15-Octadecatrien-1-ol, Di-n-octyl phthalate 1,2-(Benzenedicarboxylic acid, dioctyl ester), 2,6,10,14,18,22-Tetracosahexaene (or 2,6,10,15,19,23-hexamethyl [squalene]). Of the 18 phytochemical constituents, 14 are known to have a bearing on diabetes.

Keywords: *Vernonia amygdalina,* Phytochemicals, Butanol fraction, GC-MS Analysis.

INTRODUCTION

Plant materials play major roles in primary healthcare as therapeutic remedies in developing countries (Gagliano *et al*., 2007). One of those ailments for which plants are continuously being scrutinized for its remedy is diabetes mellitus (Tiwari and Rao, 2002). Diabetes mellitus has been ranked seventh among the leading causes of death and is considered third when its fatal complication are taken into account (Trivedi *et al*., 2004). Diabetes mellitus is a metabolic disorder resulting from defects in insulin secretion or decreased sensitivity of tissues to insulin (ADA, 2005). It is characterised by hyperglycaemia which when persistent can lead to increased production of free radicals which may ultimately result in destruction of some vital body organs like the kidney, liver, pancreas and testis and in vascular complications (Bonnefont-Rousselot *et al*., 2000; Moussa, 2008; Mgbeje *et al*., 2019a). Available synthetic remedies, apart from its high cost, have been shown to have some undesirable side effects. This has led to a search for more effective and less expensive medicinal plant alternatives. Available literature shows that there are more than 800 plant species showing anti-diabetic potential (Maton *et al*., 1993, Alarcon-Aguilera *et al*., 1998; Mgbeje *et al*., 2016a). One of these plants that have been used in ethno medicine for the treatment of diabetes is *Vernonia amygdalina* (Iwu, 1993; Nwanjo and Nwokoro, 2005;

Ojiako and Nwanjo, 2006). Its antidiabetic credential has been validated in our laboratory and elsewhere (Nwanjo and Nwokoro, 2005; Ojiako and Nwanjo, 2006; Atangwho *et al*., 2007; Ebong *et al*., 2014; Ugoanyanwu *et al*., 2015; Mgbeje *et al*., 2016a,b).

As a step towards determining the active principles of *Vernonia amydalina* involved in reversal of diabetes, and ultimately the standardization of the plant preparation in diabetes therapy, the plants extract was fractionated by sequential elution with butanol, 30%, 50% and 100% methanol (Ugoanyanwu *et al*., 2015; Mgbeje *et al*., 2016a,b). While the Flavonoid-rich 30% methanol fraction showed a better reversal of diabetes induced pancreatic cell damage (Ugoanyanwu *et al*., 2015), both 30% methanol and saponin-rich butanol fraction showed a better ameliorative effect on the diabetes induced decreases in male sex hormones and testicular damage (Mgbeje *et al*., 2016a). Of all the fractions, the saponin-rich butanol fraction on balance showed a better amelioration of diabetes induced hepatotoxicity comparable to the crude extract suggesting that it could effectively replace the crude extract in the amelioration of hepatotoxicity (Mgbeje *et al*., 2016b). Furthermore, it will be more amenable to standardization than the crude plant extract. As well as being more amenable to standardization compared to the crude extract, the fractionation is a first step towards elucidation of the specific active principle(s) involved in amelioration of diabetes induced hepatotoxicity (Mgbeje *et al*., 2016b). It is in a quest to further narrow down the active principle(s) responsible for its antidiabetic properties that the current work to elucidate the phytochemical content of the butanol fraction of *Vernonia amydalina*, using Gas Chromatography-Mass Spectroscopy (GC-MS) was carried out.

Vernonia amygdalina DEL (VA) commonly called bitter leaf belongs to the family *Asteraceae*. It is a perennial shrub with green leaves with a characteristic odour and bitter taste. It is distributed in tropical Africa and Asia where there are commonly found along drainage lines and natural forest or commercial plantation (Nwanjo and Nwokoro, 2005). It is widely used for nutritional and therapeutic purposes (Iwu, 1993; Igile *et al*., 1994; Okafor, 2005). Apart from its use as an anti-diabetic remedy, it is also used in traditional medicine as an anti-malaria, purgative, anti-parasitic and anti-helminthic, and also in the treatment of wounds (Nwanjo and Nwokoro, 2005). It is a rich source of phytochemicals, mineral and vitamins (Okafor 2005; Mgbeje *et al*., 2019b,c).

MATERIALS AND METHODS

Collection of leaf samples

The fresh leaves of *V.amygdalina* were harvested from the University Farm, University of Calabar, Cross River State, Nigeria. The plant sample was identified at the taxonomy section, Botany Department of the University and Voucher specimen (No. BCH 10013) was deposited at the Herbarium of the University.

Preparation of Plant Extract

V. amygdalina leaves were collected, washed, and air dried on the laboratory bench for 10 days. The dried leaves were ground into coarse powder using a manual blender. The Powdered leaves weighing about 2kg were soaked in 8 litres of methanol for 48hrs as previously described (Ugoanyanwu *et al*., 2015). The extract was filtered using a cheese cloth and Whatman No.1 filter paper. The filtrate was concentrated using a rotary evaporator at 45^{o}C and the residual solvent removed by placing in a water bath at 45-50^{o}C. The crude extract obtained (25g) (1.25% yield) was stored at 4^{o}C for further analysis.

Fractionation of crude extract

The crude extract was fractionated by means of column chromatography using silica gel (60-200 mesh size) as stationary phase with butanol as the mobile phase. Fractions were collected and concentrated using a rotary evaporator at 45^{o}C.

Gas Chromatography- Mass Spectrometry (GC-MS) analysis

The characterization of the Phytochemicals in the butanol fraction of *V. Amygdalina* was done using a QP2010 Gas chromatography with Thermal Desorption System, TDS 20 coupled with Mass Spectroscopy (Shimadzu, Japan) as previously described (Mgbeje *et al*., 2016c). The GC was fitted with a Restek column (0.25 mm, 60 m, XTI-5). Helium was the carrier gas at a flow rate of 1.2ml min. The MS operating conditions were: ionization voltage 70ev, ion source temperature 230°C. The identification of the components was accomplished by comparison of the retention indices, fragmentation pattern and mass spectra with spectrum of known components stored in the database of National Institute of Standard and Technology (NIST) library (http://chemdata.nist.gov).

RESULTS AND DISCUSSION

The GC-MS chromatogram of the butanol fraction of *V.amygdalina* is shown in Figure 1. Retention times, % of total area, molecular weight, and names of compounds identified from GC-MS analysis of butanol fraction of

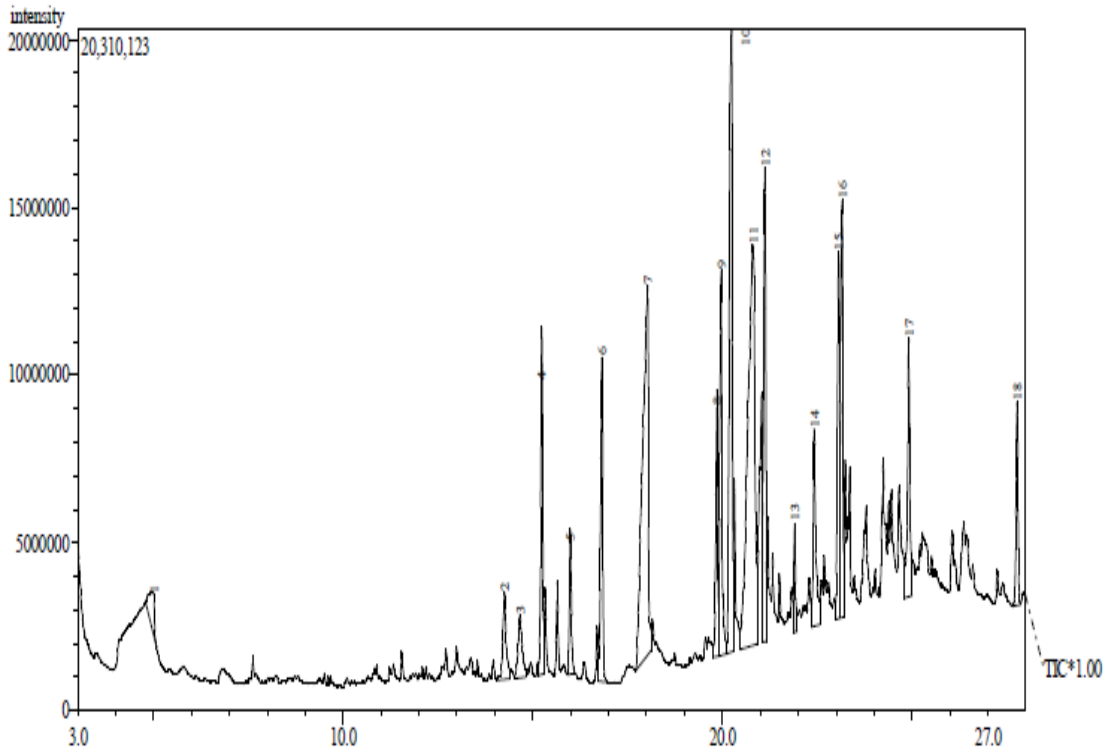

Figure 1. Chromatogram showing the different peaks (eighteen phyto-components) in the butanol fraction of *V.amygdalina.*

Table 1. Molecular weight, Retention time and names of compounds (eighteen Phyto-Components) identified from GC-MS analysis of butanol fraction of *V. amygdalina*

S/N	Retention Time (RT)	Area%	Molecular Weight	Name of Compound
1.	14.242	1.34	206	3, 5-bis 1, 1 dimethylethyl Phenol
2.	4.952	1.95	228	Tetradecanoic acid
3.	14.258	1.73	240	1, 2-epoxyhexadecane(Oxirane)
4.	14.665	4.54	270	Methylhexadecanoate (Palmitic acid)
5.	15.239	1.88	242	Hexadecanoic acid (Eicosanoic acid)
6.	15.993	4.84	294	9, 12-octadecadienoic acid (Linoleic acid)
7.	16.829	14.41	296	3, 7-dimethyldodecan-1-ol (Phytol)
8.	18.032	3.44	282	6-octadecenoic acid(Oleic acid)
9.	19.858	5.55	284	octadecanoic acid(Stearic acid)
10.	19.965	12.49	386	Cholest-5, 3-ol, 5-acetate (Cholestane)
11.	20.244	19.06	390	1,2-Benzenedicarboxylic acid (Di-n-octyl phthalate)
12	20.805	6.28	312	Hexadecanoic acid, butyl ester
13	21.125	1.36	270	16-Hexadecanoyl hydrazide
14	21.907	3.32	160	Decane, 1-fluoro- Decyl fluoride (1-Fluorodecane)
15	22.424	5.27	266	9,12-Octadecadien-1-ol,
16	23.067	5.89	264	9,12,15-Octadecatrien-1-ol,
17	23.158	4.02	390	Di-n-octylphthalat 1,2-(Benzenedicarboxylic acid, dioctyl ester)
18	24.915	2.63	410	2,6,10,14,18,22-Tetracosahexaene, 2,6,10,15,19,23-hexamethyl (squalene)

V.amygdalina is shown in Table 1. The chromatogram revealed the presences of Eighteen (18) phytochemical compounds namely: 3,5-bis 1,1 dimethylethyl Phenol, Tetradecanoic acid, 1, 2-epoxyhexadecane (Oxirane),

Methylhexadecanoate (Palmitic acid), Hexadecanoic acid (Eicosanoic acid), 9, 12-octadecadienoic acid (Linoleic acid), 3, 7-dimethyldodecan-1-ol (Phytol), 6-octadecenoic acid(Oleic acid), octadecanoic acid (Stearic acid), Cholest-5, 3-ol, 5-acetate (Cholestane), 1,2-Benzene-dicarboxylic acid (Di-n-octyl phthalate), Hexadecanoic acid butyl ester,16-Hexadecanoyl hydrazide, Decane, 1-fluoro- Decyl fluoride (1-Fluorodecane), 9,12-Octadecadien-1-ol; 9,12,15-Octadecatrien-1-ol; Di-n-octyl phthalate (1,2-Benzenedicarboxylic acid, dioctyl ester), 2,6,10,14,18,22-Tetracosahexaene (2,6,10,15,19,23-hex-amethyl [squalene]).1,2-Benzenedicarboxylic acid (Di-n-octyl phthalate) was the most abundant compound (19.06%) and 3, 5-bis 1, 1 dimethylethyl Phenol the least abundant (1.34%).

Similar GC-MS analysis have been carried out on *V. amygdalina* after hydro distillation (Ogunbinua *et al*., 2009; Adeoye *et al*., 2018), Soxhlet and microwave assisted extraction (using ethanol as solvent) (Alara *et al*., 2019) , extraction with 10% NaOH (Oladunmoye *et al*., 2019) and on fractions of a gradient solvent system comprising of petroleum ether, chloroform and methanol (after ethanol extraction) (Igwe *et al*., 2015) but this is the first report on a butanol fraction of a methanolic extract. Worthy of note is that the 11 phytocomponents of Fraction F5 of the gradient fraction system of Igwe *et al*., 2015, which synergistically caused contraction in the mammary tissue, corresponded to the first 11 phytocomponents of the 18 components of the butanol fraction in this study.

Of the 18 Phytocomponents in the butanol fraction, 14 are known to be of relevance to diabetes and may account for the fraction's hypoglycemic (Ugoanyanwu *et al*., 2015), ameliorative effect on the diabetes induced decreases in male sex and testicular damage (Mgbeje *et al*., 2016a,) and hepatoprotection (Mgbeje *et al*., 2016b). These 14 components will be discussed further only as they affect diabetes.

3, 5-bis 1, 1 dimethylethyl Phenol (3,5-Di-tert-butylphenol), an unsaturated cyclic compound, is related to Phenol-2,4-Bis(1,1-Dimethylethyl)- and Cyclotrisiloxane, Hexamethyl which have been shown to play a major role in the free radical scavenging (Prakash and Suneetha, 2014). The degree of unsaturation in these cyclic compounds are greater than that of the enediol grouping (-COH=COH-) present in L–Ascorbic acid, making them better antioxidant agents than ascorbic acid. *Tetradecanoic acid* also called Myristic acid, is a common saturated fatty acid. It prevented testosterone induced prostatic hyperplasia in rats (Veeresh *et al*., 2010) and significantly increased DGKδ2 protein expression in mouse C2C12 myotubes (Wada *et al*., 2016). Decreased expression of diacylglycerol kinase (DGK) δ in skeletal muscles attenuates glucose uptake and is closely related to the pathogenesis of type 2 diabetes. Therefore, up-regulation of DGKδ expression is thought to protect and improve glucose homoeostasis in type 2 diabetes.*1, 2-epoxyhexadecane (Oxirane)*, as it relates to diabetes, is known to have anti-inflammatory properties and reduces liver damage (Igwe *et al*., 2015). This may play a crucial role in the hepatoprotection ability of the butanol fraction.

Methylhexadecanoate (methyl Palmitate) has been shown to inhibit galactosamine-induced alterations in serum glucose concentration (as well as glutamic oxalacetic transaminase and lactic dehydrogenase activities, and sodium sulfobromophthalein retention) and to reduce Liver necrosis and inflammation in galactosamine-injected animals (Al-Tuwaijri *et al*., 1981). It also possesses a strong anti-fibrogenic (Rodríguez-Rivera *et al*., 2008; El-Demerdash, 2011; Mantawy *et al*., 2012) and anti-inflammatory (El-Demerdash, 2011) effect. The antifibrotic effect of Methyl palmitate is probably associated with its ability to reduce TGF-beta content, maybe by immunomodulation of Kupffer cells functioning (Rodríguez-Rivera *et al*., 2008) or through NF-kB inhibition (El-Demerdash, 2011) or may be partly a consequence of its antioxidant and anti-inflammatory properties (Mantawy *et al*., 2012). Inflammatory and fibrogenic markers have been shown to have a role in diabetic nephropathy and it has been suggested that they could be used as biochemical markers in diabetic nephropathy (El Mesallamy *et al*., 2012).

Hexadecanoic acid, ethyl ester has antioxidant, Hypocholesterolemic, anti-androgenic, activities (Siva-gurunathan and Xavier Innocent, 2014). Similarly, *9,12-Octadecadienoic acid (linoleic acid)* has, among other attributes, been shown to have hypocholesterolemic, hepatoprotective and anti-androgenic activities (Siva-gurunathan and Xavier Innocent, 2014). *3, 7-dimethyldodecan-1-ol (Phytol)* is important in the processing of glucose and can activate enzymes within the body that have strong positive effects on insulin level implying that phytol in the human diet could possibly help restore the metabolic functions of those with type-2 diabetes (Venkata Raman *et al*., 2012). Phytanic acid (PA) is a chorophyll metabolite from phytol with potentials in regulating glucose metabolism by regulating hepatic glucose homeostasis (Che *et al*., 2013). *6-octadecenoic acid (Oleic acid)* is classified as a monounsaturated omega-9 fatty acid. It has been associated with decreased low density lipoprotein (LDL) cholesterol, and possibly increased high density lipoprotein (HDL) cholesterol (Merck and Co., 2019). *Octadecanoic acid (Stearic acid)*, as it concerns diabetes, has been shown to lower LDL cholesterol, was neutral with respect to HDL cholesterol, and lowered the ratio of total to HDL cholesterol (Hunter *et al*., 2010). Dietary stearic acid leads to dramatically reduced visceral fat possibly by causing the apoptosis of pre-adipocytes as well as lowering blood glucose and leptin concentrations (Shen *et al*., 2014). It is likely that reducing excess visceral fat may also be very beneficial for type 2 diabetes, the metabolic syndrome, cardiovascular disease and possibly

other disease states. Stearic acid also has beneficial effects on thrombogenic and atherogenic risk factors in males (Kelly *et al*., 2001) and has been shown to reduce metastatic tumor burden (Evans *et al*., 2009).

Cholest-5, 3-ol, 5-acetate (Cholestane) is a precursor for various classes of steroid hormones in plants. It binds estrogenα (ERα) and Estrogenβ (ERβ) receptor sites (Igwe et al., 2015). Cholestane is a Phytosterols (plant sterols). Phytosterols have been shown in clinical trials to block cholesterol absorption sites in the human intestine, thus helping to reduce cholesterol absorption in humans (Ostlund *et al*., 2003). *Hexadecanoic acid, butyl ester* has anti-androgenic, Antioxidant, Hypocholestrolemic properties (Igwe at al., 2015). Similarly, *9,12-Octadecadien-1-ol* has hypocholesterolemic, hepato-protective properties (Kalaivani *et al*., 2012; Padmashree *et al*., 2018). *9,12,15-Octadecatrien-1-ol* has been shown to be anti-inflammatory and hypocholesterolemic (Srinivasan *et al*., 2014; Padmashree *et al*., 2018) while *2,6,10,14,18,22-Tetracosahexaene, (2,6,10,15,19,23-hexamethyl [squalene])*, among other attributes, has anti-inflammatory and anti-atherosclerotic activities (Venkata *et al*., 2012; Sivakumar and Gayathri 2015).

CONCLUSION

In summary, earlier work has shown that the butanol fraction of *Vernonia amygdalina* to ameliorate the diabetes induced liver damage comparable to the crude plant extract. This is in addition to its hypoglycemic property and its reversal of diabetes induced reduction in levels of male sex hormones and testicular damage. GC-MS analysis of the butanol fraction revealed 18 phyto-constituents, 14 of which had some direct bearing on diabetes. Of direct relevance to it hepatoprotection was *1, 2-epoxyhexadecane (Oxirane)*, *Methylhexadecanoate (methyl Palmitate)* and *9,12-Octadecadien-1-ol*. This work represents a step towards better standardizing the plant preparation for presentation in diabetes therapy. It also is a step towards isolating the active diabetic principles in the plant.

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