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Free Radical Scavenging Activity of Non Saponifiable Lipids from Dieffenbachia picta (Lodd.) Schott. Leaves and Identification of a Lanostane Triterpenoid

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Abstract
Dieffenbachia picta (Lodd.) Schott, mostly used as ornamental plant, has not been thoroughly investigated for its medicinal properties due largely to the presence of oxalic acid deposited as crystals of calcium oxalate on parts of the plant especially the leaves. The present study however was aimed at providing an *in-vitro* evidence for the potential radical inhibitory activity of isolates obtained from n - hexane fraction of *D. picta* leaf found to have antioxidant properties in our previous study. Isolation of compounds was achieved by gradient elution column chromatographic technique. Ultra Violet/visible (UV), Infrared (IR) and Nuclear Magnetic Resonance (NMR-1D and 2D) spectroscopic techniques were used for structural elucidation. Molecular mass was ascertained by low and high resolution Electron Impact (EI/EI-HR) mass spectrometry. Gas chromatography (GC) and gas – chromatography mass spectrometry (GC-MS) were used to characterize the lipid component. Free radical scavenging activity of the isolates was assessed using 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) method and butylated hydroxylanisole (BHA) was used as standard. Structures were proposed for the non saponifiable lipids as Tetradecyloctadecyl behenate (DP1) and 1~chlorotritriacontane (DP2) while phytol (31.44%), isopropyl palmitate (14.91%), isopropyl stearate (8.61%) were the major constituents of the oil (DP3). A lanostane triterpenoid 11β, 19-epoxy-Lanostan-3β-ol was also identified in the oil. DP1 and DP3 exhibited significant antioxidant activity with IC_{50} (μM) values of 43.5± 0.53 and 25.43±0.06 respectively comparable to the standard. The results of the present study indicated that *D. picta* is rich in antioxidant metabolites despite the presence of toxic calcium oxalate crystals.
Keywords: Tetradecyloctadecyl behenate; phytol; 11β, 19-epoxy-Lanostan-3β-ol; 2, 2-diphenyl-1-picrylhydrazyl radical; *Dieffenbachia picta*

1. Introduction

Dieffenbachia picta Schott (Dumb cane) of the family Araceae is a herbaceous plant with various traditional claims ranging from antimicrobial, antioxidant, and anti rheumatism in traditional medicine. It is native to tropical America and the West Indies. In Nigeria, it is used as an ornamental plant. The plant has been used as ornamental plant since the 1820s (Simson and Ogorzaly 1995; Oloyede et al., 2011, Li et al., 2014). It is also rarely cultivated as food plant and medicine (Johnson, 1995; Keating, 2004). Our previous studies however showed that extracts and essential oils obtained from leaves and stem of *D. picta* have antioxidant and antimicrobial properties. Phytochemical screening also confirmed the presence of alkaloids, flavonoids, sugars, resins, phenolics and reducing sugar (Oloyede et al, 2011 and 2012). Brine shrimp toxicity assay showed that the plant is toxic to brine shrimp larvae eggs (Oloyede et al, 2012) thus confirming early reports about the toxicity of *D. picta* and other plants in the Araceae family (Frausin et al., 2015). *Dieffenbachia* plant especially the leaf contain needle-shaped calcium oxalate crystals called raphides which when chewed, can cause a temporary burning sensation (Walter and Rhanna 1971; Fronhne, and Pfander 1984). Despite the fact that this plant is very poisonous when ingested; it is not devoid of medicinal properties. In the Philippines, Dumb cane is a focus of research because of its antiangiogenic content that could be used in fighting cancer cell (Edmon, 2017). Therefore, based on results obtained from our previous research activities that *D. picta* essential oil and crude extracts have inherent medicinal properties, we were prompted to carry out bioassay guided isolation on this plant. The hexane fraction being the most active (Oloyede et al, 2012) was subjected to free radical scavenging activity. Antioxidants have been known to ameliorate free radical generated diseases. Oxidative stress often results from overproduction of reactive oxygen species arising either from mitochondrial electron transport chain or excessive stimulation of NAD(P)H oxidase thereby causing deleterious conditions which can lead to damage to cell structures, lipids and membranes, proteins and DNA (Albano et al., 2012; Shaiq et al., 2013; Lone et al., 2013; Onocha et al., 2016). Therefore search for antioxidants that will combat degenerative diseases is a very important research area.

In view of the above, this present work was projected to further evaluate and report the chemical components responsible for the free radical scavenging properties of *D. picta* n-hexane fraction. This was

achieved by carrying out gradient elution column chromatography. Compounds were characterized by 1D and 2D NMR and Electron Impact (EI/EI-HR) mass spectrometry. GC and GC-MS was used to characterize other lipid component. The free radical scavenging activity of the isolates was assessed using 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) method and butylated hydroxylanisole was used as antioxidant standard.

2. Materials and Methods

2.1 Chemicals and Reagents

The following BDH chemicals and reagents: chloroform, dichloromethane, ethyl acetate, n-hexane, methanol, hydrochloric acid, ammonia solution, conc. tetraoxosulphate (VI) acid, conc. hydrochloric acid, ammonia solution, sodium potassium tartarate, potassium chloride, glacial acetic acid, were used. Those that are general purpose chemicals were distilled prior to use. Dimethylsulphoxide (M&B, England), and silica gel 30 - 260 microns (Merck, Germany) and 2, 2 - diphenyl-1-picrylhydrazyl (DPPH), and butylatedhydroxylanisole (BHA) were obtained from Sigma Chemical Co (St Louis, MO).

2.2 Plant Material

N-hexane fraction of the leave extract of *D. picta* (73 g) found to have antioxidant activity in our previous study (Oloyede et al., 2012) was used for this study. Details of collection, extraction and fractionation of samples have been described in our previous article (Oloyede et al., 2012). We however describe the isolation technique used in this present study. Notably, during extraction, approximately 60% of white crystalline deposits were obtained which were identified as oxalic acid deposited as crystals of calcium oxalate. This is said to be true to all in plants of the Araceae family and is responsible for the toxicity of some genera (*e.g. Dieffenbachia* Schott) (Frausin,et al, 2015).

2.3 Isolation Procedure

The hexane soluble fraction (73 g) was adsorbed in silica gel and subjected to gradient elution column chromatography using silica gel 230 – 400 mesh size and *n*-hexane, ethylacetate (EtOAc), and methanol MeOH as eluent in order of increasing polarity. 25 sub fractions were obtained. The sub fractions 1-9 obtained from 100% hexane afforded compound **DP1** (15 mg) which is white crystal. Factions 10-16 gave compound **DP2** (19 mg) which was obtained from 95% hexane and 5% ethylacetate (EtOAc). It was further recrystallized with EtOAc to give white crystalline substance. Other fractions obtained as yellow oil were pooled together after Thin layer chromatography (TLC) showed similar profile using EtOAc : hexane (2:1) as eluent and silica gel F_{254} precoated TLC plates as adsorbent. This was re-chromatographed in a micro column and preparative TLC using same system used for TLC, EtOAc:hexane (2:1) and other systems (EtOAc: hexane (2:0.5), EtOAc:hexane 1:1, and 100% dichloromethane). There was no further separation in the oily substance. This mixture was therefore subjected to Gas chromatography and Gas Chromatography-Mass spectrometry (GC and GC-MS). This is compound **DP3 (**21 mg).

2.4 General experimental procedures

Analytical balance KERN ALS 220-4 KERN & SOHN, Germany was used for all weighing. Precoated TLC Aluminium sheets Silica gel 60 F_{254} ((20 cm × 20 cm, 0.2 mm thick; Merck, Germany) for analytical thin layer chromatography and Alugram Sil G/UV_{254} for preparative chromatography (Macherey-Nagel MN, Germany). Silica gel (230–400 mesh) was used for column chromatography (CC). Visualization of the TLC plates was carried out under UV at 254 and 366 nm and by spraying with ceric sulfate reagent solution (1g in 10% H_2SO_4 with a dilution factor of 2 mL stock and 8 mL distilled water) with heating. Vanillin sulphuric acid (1 g and 1 mL 95% sulphuric acid in 250 mL ethanol) was also used. Buchi Rotary Evaporator fitted with Vacuum pump V-700 and B-490 heating bath was used to concentrate samples. The melting points of all crystalline solids were determined by a Buchi M-560 melting point apparatus. Ultra violet (UV) spectra were obtained in methanol on Evoltion 300 Thermo Scientific UV – visible spectrophotometer. The Infra red (IR) spectra were recorded on FT-IR-8900 Fourier Transform IR Spectrophotometer Shimadzu IR spectrometer. Optical rotations were recorded on JASCO P-2000 polarimeter, Polatronic D Schhmidt and Haensch, Germany. ID and 2D Nuclear Magnetic Resonance (NMR), ^{1}H and ^{13}C NMR, HMQC, COSY, NOESY and HMBC spectra were recorded on AVANCE AV-500 spectrometer operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C NMR, respectively. The chemical shift values (δ) are reported in ppm and the coupling constants (J) are in Hz. The EIMS, HREIMS were recorded on JEOL MS 600H-1 with a data system showing mass to charge (*m/z*). The gas chromatography (GC) was performed on a Shimadzu gas chromatograph (GC-17A) (Method name FAME-MOI, column oven temperature set 70-300 °C (maximum), injection port 220-400 °C and detector temperature 240–450 °C, control mode is split, column pressure (kPa):71, column flow rate 8.19 mL/min, linear velocity (cm/s), 50.01, column length (m): 60, column diameter (mm): 0.53, flame-ionization detector). The percentage compositions were obtained from electronic integration measurements using flame ionization detector (FID), set at 250ºC. Triple

quadrupole Acquisition Method using electron impact (EI) as ion source at 70eV was used for the GC-MS with the following parameters, injection volume: 2µL, oven equilibrium time: 0.5 min, maximum temperature 325 °C. Oven was programmed to 50 °C for 3 min, then 6°C /min to180°C for 20 min and then 6°C /min to 290°C for 40 min with a Run time of 103 min. Split mode was used and column type was ZEBRON –ZB-5 column with dimesions 360°C: 30 m x 250 µm and 0.25 µm.. Initial temperature was 50 °C, pressure 9.78 psi, flow 1.2 mL/min with average velocity 39.92 min, hold on time 1.25 min and Flow programme 1.2 mL/min for 0 min. Mass Hunter Workstation Software Qualitative analysis version 13.04.00 Agilent Technologies In.2011 was used and individual constituents of the oil were identified on the basis of their retention indices compared with data previously reported in literature.

2.5 Determination of DPPH Free Radical Scavenging Activity

The free radical scavenging activity was measured using 2,2-diphenyl-1-picryl-hydrazyl (DPPH) method. DPPH is a free radical with lone pair of electron which becomes paired on contact with an electro donor or the purple colour of DPPH becomes decolourised. There is reduction in absorbance at 515 nm in a UV – visible spectrophotometer (Gulcin et al., 2005; Oloyede et al., 2012). A solution of 0.3 mM DPPH was prepared in ethanol. Different concentration of each sample (62.5 µg - 500 µg) was prepared and five microlitres of each of the sample was mixed with 95 µl of DPPH solution in ethanol. The mixture was dispersed in 96 well plates and incubated at 37° C for 30 min. The absorbance at 515 nm was measured by micro titre plate reader (Spectramax plus 384 Molecular Device, USA) and percent radical scavenging activity was determined in comparison with the ethanol treated control. BHA is used as standard. Inhibition concentration at 50% (IC_{50}) was also calculated (Scalbert et al, 2005; Oloyede 2016).

2.6 Statistical Analysis

All experimental data were analyzed statistically by one-way analysis of variance, using the SPSS software package (version 12.0; SPSS, Inc., Chicago, Illinois, USA). All the measurements were done in triplicate and results are expressed in terms of mean ± standard deviation (Values of $P \leq 0.05$ were considered to be significant) and IC_{50} values were calculated using GraphPad Prism 5 version 5.01 (Graph pad software, Inc., La Jolla, CA, USA.) software.

3. Results

Compound DP1 (15 mg): white crystal. $[\alpha]_D^{25}$-0.08^0 (MeOH). UV (Methanol) λ_{max} nm (logε): 378 (0.234), 366 (0.238), 351 (0.242), 222 (1.0), 213 (0.999). IR (KBr) λ_{max} cm^{-1} : 3864.0, 3743.9, 3452.2, 2920.1, 2849.6, 1737.6, 1642.5, 1467.7, 1372.9, 1170.4, 1102.5, 957.8, 919.0, 724.5. ^{1}H-NMR (500MHz $CDCl_3$) δ: 4.06 (2H, t, *J*=5.6Hz), 2.29 (2H, t, *J*=7.5Hz), 1.63 (38H, t, *J*=5.0Hz), 1.29 (56H, dd, *J*=13.0, 1.5Hz), 0.89 (9H, t, *J*=7.0 Hz) EI-HRMS *m/z*. 789.45316 Calc. 788.8342 g/mol. M.pt-71.3^{0}C DP1 is Tetradecyloctadecyl behenate $C_{54}H_{108}O_2$

Compound DP2 (19 mg): white crystal. $[\alpha]_D^{25}$ -0.002^0 (MeOH). UV (Methanol) λ_{max} nm (logε): 440 (0.006), 264 (0.030), 222 (0.716), 213 (0.693). IR (KBr) λ_{max} cm^{-1}: 3745.8, 2919.6, 2849.1, 1644.4, 1466.2, 1376.2, 724.3. ^{1}H-NMR (500MHz $CDCl_3$) δ: 0.7 (18H, t, *J*=7.0Hz), 1.2 (44H, dd *J*=17.8, 10.3Hz), 3.3(4H, dd, *J*=6.3, 6.3Hz), 3.6 (1H, s). EI-HRMS *m/z.* 499.3390 Calc. 498.789 g/mol. DP2 is 1~chlorotritriacontane M.pt-69.1^{0}C. Precipitate of silver halide formed in silver nitrate test confirmed the presence of chloride.

IR of DP1 showed fatty acid ester at bands 1737.6 cm^{-1}, 2849.6 and 1170.4 µm that are useful as diagnostic of esters. Most of the remaining bands are absorption frequencies of hydrocarbon chain, especially strong C-H stretch at 2850-3000 while sharp peaks at 2919.6 and 2849.1 confirmed C-H of alkanes in DP2. Double bond equivalence (DBE) of 1 confirmed a single unsaturation in DP1. ^{1}H-NMR at 4.0 and 2.29 are indicative of protons adjacent to O-CO group which is confirmed by 2D NMR (COSY, NOESY and Dept-HSQC) C of the ester at 35 and 64 ppm. In DP2 however 2D experiment confirmed only aliphatic carbon signals except that a shift downfield occurred for every signal due to the electron withdrawing effect of the electronegative chlorine atom.

Compound DP3 (21 mg): yellow oil was subjected to GCand GC –MS analysis. Figure 1 shows the chromatogram while Table I gives the compounds detected and their relative percentages.

Thirty two compounds were detected, 14 esters (43 75%) being the dominating constituents. Others are 7 carboxylic/fatty acids (21.88%), 6 alcohols (18.75%), 3 ketones (9.38%), 1 aldehyde (3.13%) and 1 carboxylic acid (3.13%) in relation to the total constituents Figure 2 shows some of the isolated compounds from *D. picta*. Notably, a lanostane type triterpenoid 8-(1,5-Dimethylhexyl)-1,1, 7a, 10a-tetramethyl hexadecahydrocyclopenta [7,8] phenanthro [5, 4a-bc]furan-2-ol (Lanostan-3β-ol, 11β, 19– epoxy-) which is rare in plants was identified in the plant's oil at EI+ scan 67.649 min with Molecular formula $C_{30}H_{52}O_2$ and mass 444.

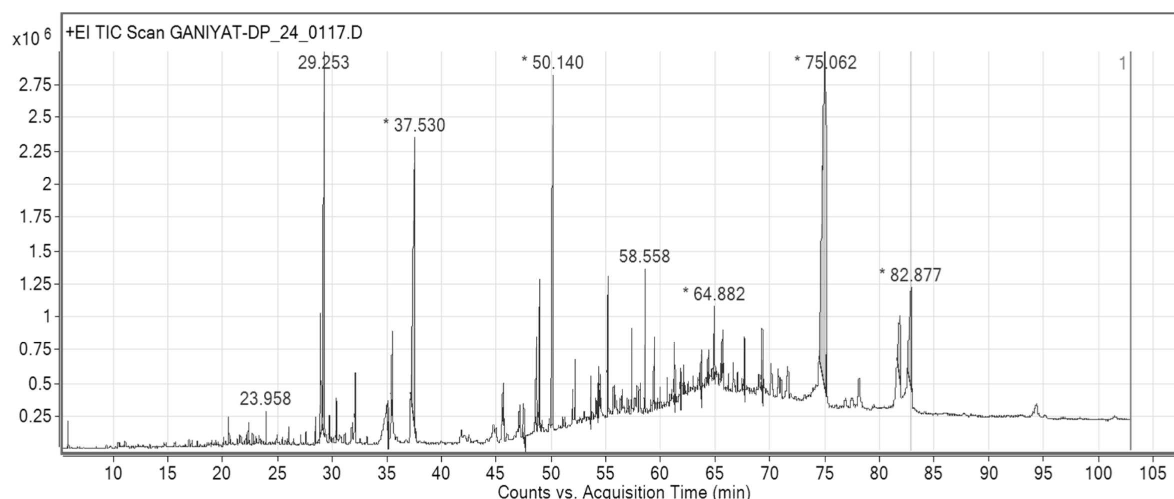

Figure 1: Gas Chromatogram showing Retention time (RT) of compound DP 3 from *D. picta*

Table 1: Compounds detected in DP3

S/N	KI	Name of compound	M. Formula	(g/mol)	Rel%	Class of compound
1.	1519	Ethyl Geranyl acetone	$C_{14}H_{24}O$.	208	0.29	Ketone
2.	1453	Geranyl acetone	$C_{13}H_{22}O$	194	0.21	Ketone
3.	1506	Isopropyl 9-oxononanoate	$C_{12}H_{22}O_3$	214	0.18	Ester
4.	1224	(6E)-3,7-Dimethyl-6-nonenal	$C_{11}H_{20}O$	168	0.1	Aldehyde
5.	1615	i-Propyl dodecanoate	$C_{15}H_{30}O_2$	242	0.28	Ester
6.	1563	Hexa-hydro-farnesol	$C_{15}H_{32}O$	228	0.16	Alcohol
7.	1836	isopropyl myristate	$C_{17}H_{34}O_2$	270	0.43	Ester
8.	1880	Oleyl Alcohol	$C_{18}H_{36}O$	268	2.44	Alcohol
9.	1754	Hexahydrofarnesyl acetone	$C_{18}H_{36}O$	268	9.76	Ketone
10.	2027	3-Eicosyne	$C_{20}H_{38}$	278	0.31	Hydrocarbon
11.	2045	Phytol	$C_{20}H_{40}O$	296	31.44	Alcohol derivative
12.	1878	Methyl palmitate	$C_{17}H_{34}O_2$	270	0.73	Ester
13.	1978	Ethyl palmitate	$C_{18}H_{36}O_2$	284	0.46	Ester
14.	1968	Palmitic acid	$C_{16}H_{32}O_2$	256	1.58	Carboxylic acid
15.	2013	Isopropyl Palmitate	$C_{19}H_{38}O_2$	298	14.91	Ester
16.	2183	Linoleic acid	$C_{18}H_{32}O_2$	280	0.7	Carboxylic acid
17.	2193	Ethyl linoleate	$C_{20}H_{36}O_2$	308	0.47	Ester
18.	2185	Ethyl Oleate	$C_{20}H_{38}O_2$	310	1.04	Ester
19.	2228	Isopropyl linoleate	$C_{21}H_{38}O_2$	322	4.88	Ester
20.	2220	i-Propyl 9-octadecenoate	$C_{21}H_{40}O_2$	324	3.46	Ester
21.	212	Isopropyl stearate	$C_{21}H_{42}O_2$	326	8.61	Ester
22.	2112	Isobutyl palmitate	$C_{20}H_{40}O_2$	312	0.34	Ester
23.	2175	Oleic acid	$C_{18}H_{34}O_2$	282	0.68	Carboxylic acid
24.	3942	1-Heptatriacotanol	$C_{37}H_{76}O$	536	3.99	Alcohol
25.	2439	Cyclohexyl palmitate	$C_{22}H_{42}O_2$	338	0.51	Ester
26.	2380	Eicosanoic acid	$C_{20}H_{40}O_2$	312	1.06	Carboxylic acid
27.	2564	Behenic acid	$C_{22}H_{44}O_2$	340	1.35	Carboxylic acid
28.	2763	Lignoceric acid	$C_{24}H_{48}O_2$	368	0.72	Carboxylic acid
29.	3094	Ethyl iso-allocholate	$C_{26}H_{44}O_5$	436	0.75	Ester derivative
30.	3036	γ-Tocopherol	$C_{28}H_{48}O_2$	416	0.49	Alcohol derivative
31.	864	Lanostan-3β-ol, 11β,19-epoxy-	$C_{30}H_{52}O_2$	444	0.91	Alcohol derivative
32.	2274	cis-10-Nonadecenoic acid	$C_{19}H_{36}O_2$	296	5.17	Carboxylic acid
				Total%	98.50%	

KI- Kovats retention index, (relative to n-alkane hydrocarbon standards.(Retention Data. NIST Mass Spectrometry Data Center., 2007)

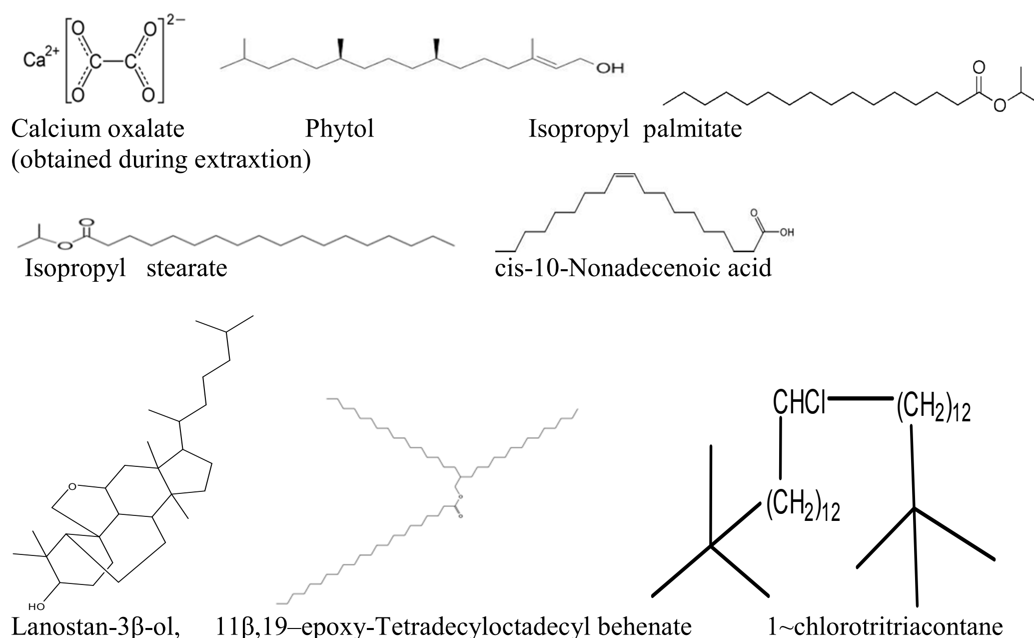

Figure 2: Major compounds responsible for free radical scavenging activity in the n-hexane fraction of *Dieffenbachia picta*

Free radical scavenging activity

In the present study, isolates from hexane fraction of *D. picta* were evaluated for *in-vitro* free radical scavenging activity. DP1 and DP3 showed significant antioxidant activity with IC_{50} (μM) values of 43.5± 0.53 and 25.43±0.06 respectively compared to the standard BHA (44.2 ± 0.09). DP2 was also moderately active with a value of 65.8± 0.83.

4. Discussion

D. picta is rich in lipids which are biologically essential organic molecules because they are the best energy source for humans. Fats are solids while oils are liquid at room temperature. They facilitate the digestive process by depressing gastric secretion and pancreatic flow (Rosenthal and Glew, 2009). The unsaturated fatty acids are not synthesized by animals and man but are supplemented from vegetable food. They are biologically relevant because they lower the levels of cholesterol and triglycerides (Shameel et al., 1995). Tetradecyloctadecyl behenate and 1~chlorotritriacontane were obtained as white crystals and were the major constituents of the plant. Phytol (31.44%), isopropyl palmitate (14.91%), isopropyl stearate (8.61%), cis-10-Nonadecenoic acid (5.17%), isopropyl linoleate (4.88%) are the major constituent of the oil. These constituents no doubt accounted for the observed antioxidant activity .Phytol is the product of chlorophyll metabolism in plants and is an acrylic diterpene alcohol which is used in the manufacture of Vitamin E and K which are important in many functions of the human body. It has been reported to regulate blood glucose and is able to reduce cholesterol levels in blood thereby reducing blood pressure levels. Antinociceptive, antimicrobial and antioxidant activities of phytol *in vivo* and *in vitro* are well documented (Yoshihiro et al., 2005; Santos, et al, 2013). Isopropyl palmitate, stearate, linoleate and myristate are esters of palmitic acid, or isopropyl alcohol and linoleic acid (linoleate). They are polar emollients used in cosmetic and topical medicinal preparations where good absorption into the skin is desired (Wen et al., 2009; Engelbrecht, et al., 2012). Another important component detected in *D. picta* is γ-Tocopherol which is the major form of vitamin E in many plants and used as food additive. γ- tocopherol is a potent defender against disease-provoking compounds in the body known as reactive nitrogen oxides. Furthermore, γ-tocopherol has been found to reduce inflammation, regulate factors that guard against certain cancers and activate genes involved in protecting against Alzheimer's disease (Wagner et al., 2004). These constituents justified our previous finding that the presence of phenolics in *D. picta* detected during phytochemical screening could be responsible for the observed scavenging activity of *D. picta* (Oloyede et al., 2012). Notably, DP1 was isolated as the haloalkane (1~chlorotritriacontane). Alkanes are waxes produced by plants and contribute to the hydrophobic properties of leaf wax. They protect the leaf from water loss via evaporation and serve as part of the plant's first barrier from the external environment. Alkanes are used as solvents, heating oils, fuels, in the synthesis of fats and as biomarkers. Forest fires, volcanic eruptions and maritime metabolic processes produce haloalkanes and many of these are utilized in medicine and technology, for example ethyl chloride (chloroethane), used as a skin coolant in tropical areas (Riley et al., 1993; Yi and Jinxian, 2011; Tipple et al., 2013; Carnahan et al., 2013). Lastly, these compounds no doubt accounted for the

observed significant free radical scavenging activity observed in *D. picta* n-hexane fraction which supports our previous findings.

5. Conclusion

It is therefore concluded that *Dieffenbachia picta* Schott (Dumb cane) used in traditional medicine may be used in modern drug development because of its antioxidant potential. It is rich in non saponifiable lipids and phytol which are generally believed to be safe for human use. Isolated compounds showed significant free radical scavenging activity *in vitro* when compared with butylated hydroxyl anisole (BHA). However, further studies are required to test these compounds for their *in vivo* activity.

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Declaration of Interest

The authors declare that there are no conflicts of interest associated with this work.

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