**Research Article** **ISSN: 2349 – 7114**

# Asian Journal of Research in Pharmaceutical Sciences and Biotechnology

**Journal home page: www.ajrpsb.com**

https://doi.org/10.36673/AJRPSB.2020.v08.i04.A18

# ISOLATION, ANTI-INFLAMMATORY AND ANTI-SNAKE VENOM ACTIVITIES OF *DANIELLIA OLIVERI* (ROLFE) HUTCH AND DALZIEL LEAF AND ROOT EXTRACTS AGAINST *NAJA NIGRICOLLIS* VENOM

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**ABSTRACT**

*Danielliaoliveri* is a plant that is used in the Nigerian traditional medicine as astringent, painkiller, anti-inflammatory, abortifacient, anti-diarrhoea, aphrodisiac, antidote, antibacterial and diuretic for decades. Some of these uses have been verified scientifically, yet, there are no records on the use of *D.oliveri* leaf and root extracts to treat snake-bite. The aim of this present study is to isolate the bioactive compound and evaluate the anti-inflammatory and anti-snake venom activities of *D.oliveri* leaf and root extracts against *N.nigricollis* venom. Isolation of compounds was carried out by silica gel column chromatography. Bioactive compounds were characterized using GC-MS and NMR spectroscopy. Carrageen an-induced paw edema was used to assess the ability of the extracts to reduced inflammations. Lyophilized *N.nigricollis* venom was dissolved in appropriate PBS solution and constituted into various concentrations needed. The standard drug was lyophilized polyvalent antiserum. Evaluation of leaf and root extracts were carried out to assess its potency on *N.nigricollis* snake venom by examining parameters such as enzymatic activities, lethality, necrotizing, paw edema-forming and hemorrhagic activities. Results showed that the extracts contain various phytochemicals like saponins, flavonoids, tannins, balsam and steroids. $LD_{50}$ of both extracts were found to be greater than 5000mg/kg b.w. The extracts greatly reduced diameter of paw edema in dose-related manner. Enzymatic activities of the venom were mostly inhibited by the pure compounds and ethyl acetate fraction on phospholipase A2 (PLA2), acetyl cholinesterase and 5-nucleotidase, while necrotizing activity paw edema area and lethality of the snake venom was reduced in dose-dependent fashion. GC-MS, HMR (1D and 2D-NMR) showed phenolic as bioactive compounds inhibiting the *N.nigricollis* venom activities in the rats. The study therefore showed that *D.oliveri* extracts possessed potent anti-venom activities against *Najanigricollis* venom, with the root extracts showing the highest anti-snake venom activities. The further justified the claims of using the leaves and roots extract to treat *N.nigricollis* snake venom in folkloric medicine.

**KEYWORDS**

*Daniellia oliveri*, *Najanigricollis*, Snake venom, Bioactive compound and Traditional medicine.

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## INTRODUCTION

*Daniella oliveri* is a plant which belongs to the family Fabaceae with 670 genera and nearly 20,000 species comprising trees, shrubs, vines and herbs. It is locally called *''Maaje''* in Hausa language in Nigeria. The plant is distributed in Senegal, Nigeria,

Cote' diviore, Eastern Sudan, Uganda, Mozambique and in most West African countries with heavy to moderate rainfalls. It is a deciduous plant of wooden savannah of Sudan and guinea savannah growing usually at 12m tall although sometimes it can grow up to 45m. The phytochemical analysis of the leaf, stem and root extracts revealed it contained flavonoids, saponins, alkaloids and other forms of glycosides (Muanda *et al*, 2011[1], I wu, 2014[2]). In traditional medicine, the decoction of the leaves is used to treatment gonorrhoea, fevers, and diabetes while bark and root decoctions are used to treat the skin disease called ''*craw-craw*'' (Abbiw, 1990[3], Burkhill, 2004[4]) and the stembark is used as anthelminthic, antidiabetic, anti-leprosy and anti-kidney failure agents (Adodo, 1997[5]). The ''*Jukun*'', ''*Ichen*'' and ''*Chamba*'' tribes in Taraba State, Nigeria claimed that the plant's leaf and root can effectively be used to treat venoms from snakebite of *Najanigricollis* (and other snakes), scorpion, frog and centipede (these claims have not been verified scientifically).

These claims on the use of the leaf and root extracts to treat the venom resulting from the bite of *N.nigricollis* (black cobra) were being investigated for the first time in this present study. Snake venom anti-serum is expensive and need good storage conditions which is be lacking in villages that are mostly snake endemic areas in Africa. Therefore, the use of medicinal plants alternatives for treatment of poisonous snake bites is of significance. Venom by definition, is a poison substance which is secreted by animals such as snakes, spiders, centipedes and scorpions that is injected into its prey or aggressors through bites or stings. In a simple term, it is a form of toxins secreted by an animal with the aim of causing harm to another animal (Peter 2004[6], Clark *et al*, 2019[7]).

There are different types of snake venoms viz: cytotoxic venom, neurotoxic venom and hemotoxic venom and each produced different effects in humans. For instance, cytotoxic Venom destroys tissue and causes pain, inflammations and wears away at the skin. This type of venom differs in potency according to the species of snake, size of the snake and the amount of venom injected. Snakes with cytotoxic venom include most of the adders and vipers, some cobra species like the Mozambique spitting cobra, black-necked spitting cobra and zebra cobra. Species whose venom is cytotoxic and can cause death include rattle snakes, puff adders, Gabon vipers, saw scaled vipers, Russell's viper, bush master, lance head vipers and many other viper species as well as the cobra species listed above. A bite from one of these snakes will cause severe inflammation and often the entire limb will swell up. Tissue damage will occur, and in the absent of an anti-venom death might occur (Sajon *et al*, 2017[8], Slagboom *et al*, 2017[9]).

Neurotoxic venom on the other hand, is very dangerous; bites will cause drowsiness, blurred vision, difficulty in speech and suddenly, paralysis which causes the lungs to be functionless (Casewell *et al*, 2019[10]). This type of venom acts very fast. If a person is bitten, anti-venom will usually be needed and the person may also have to be put on life support in case anti-venom is not readily available or if not treated quick enough. Snakes which have neurotoxic venom include: almost all of the cobras like *Najanigricollis*, mambas, coral snakes, banded kraits and yellow-bellied sea snakes. Hemotoxic venom stops blood from clotting causing internal bleeding. The bite site will start bleeding and the person will start to bleed from small cuts and mucus membranes. The person will also have symptoms like headaches, nausea and may start to vomit. Death may occur if anti-venom is not administered. Snakes which have hemotoxic venom include: the boom-slang (a back-fanged snake from Africa) and the vine snake. Some snakes have a combination of different venom types for example the berg adder. The term ''victim of snake bite'' was not used here because, snakes only bite people in self-defence when humans attempt to kill or pick up snakes (Schendel *et al*, 2019[11], Post *et al*, 2020[12], Laxme *et al*, 2019[13]).

The use of *D. oliveri* leaf and root extracts in Taraba State, Nigeria dated many centuries, and this practice is preferred to the use of conventional anti-snake venom. However, there is no clear preference

on the parts of the plant used for this purpose by these tribes in Taraba State, Nigeria. In this study, carrageenan was used to induce inflammation in rats while *Najanigricollis* venom was induced in the rats using appropriate route. Isolation of bioactive compounds in leaf and root extracts was carried out by column chromatography while their structures were elucidated uing GC-MS, and NMR spectroscopy ($^{1}$H, $^{13}$C, $^{135}$DEPT, HMBC, and HSQC). Hence, this study was aimed at isolating the bioactive compounds from the leaf and root extracts with assumed anti-snake activities, and evaluate the anti-inflammatory and anti-snake venom activities of the extracts with a view to ascertaining the claims in traditional medicine.

## MATERIAL AND METHODS

### Collection and identification of plant

Fresh leaves and roots of *Daniellia oliveri* were collected in the evening hours from Sabon-Dali, Bali Local Government Area, Taraba State. It was identified with a voucher number of FAB003 deposited in the herbarium of plant research laboratory, Bali.

### Preparation and extraction of plant materials

The leaves and roots of the *D.oliveri* were air dried under shade for two weeks, and then reduced into fine powder form using and electronic grinder (made in Japan). The powder weighing 2.5kg each were then stored separately in dry clean polythene bags and stored at 45$^{o}$C in a refrigerator (LG, made in China). The powders were extracted by cold maceration technique in 99.1% methanol (v/v) (Sigma, USA) for 48 h. The yields obtained were 16.88% and 12.44 % for leaf and root respectively, and were stored in a refrigerator at 4$^{o}$C ± 2$^{o}$C for further use.

### Phytochemical analysis of plant extracts

The methods as described by Evans (2009)[14] and Sofowora (2006)[15] were used to determine the presence of some secondary metabolites like saponins, flavonoids, alkaloids, balsam, volatile oils, terpenoid and others in the leaf and root extracts.

### Isolation and structural elucidation of compounds

Exactly 4.5g each of leaf and root extract were dissolved separately in a mixture of distilled water and methanol (30:70). The mixture was fractionated successively by liquid-liquid partition (LLP) using solvents in the following order: n-hexane (HF; 500mL), ethyl acetate (EF; 500mL), n-butanol (BF; 500mL) and aqueous phase (AF; 500mL). The fractions were all concentrated *in vacuo* using rotary evaporator (made in China) and stored at 4$^{o}$C separately for further use. The fractions were tested each bio-guided by anti-venom activities for 24 h. The ethyl acetate fraction (EF) was then subjected to silica gel column chromatography with mesh size 60-120mm. Elution was carried out by gradient elution using hexane-methanol (ratio: 100:0, 9:1, 8:2, 6:4,3:2, 1:1 and 0:100). It was followed by gradient elution of ethyl acetate-hexane (ratio: 100:0, 9:1, 8:2, 6:4,3:2, 1:1 and 0:100). A total of 30 mL sixty fractions each were collected and grouped based on their TLC into four. Each sub-fraction was bio-monitored ant-venom activity for 24 h. Sub-fraction F1.4 was re-chromatographed into silica gel column and eluted with hexane-methanol (ratio; 100:0 and 0:100) to give 12.5mg of compound 1 from leaf extract, while sub-fraction F2.1 yielded 8.04 mg compound 2. The compounds were checked for purity on TLC and HPLC (Theakston and Reid 1983[16], Jothy *et al*, 2011[17], Calderon *et a*l, 2002[18], Deepika *et al*, 2016[19], Alam and Gomes 1998[20]).

### Structural elucidation of compounds

The mass spectra (MS) data were measured on Agilent technologies A7895 GC coupled to a mass spectrum with helium carrier gas, while $^{1}$H-NMR (COSY, NOESY, HMBC, HSQC) and $^{13}$C-NMR (135-DEPT) were recorded on a Bruker ASCEND 600 MHZ NMR. Chemicalshifts were recorded as ppm (part per million) with $CDCl_3$ as the reference solvent (Deepika *et al*, 2016[19], Nkeoma *et al*, 2014[21]).

### Collection of *Naja nigricollis* venom

A black cobra (*Najanigricollis*) was captured from Daniya, Bali Local Government Area, Taraba State

and its venom was collected by milking method as previously described by Markfarlane (1967)[22], lyophilized and stored at 4°C until required. This was subsequently referred to as crude venom. The lyophilized polyvalent antiserum for snake venom which serve as standard was obtained on demand from a research institute in Nigeria.

**Laboratory animals**

Twenty-five mature Wistar albino rats of opposite sexes weighing between 100 -150g as well as thirty-nine male Swiss albino mice weighing between 15-20g, were purchased from the animal house of the department of Pharmacology, University of Jos, Nigeria. Ethical approval for the use of these was approved by the research ethical committee of the same University. The animals were housed in separate cages and cared for following the laid rules on the use of animals in research (Kilkenny *et al*, 2010[23]).

**Acute toxicity determination of extracts**

Acute toxicity ($LD_{50}$) studies of extract was carried out according to the method of Lorke (1983)[24]. Animal were given doses of extracts at 10, 100, 1000mg/kg (first phase) and 1600, 2900, 5000mg/kg b.w. (second phase) (i.p.). Animals were observed for signs of toxicity for 14 days.

**Determination of snake venom $LD_{50}$**

The $LD_{50}$ of *N.nigricollis* venom was evaluated following the procedures described by Theakston and Reid (1983)[16]. Briefly, *N.nigricollis* venom of various concentrations were dissolved into 0.2 mL PBS solution and administered to mice grouped into five mice per group (i.p.). A confidence limit put at 50% probability was used to calculate the lethal dose of the venom for deaths which occurred within the first 24 h of venom intoxication.

***In vivo* experiment for anti-snake venom studies**

**Experimental design for anti-snake venom activity of DOE**

The rats were grouped into five groups of five as follows:

Group 1 is a normal control which received snake venom only

Group 2 received snake venom + polyvalent anti-venom

Group 3 received snake venom + 100mg/kg DOE

Group 4 received snake venom + 200mg/kg DOE

Group 5 received snake venom + 400mg/kg DOE

The extracts (DOE) denoted leaf and root crude methanol extracts respectively.

**Neutralization of snake venom lethality by DOE**

To evaluate the potency DOE on *N.nigricollis* venom, twice and thrice the lethal dose (2 $LD_{50}$ and 3$LD_{50}$) of the venom was administered to the rats (i.p.) immediately after oral administration of DOE. Probit analysis of results were calculated as previously described by Theakston and Reid, 1983[16] and Chacko *et al*, 2012[25]).

**Neutralizing effects of DOE on venom hemorrhagic activity**

The minimum hemorrhagic dose (MHD) of the *N.nigricollis* venom was determined by was intradermal injection of venom dose on shaved areas within the dorsal region of the skin. After 5 min, various doses of DOE were administered orally to the rats (Theakston and Reid, 1983[16], Chacko *et al*, 2012[25], Kondo *et al*, 1960[26], Ode and Azusu, 2006[27]).

**Neutralizing effects of DOE on venom necrotizing activity**

To determine the minimum necrotizing dose (MND) of *N.nigricollis* snake venom, the venom was intradermally injected to various groups of rats on shaved portions of the skin, followed by oral administration of DOE after 5 min (Abubakar *et al*, 2000[28], Theakston and Reid, 1983[16], Chacko *et al*, 2012[25]).

**Neutralizing effects of DOE on venom edema forming activity in rats**

In order to determine the minimum edema dose (MED), 0.1mL *N.nigricollis* venom were injected into the sub-plantar area of the paw of the rat's groups followed by oral administration of DOE. The control group received only *N.nigricollis* snake venom and all rats were not fasted prior to the study. Plethysmograph was used to measure the volume of paw edema (Kumar and Basu, 1994[29], Alam and Gomes, 1998[20], Chacko *et al*, 2012[25]).

**Experimental design for *in vivo* anti-inflammatory study**

Mice of opposite sexes were divided into five groups of five mice follows:

Group 1 is the normal control group which received 5 % tween 80 solution (o.p.).

Group 2 is a positive control which received 300mg/kg aspirin (o.p.).

Group 3 received 300mg/kg b.w. DOE (o.p.).

Group 4 received 600mg/kg b.w. DOE (o.p.).

Group 5 received 1,200mg/kg b.w. DOE (o.p.).

**Anti-inflammatory activity of DOE**

The anti-inflammatory activity of *D. oliveri* extracts was determined by carrageenan-induced paw edema in mice as previously described by Kumar and Basu (1994)[29]. Briefly, Mice were given 0.1mL of 1% carrageenan in normal saline by sub-plantar injection. This was followed by oral administration of DOE 30 min later. The volume of paw edema was measure using plethysmometer. The results obtained from the treated groups were compared with that of the standard drug.

**Statistical analysis**

The acute toxicity of the *N. nigricollis* snake venom was calculated by probit analysis and expressed as µg/kg while the data were expressed as mean±SE. $P<0.05$ was considered statistically significance (one-way ANOVA). All data were analyzed using Graphpad prism version 9 software 2020.

## RESULTS

**Acute toxicity studies of extracts**

Acute intoxication *D. oliveri* leaf and root extracts showed no signs of toxicity or mortality after two weeks' administration (i.p.) at highest dose of 500mg/kg b.w. in the animals. $LD_{50}$ was found to be greater than 500mg/kg b.w. while that of the snake venom was 275.13µg/kg. Simultaneous administration of extract and venom resulted into 100 % survival rate of rats in EF while 50% survival was seen in BF an AF after 48 h (Figure No.1 and No.2). The result showed that DOE is very safe for use as oral medication in traditional medicine.

**Effects of *D. oliveri* extracts on phospholipase A2 activity**

The various fractions of leaf and roots showed significant reduction in phospholipase A2 activity except for HF of root extract with increased % inhibition activities where platelets activity increased with delayed clotting time. From the result, the EF showed the most significant reduction in PLA2, platelet and hemolysis of *N.nigricollis* snake venom-induced activities (Figure No.3).

**Effects of DOE on *N. nigricollis* venom hemorrhagic activity**

From the results obtained, EF of *D.oliveri* root extract showed highest reduction in venom induced hemorrhagic activity in rats with minimum hemorrhagic doses of 4.01±0.01 2.11±0.01 for leaf and root extracts respectively compared to the normal control with 12.02±0.04 8.16±0.02 (Table No.1).

**Effects of DOE on venom necrotizing and edema-forming activities**

The use of the drug or venom alone does not show significant reduction in lesion area caused by necrotizing activity of the *N.nigricollis* venom in the Wistar rats. However, much improved neutralization of necrotizing activity the venom was achieved in dose-dependent fashion with group 5 (venom + 400mg/kg DOE) having the lowest neutralizing area of 0.40±0.01mm as well as the highest percentage paw edema-forming activity of 58.1% (Figure No.4 and Figure No.5).

**Anti-inflammatory effects of *D. oliveri* extracts in mice**

From the result, the EF of leaf and root extracts showed significant reductions in % paw edema volumes in mice. These reductions were dose-dependent from 300-1200mg/kg b.w. DOE within 3h while, the HF showed the least potent effect on the paw edema reductions in the mice (Figure No.6).

**Phytochemical constituents, GC-MS and NMR analysis of bioactive compounds**

Analysis of the leaf and root extracts showed that the plant contains majorly flavonoids, saponins, tannins and steroids. Most of these secondary

metabolites were detected in the ethyl acetate fraction (EF) when compared with other solvents (Table No.2).

The mass spectra data showed compounds with molecular weight of 213mol/g, and a base peak at 139 with several fragmentation patterns for leaf extract and 224mol/g and a base peak at 43 with several fragmentation patterns for root extract (Figure No.19 and No.20). $^{1}$H-NMR (600 MHZ, $CDCl_3$) showed five signals for leaf extract with the following data: 1H δ (ppm) 5.30 (s, J= 1.0 Hz), 2H δ 5.70 (s, J= 0 Hz), 3H δ 7.30 (s, J= 3.0 Hz), 4H δ 7.50 (q, J = 1Hz), 5H δ 7.70 (q, J = 1.0 Hz) while the root extract showed also five signals at: 1H δ 2.83 (d, J = 0 Hz), 2H δ 3.23 (d, J = 0Hz), 3H δ 3.70 (s, J= 1.0 Hz), 4H δ 4.0 (s, J = 1.0 Hz), 5H δ 4.30 (q, J = 2.0 Hz). The $^{13}$C-NMR showed 12 and 16 carbon atoms for leaf and root extracts respectively with chemical shift values range from 105 to 183ppm for the leaf extract and 15-169 ppm for the root extract which are characteristic positions for methine, methyl and methylene carbons. There are correlations between protons and carbons atoms as shown by DEPT-135, COSY, HSQC and HMBC spectra (Figure No.7-18) which confirmed the proposed compound as dibenzofuran, 3-nitro from leaf extract and cetene from root extract (Figure No.21).

## DISCUSSION

In recent times, medicinal plants have continued to be a widely accepted healthcare choice for many people for the purpose of good health maintenance and wellbeing of humans. Plants have been used to treat mild sickness such as coughs, colds, pains, fevers, as well as more serious like diseases piles, asthma, hypertension, cancer, depression and diabetes. For instance, plants like *Ginkgobiloba* products has been to treat advanced medical condition such as memory lose by helping memory enhancement. Medicinal plants have been used since time immemorial because of their low costs as well as easy accessibility and they have passed the test of time without having any side effects. The multi-target effects of medicinal plants are the fundamental basis for their overwhelming utilization which of course, is where the Nigerian traditional medicine was anchored (Iwu, 2014[2], Peter, 2004[6], Adodo, 1997[5]). Currently, the integration of scientific procedures into traditional medicine has given a new face to use of herbal drugs for use in disease management and treatment for the betterment of humanity (Ramawat, 2009[30], Odugbemi, 2008[31]).

The prevalence of snakebite in most communities in Nigeria especially during the wet season is on the increase with about 20% resulting in deaths if no immediate treatment with potent anti-snake venom is administered to the person. The use of medicinal plants to treat snakebite has gained popularity because of their efficacies over the conventional anti-venom drugs. Moreover, medicinal plants contain arrays of pharmacologically active compounds that are potent against snake venoms (Sivaraman *et al*, 2017[32], Ode and Azusu, 2006[27]).

In this current study, acute toxicity evaluation of the leaf and root extracts showed that *D. oliveri* is safe because $LD_{50}$ value greater than 5000mg/kg body weight is biological insignificant (Lorke, 1983[24]). This obviously was the main reason for overwhelming use of these morphological parts of the plant in traditional medicine for the treatment of numerous diseases. Also, from our study, the use of the extracts in combination with the *N.nigricollis* snake venom resulted in 100% survival within the durations of 3 hours (Figure No.1 and 2). The safety of many plant extracts in treating snake venoms have been reported by various researchers unlike, the undesirable side effects of most conventional anti-snake venoms (Sajon *et al*, 2017[8], Ramawat, 2009[29], Azusu and Harvey, 2003[33]). The study further revealed the ability of the extracts to reduce *N.nigricollis* venom-induced hemlysis and clotting time of the platelets in the rats administered same dose of the snake venom (Table No.1, Figure No.3). Significant decrease in these activities was obtained from the EF of the root than the leaf extract. There was no significant difference between the effects observed from the root and leaf extracts. Because of the popular use of the leaf extract by the *Jukun* and

*Ichen* people as opposed to the root extracts used by the *Chambas* in Taraba State, we presented mainly the results from the leaf extracts. Similarly, D. oliveri extracts were able to inhibit the hemorrhagic activity of the venom with a MHDs of 4.01±0.01 and 2.11±0.01 for EF of leaf and root extracts respectively (Table No.1). Moreover, the extracts showed significant reductive effects on venom necrotizing and paw edema-forming activities in the rats. The results showed that at dose of 400mg/kg b.w. the extract produced the most potent action of these activities caused by the *N.nigricollis* venom in the animals (Figure No.4 and Figure No.5). Necrosis is that process by which an injured cell is destroyed prematurely due to the self-digestive effects by its own enzyme (autolysis). All venoms from snake bite exert various degrees of necrosis which are linked to neurological response. The necrotizing effect of *N.nigricollis* venom has been reported to result in large lesion in affected area, thereby creating cytotoxic and neurological effects in the host (Post *et al*, 2020[12], Clark *et al*, 2019[7], Laxme *et al*, 2019[13]). The ability of the extracts to halt the necrotizing effect of *N.nigricollis* venom in the rats in this present study, may be due the presence of some metabolites like saponins and flavonoids in the extracts (Deepika *et al*, 2016[19], Pithayanukul *et al*, 2005[34]). This may also account for the reason for the reductions in paw edema volumes in the rats treated with high dose of EF mixed with the venom in this study (Figure No.5). This result obtained where a higher dose of extract resulted in reduced paw edema volume has also be reported previously with some medicinal plants (Shenoy *et al*, 2013[35], Pla *et al*, 2019[36], Azusu and Harvey, 2003[33], Gutierrez *et al*, 2017[37], Cardoso *et al*, 2019[38]).

In addition to the pharmacological activities displayed by the *D. Oliveri* extracts which were attributed to the abundant phytochemicals in the extracts especially, flavonoids, saponins and tannins that were detected in large amount (Table No.2), flavonoids and polyphenols for instance have been reported to possess the capability of binding to snake venom protein as well as inhibiting the enzymatic activities of the snake venom like phospholipase A2 (Abubakar *et al*, 2000[28], Lomonte *et al*, 2009[39], Rodrigo *et al*, 2012[40]). This is the reason for the results obtained in this present study (Figure No.3). Previous studies have shown that snake venoms are complex mixture of substance which consist of many enzymes that exert pains, inflammation and necrosis at the affected areas (Cardoso *et al*, 2019[38], Lomonte *et al*, 2012[41]). The ability of the plant extracts especially the ethyl acetate fractions (EF) of leaf and root to inhibit PLA2 enzymes in the *N.nigricollis* venom may be due to its oxidizing effect on calcium ions (Slagboom *et al*, 2017[9], Rodrigues *et al*, 2004[42]).

Phospholipase A2 is made up of heterogeneous subdivision of lipolytic enzymes that possess the capability to hydrolyse the ester linkages found in phospholipids along phosphodiesterase and acylhydrolase activities. They are described as one of the most important proteins present in *N. nigricollis* snake venom and the most destructive of them all causing inflammation and many pathological effects from snake envenomation like neurotoxicity, cardiotoxicity, myotoxicity, cytotoxicity and many more (Rodrigues *et al*, 2004[42], Gutierrez *et al*, 2017[37]).They also exist in many biological fluids of humans like platelets, macrophages, muscles, placenta and spleen (Chacko *et al*, 2012[25], Clark *et al*, 2019[7], Schendel *et al*, 2019[11], Casewell *et al*, 2019[10]). The ability of the *D. oliveri* extracts to decrease the scavenging activities of PLA2 in this study further justifies the use of these plant parts to treat N. nigricollis snake venom in traditional medicine in Nigeria. The mechanism of action is yet to be studied in this current study.

From our study, the DOE was able to reduced inflammation caused by carrageenan in the mice in dose-dependent fashion (Figure No.6). At 1200mg/kg b.w. DOE, the EF of leaf extract reduced the volume of paw edema in group 5 mice. This result was statistically significantly different compared with the control normal group at $p< 0.05$ (one-way ANOVA). The reduction of inflammation by the extracts in the mice may be due to the

inhibition of inflammatory mediators through the blockage of signal pathways that regulate swelling and pain. The non-steroidal anti-inflammatory drug-like features might be due to high content of flavonoids compounds in the ethyl acetate fraction of the leaf and root extracts (Nkeoma *et al*, 2014[21], Jothy *et al*, 2011[17], Hossain *et al*, 2012[43]). Flavonoids have been reported to have shown various degrees of therapeutic potential in biological systems and this is the case in this current study.

The structural elucidation of the isolated compounds using advanced spectroscopic techniques 600 MHZNMR ($^{1}H$, $^{13}C$, 135 DEPT, COSY, HSQC and HMBC) and GC-MS (Figure No.7-20), revealed a flavonoid compound benzofuran from the leaf extract which is believed to have universal occurrence in many plants and cetene from the root extracts (Figure No.21). Benzo furan have been reported to possess anti-tumor, antibacterial, anti-oxidative and anti-viral activities. In this current study, this compound isolated from the EF sub-fraction was responsible for the observed biological activities of the leaf extract, while cetene isolated from root extract was the bioactive compound. The biological activities of cetene has not been reported before. This is the first report on its use, and the first time it was isolated from any member of the family Fabaceae. Cetene which is a hydrocarbon that is oily in nature belongs to the ethylene groups and were initially obtained from the head of sperm whales and used to manufacture cosmetics. Similarly, benzofuran derivatives have been reported to have shown anti-inflammatory and analgesic activities in mice (Gupta *et al*, 2018[44], Serna and Martinez, 2015[45]). This report further validates the anti-inflammatory activity of the extracts through the significant reduction in carrageenan-induced paw edema volume in the mice (Figure No.6).

It is possible that the observed anti-inflammatory activity of *D. oliveri* extract may be achieved through the inhibition of pro-inflammatory cytokines (Bose *et al*, 2011[46], Xie *et al*, 2014[47], Gupta *et al*, 2018[42]). Because of the numerous biological activities and potential applications of benzofuran in many areas of human's health, it has drawn more attentions from natural product researchers globally with wide range of applications especially in pharmacy, where many antiviral drugs like those of hepatitis have been discovered (Miao *et al*, 2019[48]). Despite the fact that this compound from *D. oliveri* leaf extract are cosmopolitan in many plant families like Rutaceae, Liliaceae, Cyperaceae and Asteraceae, to best of our knowledge, it has not been isolated from the family Fabaceae before.

Our study therefore, showed that *D. Oliveri* leaf and root extracts were potent by reducing the *Naja nigricollis* snake venom through the inhibition of the inflammation caused by phospholipase A2 enzyme, decreasing hemorrhagic and necrotizing activities. The bioactive compounds in these extracts were identified by advanced spectroscopies as benzofuran, 3-nitro and cetene from leaf and root extracts respectively.

**Table No.1: Effects of *D. oliveri* extracts on *N.nigricollis* venom-induced hemorrhagic activity (n = 5)**

| S.No | Group | MHD (mean±SE) | |
|---|---|---|---|
| | | Leaf | Root |
| 1 | Normal control (0.5mL normal saline) | 12.02±0.04 | 8.16±0.02 |
| 2 | HF 200mg/kg | 7.12±0.01 | 23.02±0.03 |
| 3 | EF 200mg/kg | 4.01±0.01 | 2.11±0.01 |
| 4 | BF 200mg/kg | 6.03±0.01 | 3.04±0.01 |
| 5 | AF 200mg/kg | 5.98±0.01 | 5.01±0.01 |

MHD; Minimum hemorrhagic dose, n = 5.

**Table No.2: Phytoconstituents of *D.oliveri* methanol extract sub-fractions**

| S.No | Constituents | Leaf extracts | | | | Root extracts | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | HF | EF | BF | AF | HF | EF | BF | AF |
| 1 | Alkaloids | - | + | - | - | - | - | - | - |
| 2 | Saponins | - | +++ | + | + | - | ++ | - | + |
| 3 | Tannins | - | + | - | - | - | ++ | + | + |
| 4 | Flavonoids | - | +++ | - | + | - | - | + | ++ |
| 5 | Steroids | - | ++ | + | + | - | ++ | - | + |
| 6 | Anthracenes | - | - | - | - | - | - | - | + |
| 7 | Volatile oil | ++ | - | - | - | - | - | - | - |
| 8 | Balsam | - | - | - | - | ++ | - | - | - |
| 9 | Cardiac glycosides | - | - | - | - | - | + | - | - |

+ = detected in trace amount, ++ = detected in moderate amount, +++ = detected in large amount, - = not detected. HF; hexane fraction, EF; ethyl acetate fraction, BF; butanol fraction, AF; aqueous fraction.

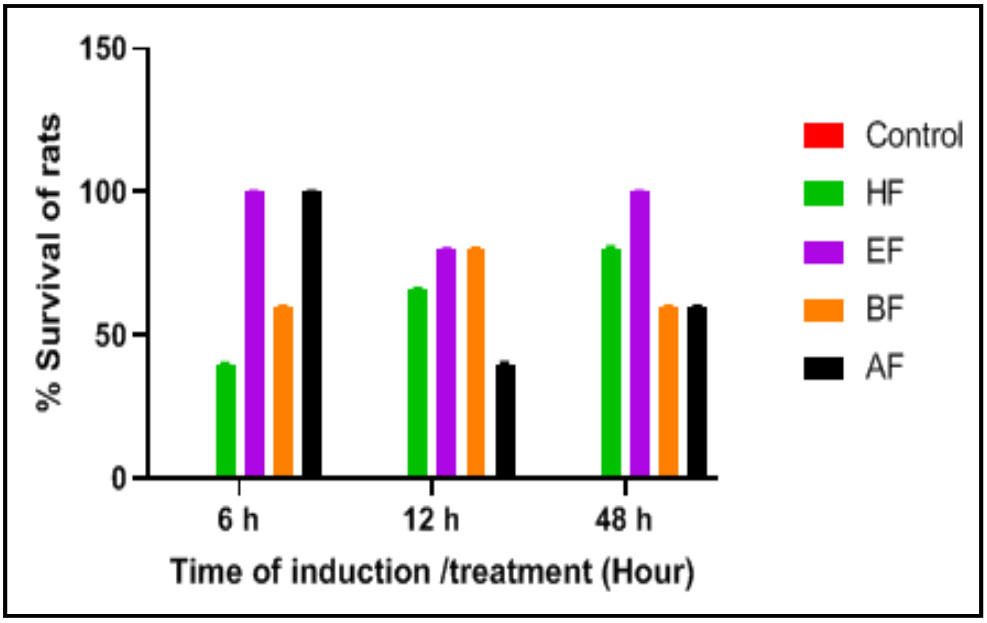



**Figure No.1: Effects of various fractions of *D.oliveri* extract on lethality of *Najanigricollis* venom in rats. D/$H_2O$; distilled water, HF; hexane fraction, EF; ethyl acetate fraction, BF; butanol fraction, AF; aqueous fraction, n = 5; p<0.05**

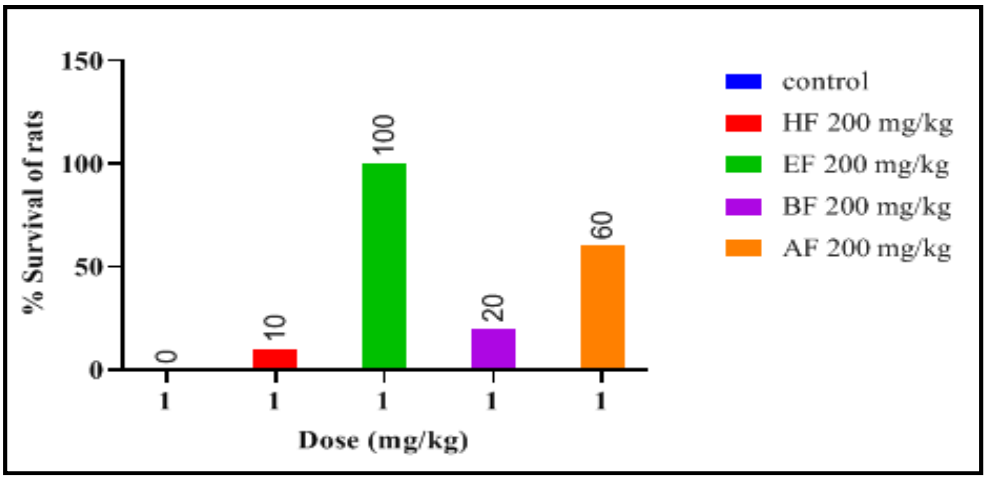



**Figure No.2: Effects *D.oliveri* extracts on survival rate of rats from *N.nigricollis* venom**

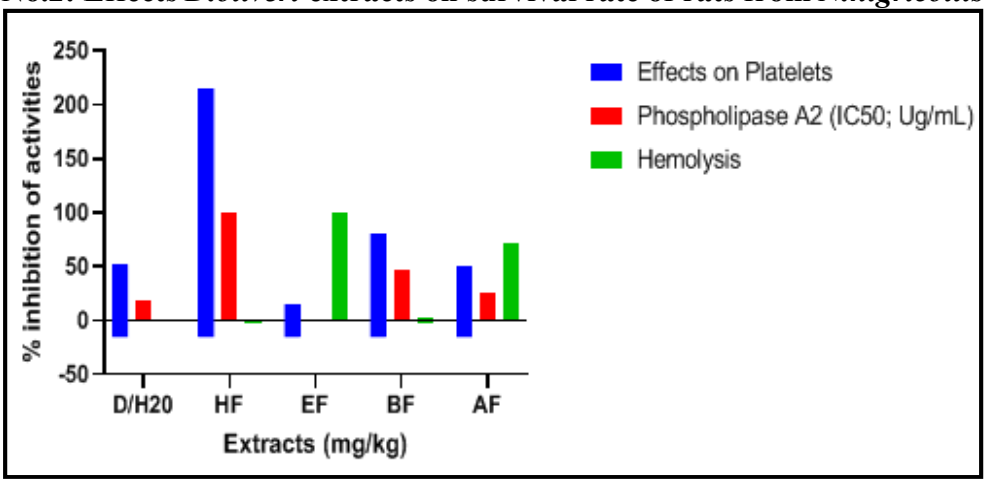



**Figure No.3: Anti-snake venom activities of various fractions of *D.oliveri* extracts. D/$H_2O$; distilled water, HF; hexane fraction, EF; ethyl acetate fraction, BF; butanol fraction, AF; aqueous fraction. P< 0.05 (one-way ANOVA)**

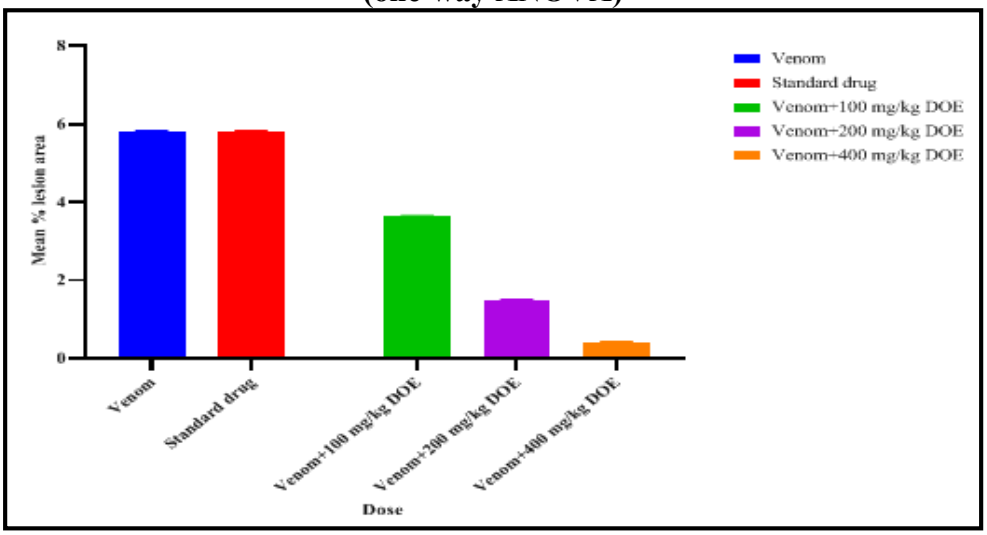



**Figure No.4: Effects of *D.oliveri* crude leaf extract on venom-induced necrosis in rats**

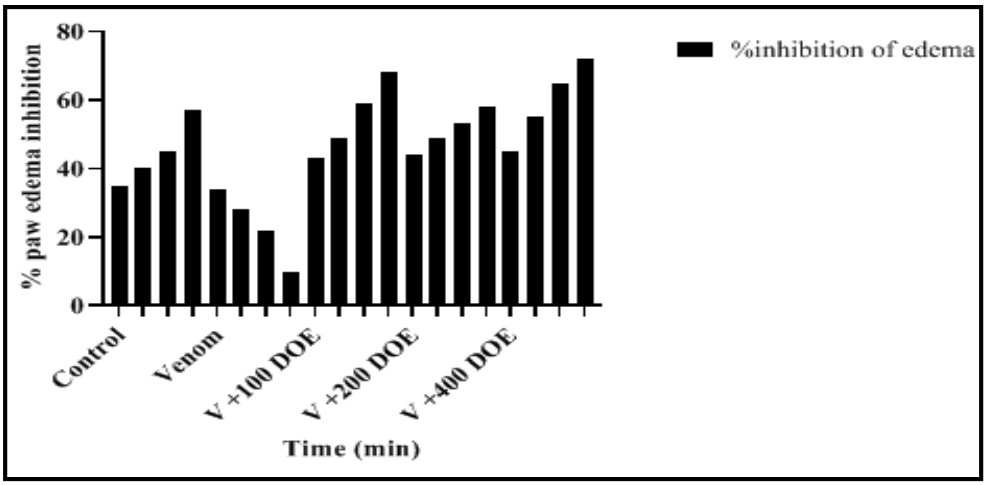



**Figure No.5: Effect of *D.oliveri* crude leaf extract on venom-induced paw edema in rats**

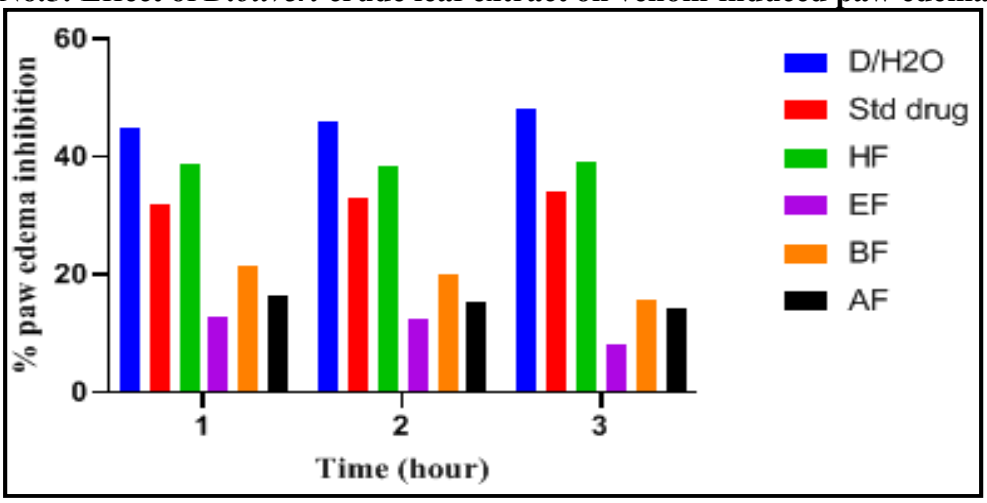



**Figure No.6: Anti-inflammatory effects of *D.oliveri* extracts in mice. D/$H_2O$; distilled water, HF; hexane fraction, EF; ethyl acetate fraction, BF; butanol fraction, AF; aqueous fraction. P< 0.05 (one-way ANOVA)**

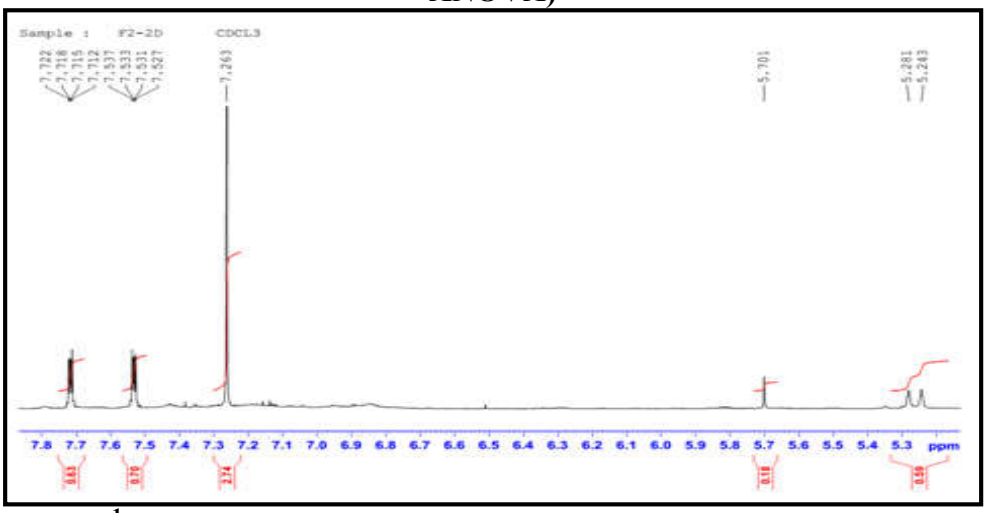



**Figure No.7: $^1$H-NMR spectra of bioactive compound from leaf extract of *D.oliveri***

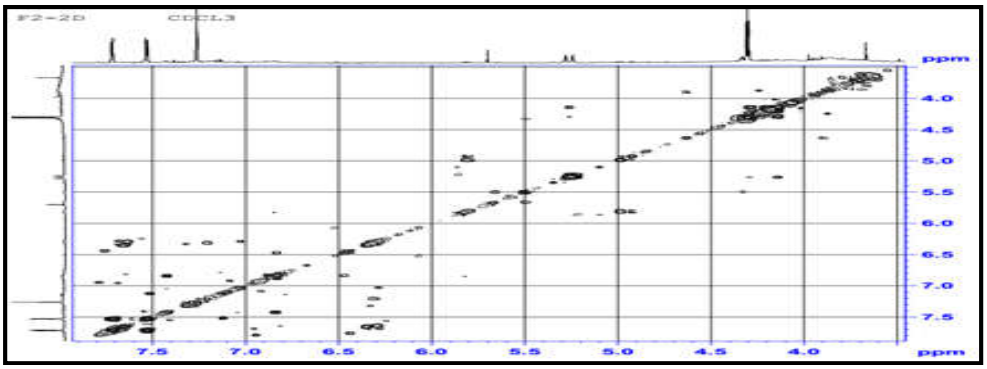



**Figure No.8: COSY-NMR spectra of bioactive compound from leaf extract of *D.oliveri***

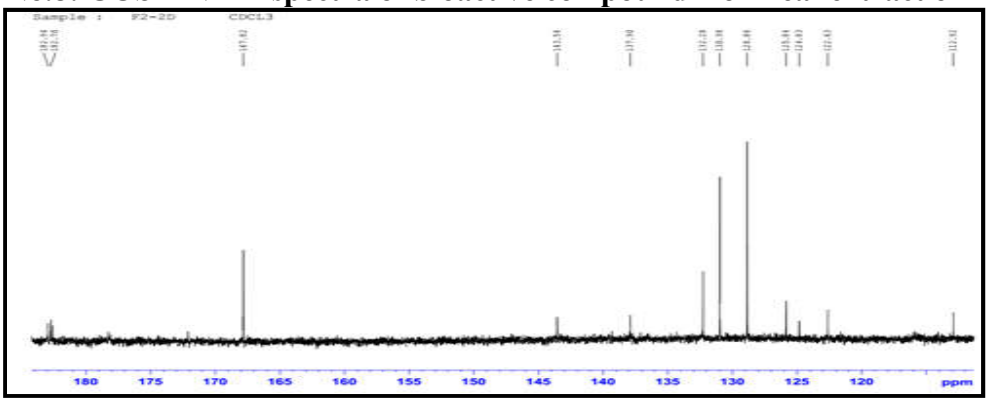



**Figure No.9: 13C-NMR spectra of bioactive compound from leaf extract of *D.oliveri***

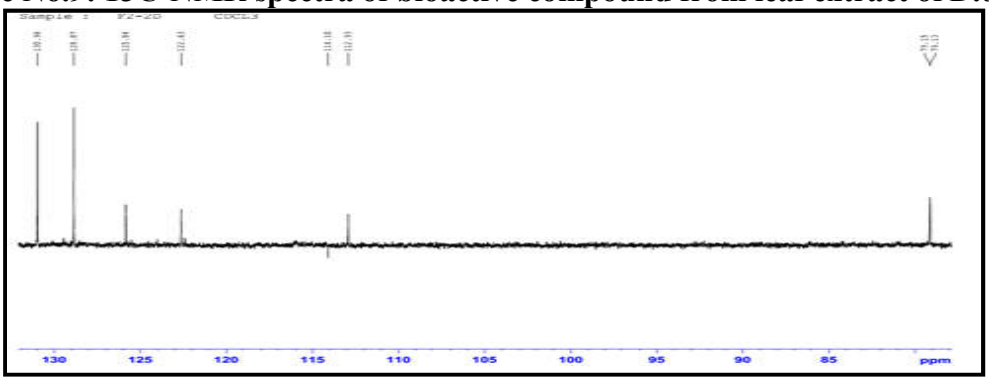



**Figure No.10: 135 DEPT- NMR spectra of bioactive compound from leaf extract of *D.oliveri***

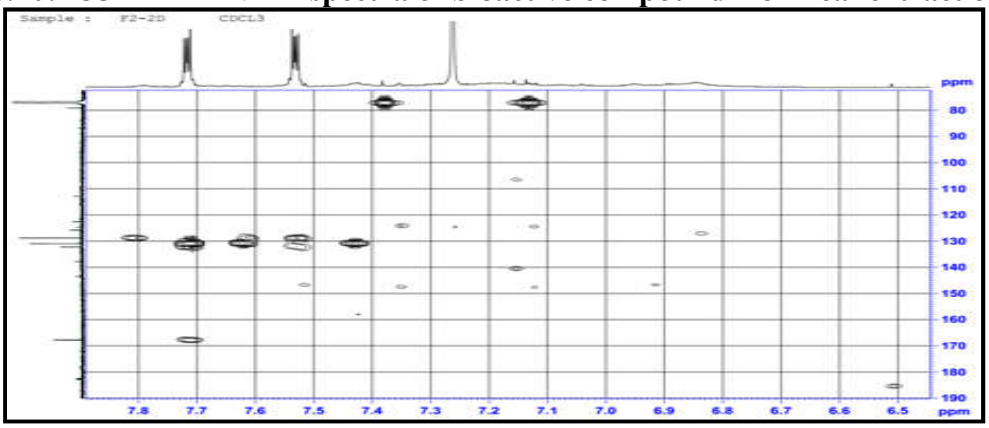



**Figure No.11: HMBC spectra of bioactive compound from leaf extract of *D.oliveri***

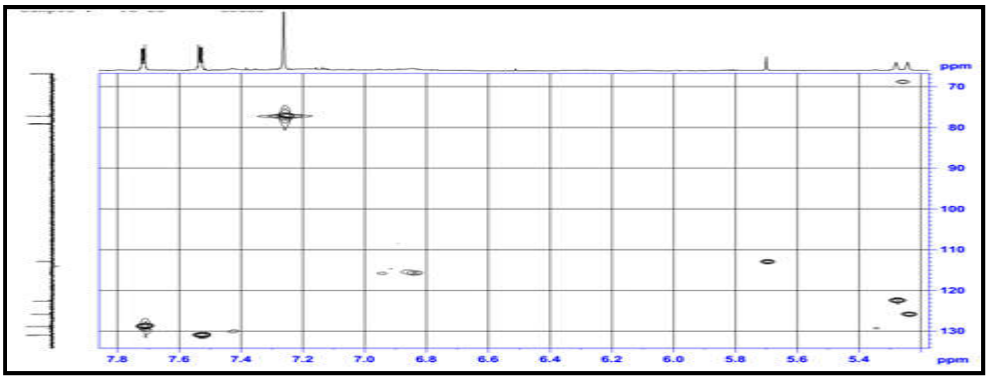


**Figure No.12: HSQC spectra of bioactive compound from leaf extract of *D.oliveri***

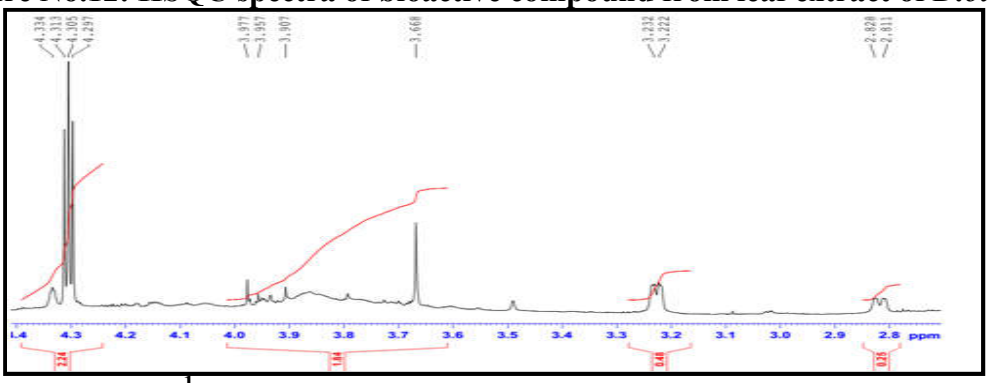


**Figure No.13: $^{1}$H-NMR spectra of compound from root extract of *D.oliveri***

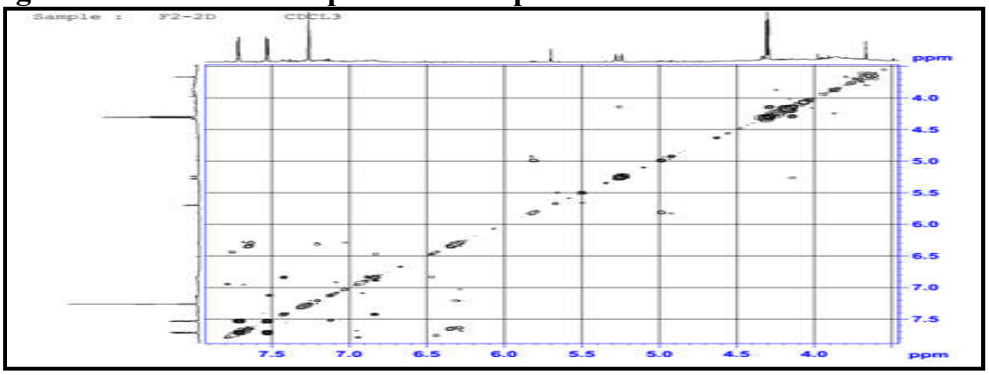


**Figure No.14: COSY-NMR spectra of compound from root extract of *D.oliveri***

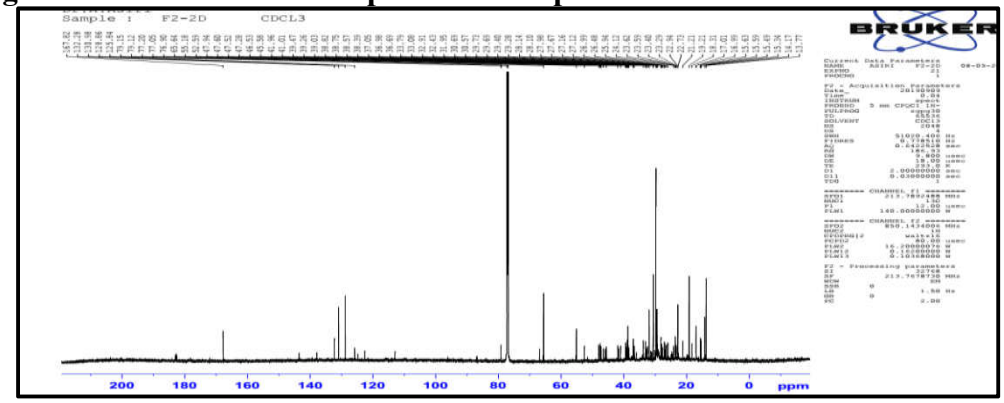


**Figure No.15: 13C-NMR spectra of compound from root extract of *D.oliveri.***

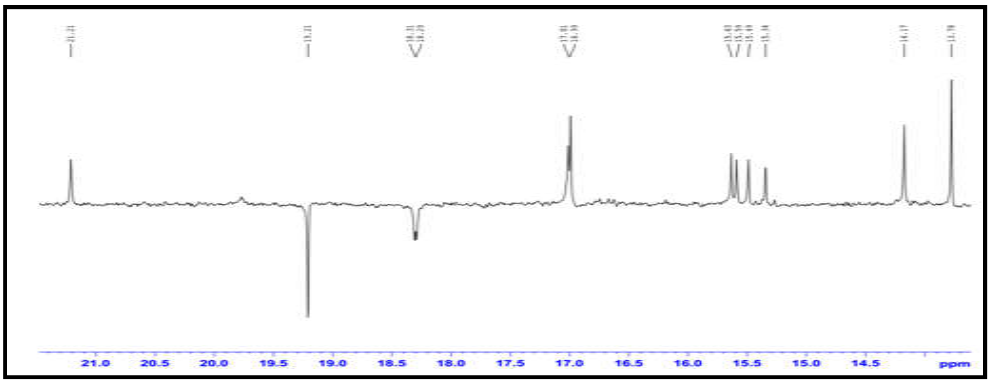



**Figure No.16: 135 DEPT-NMR spectra of compound from root extract of *D.oliveri***

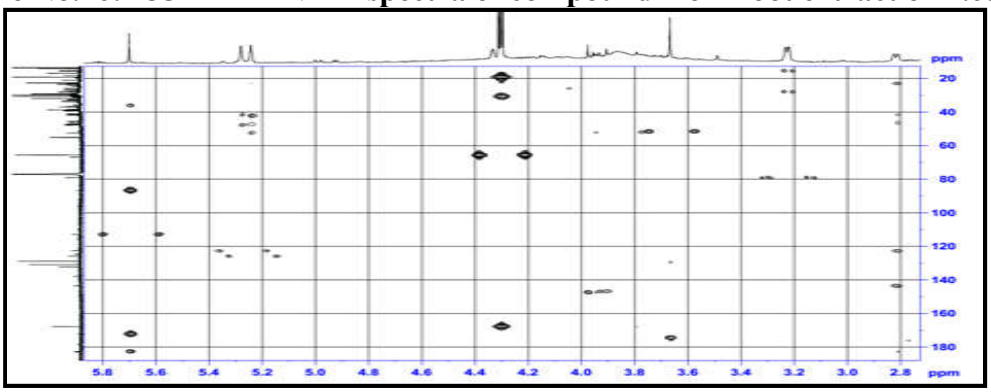



**Figure No.17: HMBC spectra of compound from root extract of *D.oliveri***

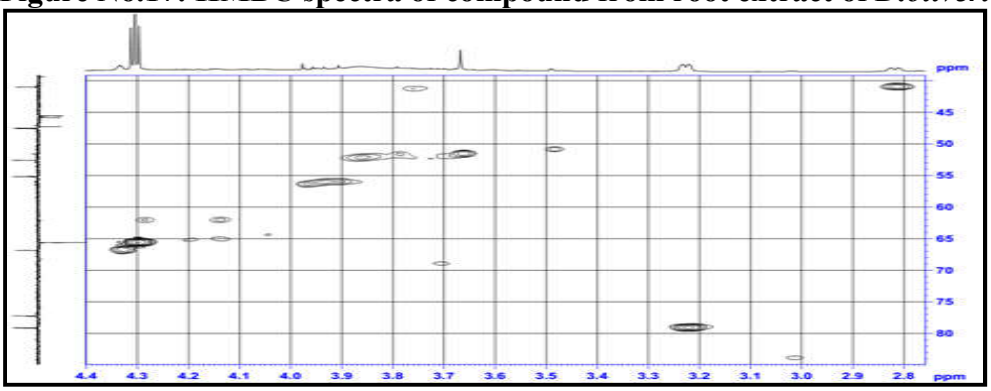



**Figure No.18: HSQC spectra of compound from root extract of *D.oliver***

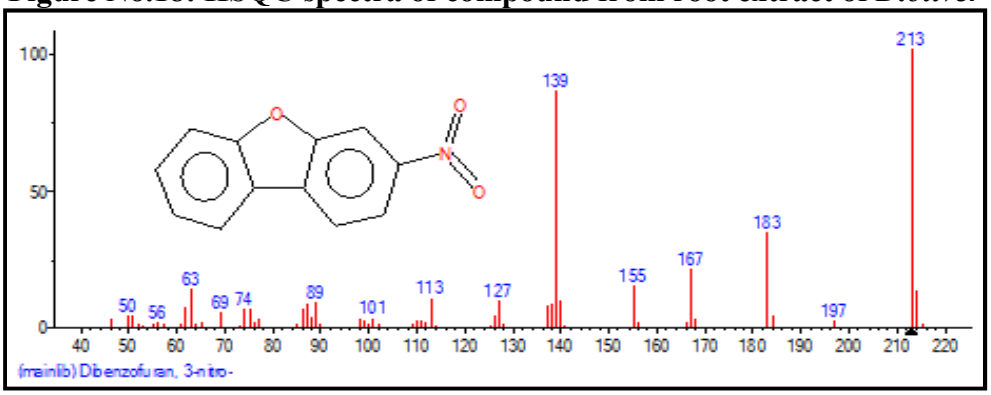



**Figure No.19: Mass spectra of compound from leaf extract of *D.oliveri***

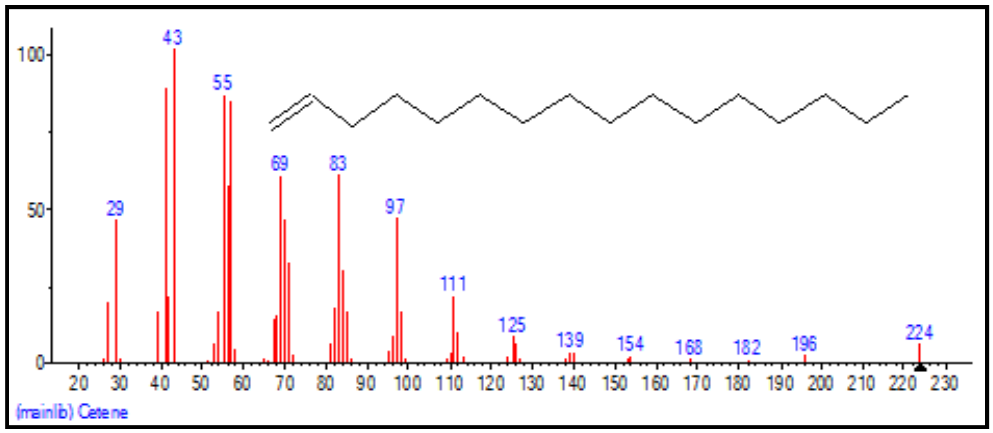
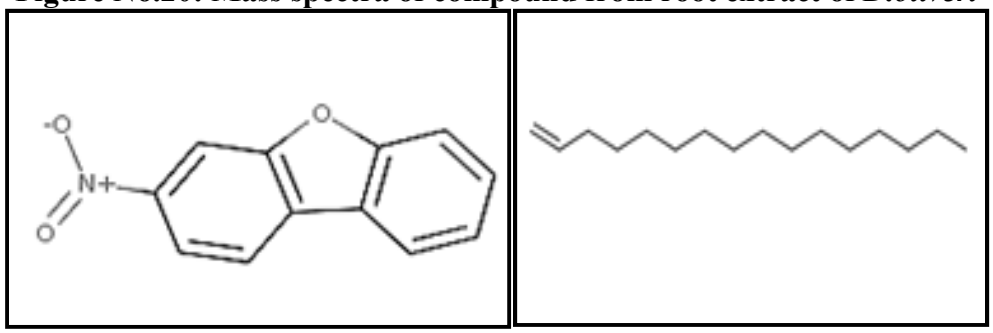



**Figure No.20: Mass spectra of compound from root extract of *D.oliveri***




**a) Dibenzofuran, 3-nitro from leaf** **b) Cetene from root extract**

**Figure No.21: Chemical structures of bioactive compounds from *D.oliveri* leaf and root extracts**

## CONCLUSION

The findings from this current study has shown that D. oliveri leaf and root extracts possess significant anti-snake venom activities against *N.nigricollis* snake venom. This further justifies the use of the extracts to treat toxins from the bite of *N.nigricollis* in folklore medicine especially in Taraba State, Nigeria. Flavonoid compound benzofuran, 3-nitro and a hydrocarbon cetene were fingered to be responsible for these biological activities exhibited by the extracts in the animals. This is the first report on the use of *D.oliveri* extracts to treat snake venom from *N.nigricollis*. The mechanism of the observed biological activities was not known and requires further research.

## AUTHORS CONTRIBUTION

Cletus A. Ukwubile: designed the study, performed the experiments, collected the plant, identified the snake, analyzed the data, prepared the first manuscript draft and searched the literature; Fave Y. Tata: performed the experiments, searched the literatures, revised the manuscript and performed the statistical analysis; Fatima Abdu and Jude A. Odugu: performed the experiment, reviewed the manuscript for final submission, performed the statistical analysis and involved in data collection; James K. Luka: performed the experiments, collected the plant parts, carried out ethnobotanical survey, and procured the snake venom.All authors read, checked and approved the final manuscript for submission.

## COMPETING INTEREST

We have none to declare.

## ETHICAL APPROVAL

Approval for the use of these animals was obtained from the University of Jos, Nigeria.

**ACKNOWLEDGEMENT**

We are thankful to the traditional and herbal medicine practitioners in Donga community in Taraba State, Nigeria for letting us know the ethnomedicinal use of this plant and Mr Ismaila Mejida of the University of Lagos Central Research Lab, for helping us with the spectroscopic aspects of this work.

**CONFLICT OF INTEREST**

We declare that we have no conflict of interest.

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