

## *In vivo* and *in silico* studies of *Dennettia tripetala* essential oil reveal the potential harmful effects of habitual consumption of the plant seed

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### ABSTRACT

*Dennettia tripetala* G. Baker (Annonaceae), is a plant with nutritional, social economy, and medicinal values. Its rising medicinal profile makes this plant a prospect in drug discovery. However, the reported strong addictive potential among habitual consumers makes the need to establish its safety imperative. In this report, we evaluated the safety profile of the essential oil of the seed of *D. tripetala* (EODS) in nulliparous female Wistar rats using *in vivo* single and repeated dose toxicity profiling, as well as *in silico* toxicity profiling of its known seed oil derived phytoconstituents. Our results showed consistent significant dose-dependent alterations in relative body weights, organ-body and organ-brain weight ratios, haematological and biochemical indices, as well as liver and kidney histoarchitectures, following single and repeated oral administrations. Significant alterations in liver and kidney histoarchitectures were consistent with the observed significant increase in AST/ALT ratio, suggesting deleterious effects of EODS on the kidney and liver. However, the lack of alterations in the histoarchitectures of the hippocampus and hypothalamus suggests that the brain may not have been adversely affected. Also, the *in silico* analysis suggests that hepatotoxic effects of EODS may be linked to Benzyl nitrile, Humulene, Linalool, (Z)- $\beta$ -Ocimene. In addition, the failure of  $\beta$ -Phenyl nitroethane, the most abundant phytoconstituent of EODS, to pass phases I and II *in silico* toxicity screening, and the presence of Caryophyllene oxide, a known toxic compound, coupled with the predicted binding of both to DNA and protein, low LD<sub>50</sub> and high percent mortality at 250 mg/kg of repeated doses, further confirmed the potentially toxic nature of EODS. We concluded that based on our *in vivo* and *in silico* observations, there is an urgent need for public education to regulate the excessive consumption of the seeds of *D. tripetala*.

### 1. Introduction

Among the medicinal plants that have found usefulness in ethnomedicine and attracted scientific interests is *D. tripetala* G. Baker (Annonaceae). *D. tripetala*, also known as pepper fruit tree, is widely found in the Southern part of Nigeria [1,2] and the spicy *D. tripetala* mature fruits constitute the main edible portions [1,2]. The seeds are popularly consumed singly or taken with kola nut, garden egg, or palm wine, particularly during cultural entertainment of guests and traditional ceremonies, such as weddings, festivals, and naming ceremonies

[1]. Seed of *D. tripetala* has generally been reported to be used as a spice in flavoring and seasoning foods such as meat, vegetable, soup, and sausage [1–3].

The seed of *D. tripetala* is consumed not just for its spicy taste but for its medicinal value [2]. Different parts of the plants are commonly used by the traditional caregivers in combination with other medicinal plants to treat various ailments including fever, cough, sore throat, infantile convulsion, typhoid, cough, worm infestation, toothache, diabetes, nausea, and vomiting, stomach upset and as agent for masking mouth odour [2,3]. Seeds of *D. tripetala* are applied to diets of pregnant and

**Abbreviations:** OECD, Organization for Economic Co-operation and Development; TG, test guidelines; FOB, functional observatory batteries; ALT, alanine aminotransferase; AST, aspartate aminotransferase; WBC, white blood count; RBC, red blood count; Hb, hemoglobin concentration; HCT, hematocrit; PLT, platelets; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW-CV and RDW-SD, red blood cell distribution width variation coefficient and standard deviation respectively; MPV, mean platelet volume; PDW, platelet distribution width; PCT, plateletcrit.

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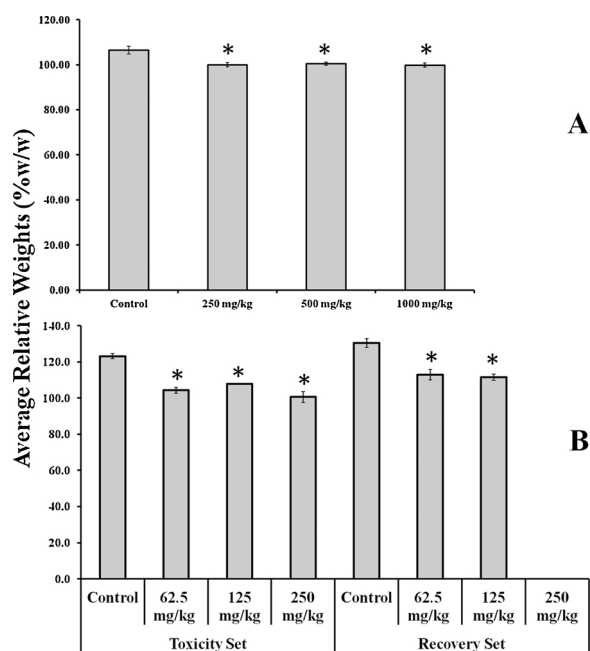
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postpartum women to aid uterine contraction and as an effective insecticide against weevils in grain protection while the fruits are popularly used as stimulants [1,2]. Previous research works have provided evidence in support of the hypoglycemic, antihyperlipidemic, renoprotective, hepatoprotective, and nephroprotective potentials of *D. tripetala* seed extract in rats [2,4]. Also, the essential oil of *D. tripetala* was reported to have elicited behavioural, analgesic, anti-inflammatory, antimicrobial, anticholinesterase, sedative, and muscle relaxant properties in rodents [5–9]. For a more comprehensive review of the uses and medicinal properties of *D. tripetala*, the reader is referred to Iseghohi (2015) [2].

The important nutritive substances of *D. tripetala* fruit/seeds are minerals, vitamins, fibers, protein, carbohydrates, as well as the antioxidant vitamins A, C, and E [1,2]. Phytochemical screening of the leaf and fruit of *D. tripetala* revealed the presence of carbohydrates, tannins, alkaloids, terpenes, flavonoids, phenols, resins, glycosides, and sterols [10–12]. The chemical analysis of the essential oil derived from the fresh fruits, dried fruits, dried seeds, and fresh leaves of *D. tripetala* revealed  $\beta$ -ocimene, linalool,  $\beta$ -phenylnitroethane, and humulene as their common constituents [8]. Consistent with an earlier report [11],  $\beta$ -phenylnitroethane was found to be the predominant constituent of the essential oil of all the parts, ranging from 61.6 % in fresh fruit to 87.4 % in dried seeds of *D. tripetala* [8]. Interestingly,  $\beta$ -phenylnitroethane has earlier been isolated from the seed of *D. tripetala*, and shown to exhibit hypnotic, anticonvulsant, and anxiolytic effects, as well as high memory enhancing activities [8,9].

Despite the many nutritional, social economy, and medicinal usefulness of this plant, there is strong evidence that the plant indeed possesses strong addictive potential among habitual consumers, confirming abusive use of the plants in some local communities in western Nigeria [3,7]. In this study, we evaluated the effects of the graded doses of the essential oil of the seed of *D. tripetala* on haematologic and plasma biochemical indices, and determine the histopathologic effects on rats'



**Fig. 1.** Comparison of average relative weight change following single and repeated oral administration of the oil of seed of *Dennettia tripetala* oil in rats. A is a single dose and B is repeated dose toxicity evaluations. Relative weight change was calculated as a percentage of weight relative to day 0 for each group. The value for 250 mg/kg Recovery Set was 0, indicating no animal in this group survive to recovery phase. A Comparison was done using Student's T-test with a significant level set at  $p < 0.05$ . \*Significant difference when compared with control.

liver and kidney following single and repeated doses and rats' brain (hippocampus and hypothalamus) following repeated doses. We also performed the *in silico* assessment of the toxicity potential of the selected phytoconstituents earlier obtained from the oil of the seed of *D. tripetala* [8].

## 2. Materials and methods

### 2.1. Plant materials

Dried seeds of *D. tripetala* were purchased from Owena market, Owena Town, Ondo-East Local Government and Central market, Ondo town, Ondo-West Local Government Area, Ondo State, Nigeria, in August and October 2018. The dried seeds of *D. tripetala* were identified by Mr. A. Ogunmoriyo, Herbarium Officer, Department of Botany, Obafemi Awolowo University (OAU), Ile-Ife, where voucher specimen with number IFE 15356, was earlier deposited. The plant's name was also checked against [www.theplantlist.com](http://www.theplantlist.com) for authentication.

### 2.2. Extraction and preparation of the essential oils

The dried seeds of *D. tripetala* were air-dried and mashed into coarse powders using pestle and mortar and subjected to hydrodistillation

**Table 1**

Organ - body and organ - brain weight ratios following single and 28-day repeated oral administration of *Dennettia tripetala* oil in rats.

	Acute Toxicity				
	Kidney: Body	Liver: Body	Brain: Body	Kidney: Brain	Liver: Brain
<b>Control (n = 6)</b>	0.0039 ± 0.0003	0.0348 ± 0.0007	0.0128 ± 0.0006	0.3049 ± 0.0023	2.7430 ± 0.0143
<b>250 mg/kg (n = 6)</b>	0.0039 ± 0.0004	0.0440 ± 0.0009*	0.0108 ± 0.0008	0.3544 ± 0.0015*	4.1090 ± 0.0205*
<b>500 mg/kg (n = 6)</b>	0.0041 ± 0.0003	0.0382 ± 0.0004*	0.0125 ± 0.0005	0.3308 ± 0.0032*	3.0700 ± 0.0130*
<b>1000 mg/kg (n = 6)</b>	0.0038 ± 0.0003	0.0390 ± 0.0002*	0.0136 ± 0.0005	0.3797 ± 0.0024*	3.9030 ± 0.0263*
	Repeated Dose Toxicity Set				
	Kidney/ Body	Liver/ Body	Brain/ Body	Kidney/ Brain	Liver/ Brain
<b>Control (n = 6)</b>	0.0027 ± 0.0001	0.0313 ± 0.0006	0.0108 ± 0.0001	0.2500 ± 0.0030	2.8968 ± 0.0201
<b>62.5 mg/kg (n = 6)</b>	0.0028 ± 0.0001	0.0345 ± 0.0003*	0.0111 ± 0.0007	0.2722 ± 0.0045*	2.7916 ± 0.0150*
<b>125 mg/kg (n = 6)</b>	0.0031 ± 0.0004	0.0375 ± 0.0008*	0.0123 ± 0.0003*	0.3760 ± 0.0053*	3.4291 ± 0.0160*
<b>250 mg/kg (n = 3)</b>	0.0061 ± 0.0007*	0.0622 ± 0.0013*	0.0170 ± 0.0013*	0.3571 ± 0.0046*	3.8333 ± 0.0130*
	Repeated Dose Recovery Set				
	Kidney/ Body	Liver/ Body	Brain/ Body	Kidney/ Brain	Liver/ Brain
<b>Control (n = 6)</b>	0.0026 ± 0.0001	0.0318 ± 0.0002 <sup>#</sup>	0.0100 ± 0.0005	0.2528 ± 0.0046	2.8802 ± 0.0188
<b>62.5 mg/kg (n = 6)</b>	0.0040 ± 0.0004 <sup>*,#</sup>	0.0466 ± 0.0011 <sup>*,#</sup>	0.0129 ± 0.0006*	0.3173 ± 0.0047 <sup>*,#</sup>	3.6852 ± 0.0170 <sup>*,#</sup>
<b>125 mg/kg (n = 6)</b>	0.0048 ± 0.0002 <sup>*,#</sup>	0.0487 ± 0.0012 <sup>*,#</sup>	0.0126 ± 0.0003*	0.3789 ± 0.0049*	3.8061 ± 0.0190 <sup>*,#</sup>
<b>250 mg/kg (n = 0)</b>	N/A				

N/A - Due to high mortality at 250 mg/kg, none is available for recovery.

\* Significant difference when compared with respective control.

<sup>#</sup> Significant difference when compared with respective Toxicity set.

**Table 2**Changes in haematological indices following single oral administration of the essential oil of the seed of *Dennettia tripetala* oil in rats.

	Control (n = 6)	250 mg/kg (n = 6)	500 mg/kg (n = 6)	1000 mg/kg (n = 6)
WBC (10 <sup>9</sup> /L)	4.00 ± 0.60	6.64 ± 1.43	4.65 ± 0.75	9.06 ± 1.33*
HGB (g/L)	132.80 ± 3.80	132.20 ± 3.39	137.67 ± 2.28	133.00 ± 2.10
RBC (10 <sup>12</sup> /L)	8.00 ± 0.20	7.54 ± 0.21	7.88 ± 0.15	7.76 ± 0.13
HCT (%)	40.20 ± 1.30	42.40 ± 1.07	44.92 ± 0.95*	44.56 ± 1.00*
MCV (fL)	50.10 ± 0.90	56.34 ± 0.35*	57.08 ± 0.50*	57.48 ± 1.04*
MCH (pg)	16.50 ± 0.30	17.48 ± 0.23*	17.43 ± 0.20*	17.88 ± 0.27*
MCHC(g/L)	329.80 ± 2.90	311.20 ± 2.25*	306.33 ± 2.96*	298.00 ± 3.51*
RDW-CV (%)	15.30 ± 0.50	16.82 ± 0.24*	17.77 ± 0.73*	18.02 ± 0.59*
RDW-SD (fL)	28.10 ± 0.80	32.14 ± 0.67*	33.73 ± 1.33*	34.96 ± 1.10*
PLT(10 <sup>9</sup> /L)	622.00 ± 7.60	516.00 ± 5.36*	539.83 ± 6.62*	545.00 ± 8.49*
MPV(fL)	8.40 ± 0.10	8.34 ± 0.12	8.10 ± 0.13	8.16 ± 0.22
PDW	14.70 ± 0.10	14.71 ± 0.08	14.47 ± 0.11	14.48 ± 0.20
PCT (%)	0.460 ± 0.079	0.429 ± 0.037	0.345 ± 0.035	0.440 ± 0.022

\* Significant difference when compared with respective control.

using Clevenger-type apparatus as earlier described [8,9]. The yield of the characteristic pungent aromatic essential oil of the seed of *D. tripetala* (EODS) was 7.5 ml/kg of the coarse dried seeds. The oil was stored in an amber glass bottle and kept in a refrigerator until use. The oil was emulsified with Tween 80 to a 5 % v/v final concentration

**Table 3**Changes in haematological indices following 28-day repeated oral administration of essential oil of the seed of *Dennettia tripetala* oil in rats.

	Control (n = 6)	62.5 mg/kg (n = 6)	125 mg/kg (n = 6)	250 mg/kg (n = 3)
WBC (10 <sup>9</sup> /L)	6.60 ± 0.53	6.40 ± 0.90	7.32 ± 0.45	10.00 ± 0.65*
HGB(g/L)	135.43 ± 1.98	131.00 ± 1.48	136.83 ± 1.94	73.50 ± 1.38*
RBC(10 <sup>12</sup> /L)	7.06 ± 0.42	8.14 ± 0.12*	8.47 ± 0.15*	5.70 ± 0.19*
HCT (%)	39.84 ± 0.27	40.50 ± 0.50	41.62 ± 1.16	32.40 ± 0.40*
MCV (fL)	57.31 ± 1.56	49.80 ± 0.70*	49.18 ± 0.68*	48.85 ± 1.35*
MCH (pg)	19.54 ± 1.19	16.10 ± 0.30*	16.08 ± 0.23*	15.05 ± 0.45*
MCHC(g/L)	340.00 ± 1.16	323.20 ± 1.39*	328.33 ± 1.02*	329.50 ± 0.50*
RDW-CV(%)	17.17 ± 0.43	16.40 ± 0.60	16.07 ± 0.30*	15.75 ± 0.35*
RDW-SD(fL)	36.16 ± 0.86	29.50 ± 1.00*	27.90 ± 0.56*	28.25 ± 1.05*
PLT(10 <sup>9</sup> /L)	449.29 ± 6.03	515.20 ± 6.36*	585.33 ± 3.37*	345.00 ± 2.00*
MPV(fL)	8.26 ± 0.22	8.10 ± 0.10	8.35 ± 0.13	9.25 ± 0.05*
PDW	12.70 ± 0.08	14.70 ± 0.05*	14.67 ± 0.07*	15.30 ± 0.04*
PCT (%)	0.37 ± 0.05	0.42 ± 0.02	0.49 ± 0.03*	0.59 ± 0.02*
	Control (n = 6)	62.5 mg/kg (n = 6)	125 mg/kg (n = 6)	250 mg/kg (n = 0)
WBC (10 <sup>9</sup> /L)	9.83 ± 0.16 <sup>#</sup>	4.72 ± 0.18*	7.53 ± 0.28*	
HGB(g/L)	134.67 ± 1.39	145.20 ± 1.48 <sup>#</sup>	118.67 ± 1.88 <sup>#</sup>	
RBC(10 <sup>12</sup> /L)	7.13 ± 0.30	8.84 ± 0.13 <sup>#</sup>	7.40 ± 0.19 <sup>#</sup>	
HCT (%)	39.67 ± 1.20	45.56 ± 0.67 <sup>#</sup>	38.52 ± 1.07	
MCV (fL)	56.15 ± 0.50	51.58 ± 0.45 <sup>#</sup>	49.87 ± 0.72*	
MCH (pg)	19.07 ± 0.34	16.36 ± 0.12*	15.97 ± 0.32*	
MCHC(g/L)	340.00 ± 1.64	318.40 ± 1.29 <sup>#</sup>	320.83 ± 1.29 <sup>#</sup>	N/A
RDW-CV(%)	18.23 ± 0.37	16.06 ± 0.25*	18.58 ± 0.45 <sup>#</sup>	
RDW-SD(fL)	37.07 ± 0.13	30.10 ± 0.49*	31.00 ± 0.57 <sup>#</sup>	
PLT(10 <sup>9</sup> /L)	506.00 ± 4.32 <sup>#</sup>	483.80 ± 2.77 <sup>#</sup>	526.83 ± 5.93 <sup>#</sup>	
MPV(fL)	8.13 ± 0.12	8.26 ± 0.24	8.80 ± 0.30*	
PDW	12.67 ± 0.96	14.74 ± 0.14*	14.92 ± 0.16*	
PCT (%)	0.41 ± 0.04	0.40 ± 0.03	0.39 ± 0.06	

N/A - Due to high mortality at 250 mg/kg, none is available for recovery.

\* Significant difference when compared with respective control.

<sup>#</sup> Significant difference when compared with respective Toxicity set.

shortly before oral administration.

### 2.3. Animal care and environmental conditions

Healthy adult nulliparous female Wistar rats (130–150 g) were bred in the animal house, Department of Pharmacology, Faculty of Pharmacy, OAU, Ile-Ife, Nigeria. Rats were housed in standard plastic cages and allowed free access to standard laboratory pellets (Grand Cereals, United African Company Plc., Nigeria) and water *ad libitum* except for an overnight fast before the start of the experiment. The procedure for the animal care was based on the “Guide for the Care and Use of Laboratory Animals – Eighth Edition” [13] and the National Centre for the Replacement, Refinement, and Reduction of animals in research (NC3Rs) guidelines [14] on humane animal care and use.

### 2.4. In vivo experiment design and procedures

#### 2.4.1. Determination of median lethal dose (LD<sub>50</sub>) and sighting study

The median lethal dose (LD<sub>50</sub>) was determined using a modification of Lorke’s method [15]. Briefly, a preliminary check was conducted with 1000 and 1600 mg/kg of EODS, being the highest and lowest doses of Lorke’s phases I and II respectively, using one rat per dose. Following confirmation of death at 1600 mg/kg, one rat each was orally administered a range of doses (250, 500, 750, 1000, 1250, and 1500 mg/kg) lower than 1600 mg/kg, for both LD<sub>50</sub> determination and sighting studies. The maximum tolerable and minimum toxic doses were confirmed and LD<sub>50</sub> was calculated as per Lorke’s method [15]. Sighting study was conducted to determine humane end-point criteria by monitoring functional observation battery (FOB) [16,17], which included piloerection, eye colour, gait, sedation, skin colour, respiration distress, tremor, convulsion, loss of righting reflex, and death, as earlier described [18].

#### 2.4.2. Single-dose acute toxicity tests

Single-dose toxicity profiling was carried out in line with OECD

**Table 4**Changes in biochemical indices following single and 28-Day repeated oral administration of *Dennettia tripetala* oil in rats.

	Acute Toxicity				
	Creatinine (mg/dl)	ALT (IU/L)	AST (IU/L)	AST/ALT	Urea (mmol/L)
Control (n = 6)	2.378 ± 0.062	9.746 ± 0.792	9.982 ± 0.894	0.972 ± 0.062	6.163 ± 0.478
250 mg/kg (n = 6)	3.251 ± 0.016*	10.482 ± 0.39	19.523 ± 0.522*	2.008 ± 0.067*	6.702 ± 0.952
500 mg/kg (n = 6)	3.030 ± 0.064*	13.612 ± 0.852*	22.515 ± 0.458*	1.680 ± 0.071*	6.686 ± 1.025
1000 mg/kg (n = 6)	3.018 ± 0.085*	15.083 ± 0.724*	74.038 ± 1.461*	4.858 ± 0.136*	7.999 ± 0.657*
	Repeated Dose Toxicity Set				
	Creatinine (mg/dl)	ALT (IU/L)	AST (IU/L)	AST/ALT	Urea (mmol/L)
Control (n = 6)	3.300 ± 0.041	37.680 ± 0.153	15.902 ± 1.329	0.423 ± 0.037	4.888 ± 0.733
62.5 mg/kg (n = 6)	7.675 ± 0.347*	67.977 ± 1.274*	33.069 ± 2.052*	0.485 ± 0.041	8.158 ± 0.037*
125 mg/kg (n = 6)	8.288 ± 0.026*	92.327 ± 1.840*	98.262 ± 3.503*	1.063 ± 0.017*	9.420 ± 0.061*
250 mg/kg (n = 3)	8.467 ± 0.906*	88.196 ± 0.997*	218.546 ± 3.595*	2.477 ± 0.048*	19.667 ± 1.061*
	Repeated Dose Recovery Set				
	Creatinine (mg/dl)	ALT (IU/L)	AST (IU/L)	AST/ALT	Urea (mmol/L)
Control (n = 6)	3.200 ± 0.039	25.599 ± 0.989	18.078 ± 0.777	0.723 ± 0.034	3.466 ± 0.262
62.5 mg/kg (n = 6)	6.117 ± 0.469 <sup>#</sup>	65.567 ± 0.422*	62.233 ± 2.574 <sup>#</sup>	0.950 ± 0.045 <sup>#</sup>	5.767 ± 0.176 <sup>#</sup>
125 mg/kg (n = 6)	6.988 ± 0.026 <sup>#</sup>	93.704 ± 0.921*	136.072 ± 1.628 <sup>#</sup>	1.452 ± 0.016 <sup>#</sup>	7.360 ± 0.282 <sup>#</sup>
250 mg/kg (n = 0)	N/A				

N/A - Due to high mortality at 250 mg/kg, none is available for recovery.

\* Significant difference when compared with respective control.

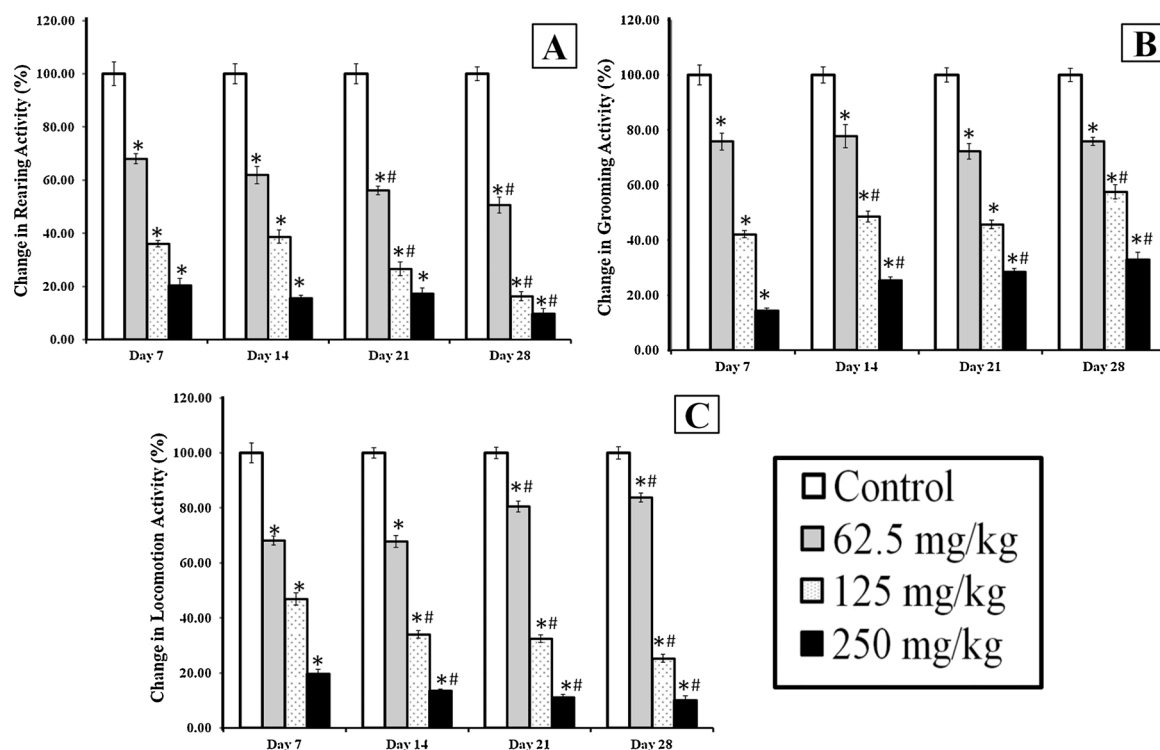
<sup>#</sup> Significant difference when compared with respective Toxicity set.

guidelines 423 [19] as earlier reported [18,20]. In this study, four groups of rats containing randomly selected 6 rats per group were orally administered 250, 500, 1000 mg/kg body weight of emulsified EODS and 5% v/v Tween 80 (control) after an overnight fast. Monitoring of weights and FOB by cage side observation were as previously described [18,20].

#### 2.4.3. Repeated dose toxicity profiling

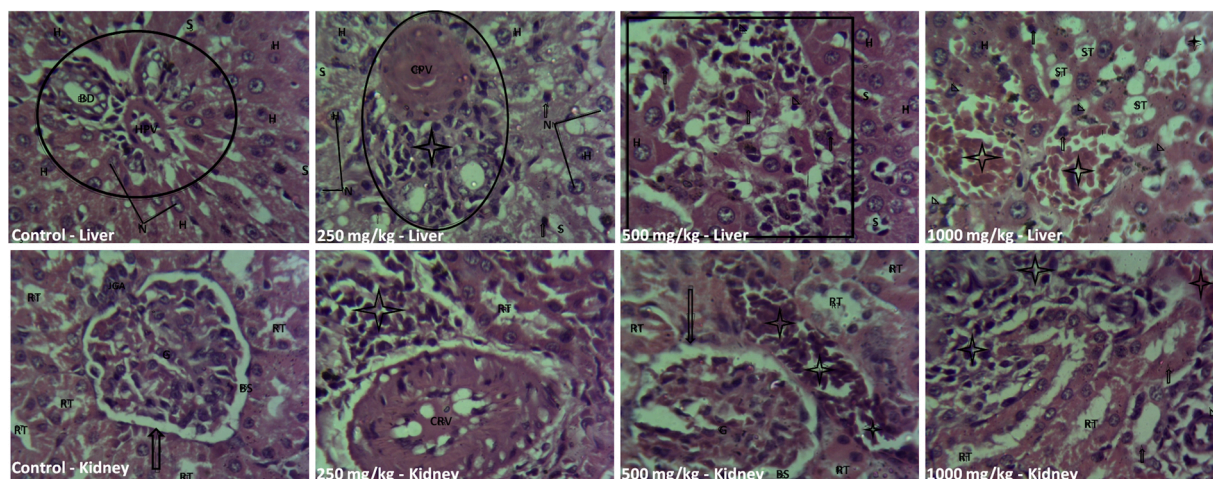
The repeated dose toxicity profiling was conducted in accordance

with the 28 days repeated dose OECD TG 407 [21] as previously reported [18,20]. Forty-eight (48) rats were randomly allotted into four groups of 12 rats each and daily orally administered with 62.5, 125, and 250 mg/kg body weight of emulsified EODS and 5% v/v Tween 80 for control, respectively, for 28 days. The highest dose of the test agent was not greater than one-fifth of the LD<sub>50</sub> as earlier suggested [18,20] and the administered volume was not more than 5 ml/kg. On day 29, the animals in each group were randomly and equally divided into toxicity set (TS) and recovery set (RS). Body weight and FOB monitoring with



**Fig. 2.** The effects of 28-day repeated oral administration of the oil of seed of *D. tripetala* on novelty-induced rearing, grooming, and locomotion in rats. Comparisons were done using Student's T-test with a significant level set at  $p < 0.05$ . \*Significant difference when compared with control. #Significant difference when compared with Day 7.





**Fig. 3.** Photomicrographs of Liver and Kidney following single oral administration of the oil of seed of *Dermnetia tripetela*. LIVER: H, hepatocytes; S, sinusoids; The Portal Region (CIRCLE); HPV, hepatic portal vessels; BD, bile duct; ST, hepatic steatosis; CPV, congested portal vessel. STAR: Mild periportal infiltration with inflammatory cells or Congested sinusoids. ARROW: Foci of degenerating hepatocytes with marked infiltration of inflammatory cells or degenerating hepatocytes and infiltration of chronic inflammatory cells. KIDNEY: G, glomeruli; BS, Bowman's capsule and Bowman's space; RT, renal tubules; STAR, Interstitium; and CRV, renal vessel. H&E staining was used and Photomicrographs were taken using Leica DM750 Camera Microscope (Magnification x 400).

cage side observation was as earlier described [18,20].

#### 2.4.4. Repeated dose novelty induced behaviours

The cumulative effects of repeated doses on novelty-induced locomotion, rearing and grooming were evaluated in rats on the 7th, 14th, 21 st, and 28th day of treatment using established procedures [22].

#### 2.4.5. Sample collection

All surviving rats were humanely euthanized by cervical dislocation and samples were collected on day 14 (acute), day 29 (TS), and day 49 (RS). Blood samples were collected into K3 EDTA tubes for haematological and biochemical analysis by cardiac puncture. Brain, kidney, and liver were removed and weighed, and liver and kidney were further preserved in 10 % formal saline for histological assessment [20].

#### 2.4.6. Evaluation of haematological parameters

Blood samples were analyzed for complete blood count using Mindray BC 2800 Haematology Auto-Analyzer [18,20,23].

#### 2.4.7. Evaluation of plasma biochemistry

Blood samples in K3 EDTA sample bottles were subjected to centrifugation at 3000 g for 5 min to obtain the plasma. Quantitative determination of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities, as well as the urea and creatinine levels, were done using standard laboratory kits (Randox Laboratories Limited, Crumlin, County Antrim, BT294QY, United Kingdom), as per the manufacturer's instructions.

#### 2.4.8. Histopathology examination

Histological assessments of liver and kidney histoarchitectures following single and repeated doses were conducted using standard procedures as previously described [20,24]. Prepared slides from not less than three specimens per dose level were mounted in DPX (Distrene Plasticizer and xylene) with a coverslip. Hematoxylin and eosin (H&E) staining procedure was used. Stained sections were reviewed and photomicrographs were taken using Hirocam digital microscope eyepiece (C and A Scientific) interfaced with an Olympus microscope (×400).

### 2.5. In silico toxicity assessment of selected phytoconstituents of EODS

The 3D SDF files and SMILES representation of the eight selected phytoconstituents reported to be present in the seed oil of *D. tripetala* [8]

were downloaded from PubChem database [25,26]. *In silico* toxicity assessment was done using VEGA *in silico* platform version 1.1.5-b22 [27,28], Toxicity Estimation Software Tool (T.E.S.T) version 4.2.1 [29], and Toxtree version 3.1.0 [30] software, and GUSAR online platform [31] with the globally harmonized system (GHS) of toxic agent classification, as earlier described [32]. Also, the compounds were submitted to pkCSM [33] and vNN ADMET [34] online servers for further physicochemical properties and toxicity assessment.

### 2.6. Statistical analysis and data presentation

Significant differences were determined using the Student's T-test and one-way analysis of variance (ANOVA) followed by Dunnett's posthoc test. Differences were considered significant at  $p < 0.05$ . All data were expressed as mean  $\pm$  standard error of mean (SEM).

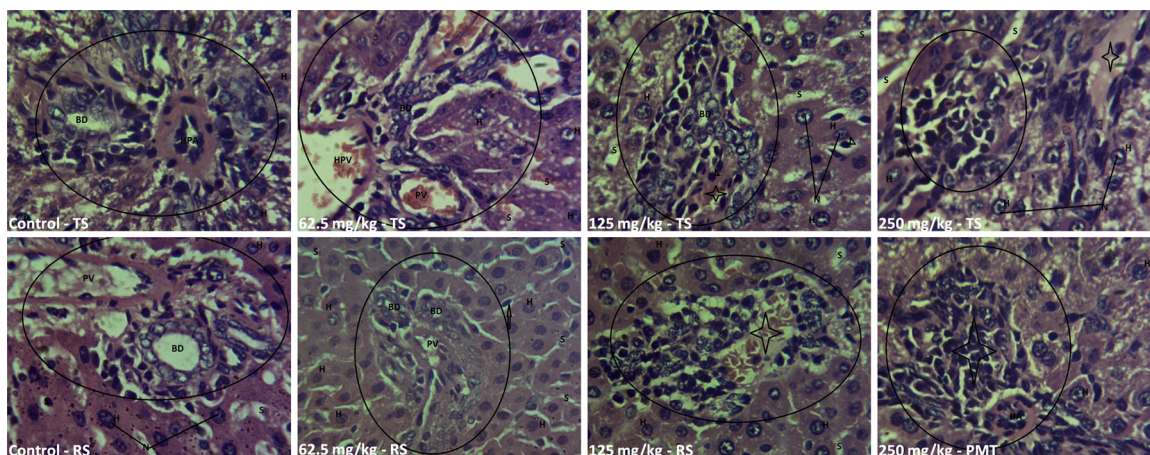
## 3. Results

### 3.1. Preliminary assessment of the toxicity potential of orally administered EODS

The preliminary assessment involves LD<sub>50</sub> determination, sighting study, and monitoring of functional observation battery (FOB) by cage side observation. Sighting study was aimed at determining humane endpoint criteria. The LD<sub>50</sub> of the essential oil of the seed of *D. tripetala* (EODS) was found to be 1369 mg/kg p.o., similar to an earlier report [3]. Sighting study showed that the combination of intense sedation, loss of writhing reflex, and respiratory distress can be regarded as humane endpoint criteria (Table S1), being the obvious signs that preceded death. Cage side observation with FOB revealed that the observed sedation and loss of righting reflex were more intense with repeated dosing, with 75 % of the animals having respiratory distress and died at 250 mg/kg. Thus, there was no recovery group for the group of 250 mg/kg repeated dose.

### 3.2. The effects of the EODS on body weights and organ weight ratios

The effects of EODS on weights following single and repeated oral administration on rats revealed a significant ( $p < 0.05$ ) reduction in average relative weight at all dose levels (Fig. 1). The Liver:body, kidney:brain, and liver:brain ratios were significantly higher than the control in all tested doses following single oral administration of EODS



**Fig. 4.** Photomicrographs of Liver following 28-day repeated oral administration of the oil of seed of *Derrnetia tripetela*. TS, Toxicity set; RS, Recovery set; PMT, Post-mortem toxicity; H, hepatocytes; S, sinusoids; HPA, hepatic portal vessels; BD, bile duct; PV, portal vessels; N, Nucleus; CIRCLE: The portal region; STAR: periportal hepatitis and/or and vascular congestion.; ARROW: mild hepatitis. H&E staining was used and Photomicrographs were taken using Leica DM750 Camera Microscope (Magnification x 400).

(Table 1). Following repeated doses, there were dose-dependent increases in organ:body and organ:brain weight ratios that were significant ( $p < 0.05$ ) at 250 mg/kg for kidney:body, 125 and 250 mg/kg for brain:body, and at all tested doses for liver:body, kidney:brain and liver:brain weight ratios. In general, the significant increases ( $p < 0.05$ ) were maintained during recovery, and to a large extent significantly higher than the toxicity set, suggesting a form of persistent effects of EODS on rats (Fig. 1B and Table 1).

### 3.3. Effects of oral administration of the EODS on hematological indices in rats

There were widespread significant alterations in most haematological indices of toxicological importance following single and repeated doses (Tables 2 and 3). The number of altered indices increases with an increase in dose, suggesting dose-dependent toxic effects. This is more pronounced with repeated doses having 70–100 % altered indices in all tested doses of TS and RS (Table 3). The RS is mostly significantly different from TS and the control, respectively indicating the extent of recovery and persistent effects of repeated administration of 62.5 and 125 mg/kg (Table 3).

### 3.4. Effects of oral administration of the EODS on biochemical indices of liver and kidney functions

In this study, creatinine, ALT, and AST were significantly increased ( $p < 0.05$ ) dose-dependently following single-dose oral administration of EODS (Table 4). AST/ALT ratio was significantly increased at all tested doses, while urea was only significantly increased at 1000 mg/kg

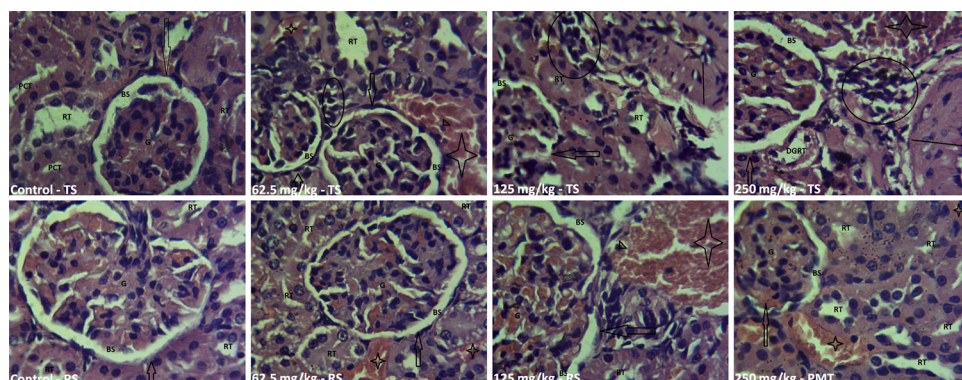
(Table 4). The effects appear more pronounced following repeated administration with all the biochemical indices showing significant increases at all dose levels when compared to control and single-dose levels. Also, AST/ALT ratios were significantly greater than 1 at 125 and 250 mg/kg (TS) and 125 mg/kg (RS), suggesting toxicological effects of persistent nature.

### 3.5. Effects of oral administration of the EODS on novelty induced rearing, grooming, and locomotion in rats

Following repeated oral administration of EODS to rats, rearing, grooming, and locomotion activities were significantly ( $p < 0.05$ ) dose-dependently reduced (Fig. 2). Rearing was significantly reduced with repeated doses on days 21 (62.5 and 125 mg/kg) and 28 (all tested doses) when compared to day 7 (Fig. 2A). Also, there were proportionally significant ( $p < 0.05$ ) increases in grooming on days 14 (125 and 250 mg/kg), 21 (250 mg/kg) and 28 (125 and 250 mg/kg) when compared with day 7 (Fig. 2B). Furthermore, locomotion was proportionally significantly ( $p < 0.05$ ) increased on day 14 (125 and 250 mg/kg), and days 21 and 28 in all tested doses when compared with day 7 (Fig. 2C).

### 3.6. Effects of oral administration of EODS on liver, kidney and brain histoarchitectures in rats

The liver histoarchitectures of the control groups following single-dose and repeated oral administration of EODS (Figs. 3–5) revealed tissue composed predominantly of the hepatic parenchymal and portal regions. The hepatocytes appear polygonal, disposed in sheet with a well-outlined nucleus, separated by the sinusoids with thin endothelial



**Fig. 5.** Photomicrographs of Kidney following 28-day repeated oral administration of the oil of seed of *Derrnetia tripetela*. TS, Toxicity set; RS, Recovery set; PMT, Post-mortem toxicity; G, glomeruli; BS, bowman's capsule and bowman's space; RT, renal tubules; DCT, distal convoluted tubule; PCT, proximal convoluted tubule; ARROW: renal corpuscle. STAR: Interstitial congestion; CIRCLE: Inflammatory cells. H&E staining was used and Photomicrographs were taken using Leica DM750 Camera Microscope (Magnification x 400).



lining, and free from collections and inflammatory cells. The portal region was composed of branches of the hepatic portal vessels, while the bile duct appeared normal (Figs. 3 and 4). Similarly, the kidney histoarchitectures revealed renal tissue consisting of the renal corpuscle which is made up of the glomerulus surrounded by the podocytes and separated by a defined Bowman's space. Also shown are the renal tubules, consisting of several segments, and are lined by columnar-cuboidal epithelium with the proximal convoluted tubules showing densely packed microvilli forming a brush border. The interstitium is also free from inflammatory cells, and congestion (Figs. 3 and 5).

However, in general, following administration of EODS, histopathology of the liver and kidney showed mild to severe features on the tissues microanatomy, such as cellular adaptation features, inflammatory responses, and cellular death (Figs. 3–5). Specifically, following single-dose oral administration of EODS, the portal region of the liver showed mild periportal infiltration with inflammatory cells and congested portal vessel with 250 mg/kg. With 500 mg/kg the foci of degenerating hepatocytes were marked by infiltration of inflammatory cells. Also seen at 1000 mg/kg were marked degenerating hepatocytes, congested sinusoids, hepatic steatosis, and infiltration of chronic inflammatory cells (Fig. 3). Furthermore, the kidney at 250 mg/kg, showed moderate interstitial nephritis and congested renal vessel, and renal tubules appear unremarkable. With 500 mg/kg, the interstitium shows marked congestion. The intensity of the effects was more at 1000 mg/kg, showing marked infiltration of the interstitium with inflammation cells (interstitial nephritis) and interstitial oedema, and some tubules show mild tubular degeneration (Fig. 3).

Meanwhile, following repeated oral administration (Figs. 4 and 5), the histoarchitectures of the liver at 62.5 mg/kg appear essentially normal, similar to control. On the other hand, at 125 mg/kg, the portal region showed mild hepatitis and vascular congestion (TS and RS), and periportal hepatitis and congestion at 250 mg/kg (TS and PMT - Post-mortem toxicity) (Fig. 4). Similarly, the histoarchitectures of the kidney showed that all test groups (62.5, 125, and 250 mg/kg TS, RS, and PMT) were marked with infiltration of the interstitium by inflammatory cells, and marked interstitial congestion. In addition, 125 mg/kg (TS) also showed medial hypertrophy in the branch of the renal artery, and 250 mg/kg (TS) has the branch of the renal artery showing medial hypertrophy, and degenerative changes in some tubules (Fig. 5).

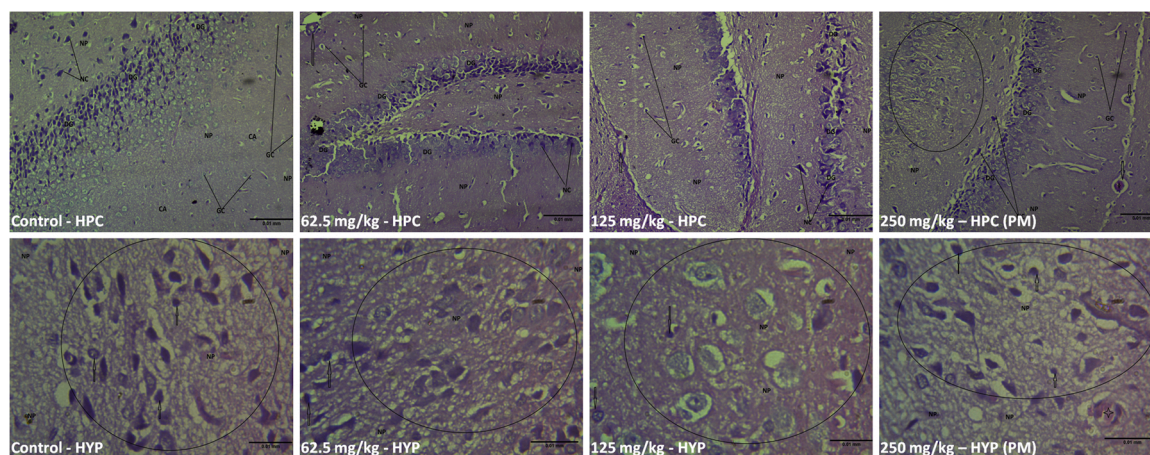
Furthermore, histological assessment of the brain, following repeated doses, showed sections of the hippocampus composed of the cornu ammonis (CA) regions made of the giant pyramidal cells and the dentate gyrus containing the granular cells (Fig. 6). The glia cells were seen distributed sparsely within the neuropil, and the capillaries appeared

distinctly without congestion, erosion, or any form of microangiopathy. On the other hand, except at 250 mg/kg (post mortem) where capillaries appear congested, the histoarchitecture of the hypothalamus shows clusters of hypothalamic nuclei, consisting of nerve cells (Fig. 6). Glial cells were seen dispersed within the Neuropil and the neurons appeared polyhedral and are organized in clusters. Also, the capillaries appear unremarkable and there is no evidence of chromatolysis/peripheral chromatolysis or Gliosis. Thus, brain histoarchitectures appear essentially normal, unaffected by the toxic effects of EODS (Fig. 6).

### 3.7. *In silico* evidence of dose-dependent organ toxicity profile of EODS

Basic physicochemical properties and cytochrome P450 enzymes effects, as provided by PubChem database [35] and pkCSM online platform [33] are provided in Table S2. Detailed analysis of VEGA, T.E. S.T, and Toxtree [27–30] toxicity predictions are presented in Tables S3 - S5. The EODS compounds showed good intestinal absorption and low skin permeability (except Caryophyllene Oxide) (Table S2). They displayed a varying degree of CNS permeability. While benzylnitrile and  $\beta$ -Phenylnitroethane showed potential to inhibit CYP1A2, only Caryophyllene Oxide is predicted to be able to inhibit CYP1A2, CYP2C19, and CYP2C9 (Table S2). Furthermore,  $\beta$ -Phenylnitroethane, the most abundant bioactive constituents, benzylnitrile, and caryophyllene oxide were considered to show high potential for toxic effects by Cramer rules (Table 5). Depending on the dose and route of administration, Gumar GHS classification showed that all the selected compounds have the potential to induce toxic effects. However, while only caryophyllene oxide was predicted to be of toxicological concern as severally reported [35], the predicted potential binding of these three compounds to DNA and protein (Table 5) may have a profound influence on their toxicity potentials.

In addition, all the compounds showed skin sensitization potential, our *in silico* analysis suggests that hepatotoxic effects of EODS may be linked to the potential toxic activities of benzylnitrile, humulene, linalool, and (Z)- $\beta$ -Ocimene (Tables 6 and S2), depending on the applicability of restricted or unrestricted domains [34]. Interestingly, only caryophyllene oxide belongs to category D of mutagenic, developmental, and carcinogenic toxicity classification, consistent with several reports documented by PubChem database [35].  $\beta$ -Phenylnitroethane, humulene, and (Z)- $\beta$ -Ocimene also failed phases I and II consensus toxicity analysis, having shown developmental and carcinogenic potentials, thus categorized as class II (C).



**Fig. 6.** Photomicrographs of Hippocampus (HPC) and Hypothalamus (HYP) sections of the Brain following 28-day repeated oral administration of the oil of *Dermnetia tripetela*. HPC, Hippocampus; HYP, Hypothalamus; PM, Post-mortem; CA, Cornu Ammonis; DG, Dentate gyrus; GC, the Glia cells; NP, Neuropil; ARROW: Glial cells. STAR: Congested capillaries; CIRCLE: Nerve cells. H&E staining was used and Photomicrographs were taken using Leica DM750 Camera Microscope (Magnification x 400).

**Table 5**  
Predicted Toxicity Classifications and Binding potentials for selected EODS Compounds.

S/No.	I.D.	Cramer	GHS LD <sub>50</sub> CLASSIFICATION				Safety Concern?	Binding Potential	
			Oral	Intraperitoneal	Subcutaneous	Intravenous		DNA	Protein
1	β-Phenyl nitroethane	III	4	4	3	3	NO	YES	YES
2	Linalool	I	5	4	4	4	NO	NO	YES
3	β-Caryophyllene	I	5	4	4	3	NO	NO	YES
4	Caryophyllene Oxide	III	5	4	4	3	YES	YES	YES
5	Benzyl nitrile	III	4	5	4	3	NO	YES	YES
6	Elemol	I	5	4	3	3	NO	NO	YES
7	Humulene	I	5	4	4	3	NO	NO	YES
8	(Z)-β-Ocimene	I	5	5	4	4	NO	NO	YES

Cramer classification uses scale I to III, where I is low, II is intermediate and III is high. 1–5 represents Category 1–5 of Globally Harmonized System (GHS) [32] toxic level Classification. The prediction was done using Toxtree software [30] and Gumar online platform [31]. The reported % composition in seed oil are 87.4, 8.8, 2.1, 0.6, 0.5, 0.2 for phytochemicals 1–6 respectively, and Trace for 7 and 8 [8].

**Table 6**  
*In silico* Toxicity screening of the selected EODS compounds.

S/No.	NAMES	PHASE I & II CONSENSUS TOXICITY ANALYSIS				<i>In vivo</i> Micronucleus	Hepatotoxicity	Skin Sensitisation
		Mutagenicity	Developmental	Carcinogenicity	REMARKS			
1	β-Phenyl nitroethane	NO	YES	YES	FAIL	Class II (C)	NO*	YES
2	Linalool	NO	YES	NO	PASS	Class II (A)	YES	YES
3	β-Caryophyllene	NO	YES	NO	PASS	Class II (A)	NO	YES
4	Caryophyllene oxide	YES	YES	YES	FAIL	Class I (D)	NO	YES
5	Benzyl nitrile	NO	YES	NO	PASS	Class II (A)	YES*	YES
6	Elemol	NO	YES	NO	PASS	Class II (A)	NO	YES
7	Humulene	NO	YES	YES	FAIL	Class II (C)	YES	YES
8	(Z)-β-Ocimene	NO	YES	YES	FAIL	Class II (C)	YES	YES

Class I are compounds that tested positive to at least one micronucleus assay, Class II are compounds that tested negative to all micronucleus assays. Class II (A) or Class II (C) are category A or C compounds which belong to Class II and passed or failed Phases I and II toxicity assessment respectively; Class I (D) are category D compounds that belong to Class I and failed Phases I and II consensus. \*Positive (YES) or Negative (NO) for Hepatotoxicity using both restricted and unrestricted applicability domains in vNN ADMET webserver (others could only be predicted with unrestricted applicability domains). The reported % compositions in seed oil are 87.4, 8.8, 2.1, 0.6, 0.5, 0.2 for phytochemicals 1–6 respectively, and Trace for 7 and 8 [8].

#### 4. Discussion

The essential oil of *D. tripetala* have been shown to demonstrate numerous noteworthy neuropharmacological activities in several animal models [5–8]. Considering the habitual consumption of the seed of this plant among the people in the locality [3], there is a growing concern about its toxicity on continuous use, hence the safety profile of this oil was evaluated after repeated oral administration in rats. The findings of this study revealed the high toxicity potential of this oil.

The cage side observations with FOB revealed that the toxic effects of EODS depend on dose and dosage frequency, going by increases in the number of animals showing behavioural changes with increasing doses and frequency. In addition, the clear signs of intense sedation, loss of writhing reflect and respiratory distress associated with death at 250 mg/kg may serve as a pointer to more serious underlying alterations in vital physiologic functions at the cellular and biochemical level. Similarly, evidence of the toxic effects of the oil is further supported by the significant reduction in relative weight change (Fig. 1) and organ/body and organ/brain weight ratios (Table 1) in most of the tested single and repeated doses, suggesting a potential for multi-organs toxicity [18,20,36,37], including hepatotoxicity, haematotoxicity, neurotoxicity, renal toxicity, tubular hypertrophy and/or chronic progressive nephropathy [36–38].

In addition, our results also revealed widespread significant alterations in most haematological indices, which increases with dose, more pronounced with repeated dosing, and persist during the recovery period (Tables 2 and 3), indicating adverse effects of EODS on rats, which may have resulted from direct effects on the blood components and/or indirect effects on other vital organs [39,40]. For instance, apart from commonly used RBC and differential WBC counts, others, such as MPV and PDW, are considered important predictors of mortality and diagnosis of persistent organ failure [41–43]. Also, RDW-CV and

RDW-SD are useful in detecting underlying adverse effects of a tested agent, differentiating iron deficiency anaemia and thalassemia, and predicting adverse effects, including mortality [41,42]. Therefore, the observed changes in haematological indices could further confirm the potential of EODS in multi-organs toxicity.

Furthermore, the observed dose-dependent widespread alteration in AST, ALT, and AST/ALT ratios following single and repeated administration of EODS in rats (Table 4), may be a reflection of underlying hepatocellular injury, cellular damage and/or leakages, and loss of functional integrity of hepatic cell membrane [44,45]. In fact, progressive liver functional impairment or severity of hepatic damage is said to be associated with an increase in AST/ALT ratio [46–49]. Similarly, the significant alteration in creatinine and urea by EODS suggest an indication of kidney injury [50–52]. Urea and creatinine are markers of kidney function, and earlier reports have attributed an increase in serum creatinine content to glomerular and tubular dysfunction [50–52]. Though there appears to be a very slow potential for recovery, the observed persistent increases in AST, ALT, AST/ALT ratio, creatinine and urea following the recovery period is a pointer to the potential of EODS to induce long-term persistent toxicological effects (Table 4). However, the observed difference between the control groups of acute and repeated dosing may be due to differences in the treatment regimen, repeated animal handling, and the effect of repeated exposure to the control vehicle (Tween 80).

A better appreciation of the toxic effects of EODS is seen in the altered histoarchitectures of the liver and kidney (Figs. 3–5). While kidney was affected in all tested doses (single and repeated), it is surprising to note that, even though a single assort produced marked histological changes in the liver, repeated administration of 62.5 mg/kg produced essentially normal liver histoarchitectures, suggesting that dose is of importance in the toxic effects of EODS. However, though available reports [1–9] have documented the central nervous system



effects of EODS, it interesting to note that contrary to the observed toxic effects of the oil on the kidney and liver, histological assessment of the brain did not reveal any significant alteration in the histoarchitectures of hippocampus and hypothalamus, except for capillaries that appeared congested at 250 mg/kg (post mortem) (Fig. 6), suggesting lack of severe toxic effect on the brain. It is possible that the neuroprotective effects of vitamins and other micronutrients found to be in high quantities in *D. tripetala* [2] may have helped to ameliorate the neurotoxic effects, such as sedation, drowsiness, CNS and respiratory depression, observed prior to death. Meanwhile, it should be noted that while the toxic effects on the liver and kidney increase with dose and frequency, persisted effects, as observed during recovery suggest serious deleterious effects on the kidney and liver. These effects are consistent with the observed alterations in haematological and biochemical indices, especially, changes in AST/ALT ratio as earlier reported [46–49]. Thus, it can be proposed that, though lack of induction or inhibition of major cytochrome P450 enzymes by most of the phytoconstituents, as predicted by *in silico* profiling, may help to explain the observed effects at a lower dose, the predicted hepatotoxic effects of benzylnitrile, humulene, linalool, and (Z)- $\beta$ -Ocimene compounds may be dose-dependent. However, the predicted categorization of  $\beta$ -Phenylnitroethane, the most abundant phytoconstituent of EODS, as Class II (C), having failed developmental and carcinogenicity tests, and tested positive to micronucleus assay, coupled with the presence of Caryophyllene oxide, a compound with known safety concern [25,26], and their predicted binding to DNA and protein, should raise serious concern on the potentially harmful effects of indiscriminate consumption of the seed of *D. tripetala*. Meanwhile, the available experimental oral toxic classification of  $\beta$ -Phenyl nitroethane (GHS 4), Linalool (GHS 4), Caryophyllene Oxide (GHS 4), Benzylnitrile (GHS 3), and Elemol (GHS 4) [25,2], are similar to the predicted values (Table 5).

Meanwhile, it should be noted that the attempt to accurately predict or determine the toxicity of compound mixtures, such as the essential oil of the seed of *D. Tripetala* in this study, is not without challenges. These challenges are related to the potential for additivity, synergism, potentiation, and/or antagonism of its many chemical components at pharmacodynamic and /or pharmacokinetic levels, as well as mimicking actual plasma concentration of the oil when consumed [53]. While we have attempted to correlate observed *in vivo* toxicity effects with the individual *in silico* predictions and/or known toxicity potential of reported phytochemicals, it should be noted that additivity, synergism, potentiation, and/or antagonism may play a role in the observed toxic effects of the oil. These potential effects, relating to compound mixtures, requires further investigations.

## 5. Conclusion

The results here presented showed clear consistencies in the effects of the EODS among the indices of weights and weight ratios, haematology, biochemical, and histopathology following single and repeated dose toxicity studies. Our data demonstrated that the toxic effects of EODS are dependent on dose and frequency of administration, and there is a high potential for persistent toxic effects on vital organs, especially liver and kidney. While 62.5 mg/kg EODS may appear relatively safe with no major effects on organs histoarchitectures, the significant alteration in key haematological and biochemical indices even at this dose is a call for toxic concern over habitual consumption of the seeds of *D. Tripetala*. The low LD<sub>50</sub>, dose dependent increase in toxic effects and high percent mortality at 250 mg/kg following repeated doses, not only confirm the potentially toxic nature of EODS, but show that the only safe route is to avoid habitual consumption of the seeds. Therefore, we propose, based on the *in vivo* and *in silico* observations here reported, an urgent need to regulate the excessive consumption of the seeds of *D. Tripetala*, for instance, through public education.

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## Conflict of Interest

The authors declare no conflict of interest.

## CRedit authorship contribution statement

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## Declaration of Competing Interest

The authors report no declarations of interest.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.toxrep.2021.07.019>.

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