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RESEARCH ARTICLE

TOXICOLOGICAL PROFILE OF *DIAPHANANTHE BIDENS* LEAF EXTRACT AND FRACTIONS IN SWISS ALBINO RATS

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Abstract

Traditional medications are prone to be consumed without consideration of their possible toxicological profile. This study investigated the toxicological profile of aqueous ethanol extract of *Diaphananthe bidens* leaf and its fractions in Swiss albino rats. The LD_{50} and subchronic toxicities were determined by analyzing biochemical, haematological, reproductive hormone, and histopathological parameters. The extract was fractionated using n-hexane, n-butanol, and ethyl acetate, and phytochemicals were measured. The OECD 425 guidelines guided the 90-day subchronic toxicity testing of the extract. Blood samples were collected on the 91st day for haematology, biochemical, and reproductive hormone analysis using standard kits. The extract possessed an LD_{50} of 9450 mg/kg, and phytochemical analysis showed the presence of tannins, alkaloids, glycosides, saponins and terpenoids in varying degrees. Ethyl acetate was found to be the most bioactive at 328.2 mg/GAE/g, with a 40.32% yield. The effective dose of the extract, n-hexane, ethyl acetate, n-butanol, and aqueous fractions was recorded at 301, 8069, 186, 433, and 759 mg/kg, respectively. The extract and ethyl acetate significantly ($P<0.05$) lowered levels of ALT, AST, and ALP in the blood compared to groups given a vehicle and significantly ($P<0.05$) elevated plasma PCV, Hb, WBC and RBC counts after administration of extract and ethyl acetate therapeutic doses (186, 372 and 744 mg/kg). The histopathological analysis revealed normal liver and kidney architectures. In conclusion, the leaf extract and fractions of *Diaphananthe bidens* appear relatively safe following a sub-chronic administration.

Introduction:-

Indigenous medical systems have long utilized plants for therapeutic purposes(Mpofana *et al*., 2023), particularly in developing nations where natural medicines are considered less harmful, and more easily accessible than synthetic ones(Nouioura *et al*., 2023). In impoverished countries,a significant portion of the global populace,

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approximately80% relies on medicinal plants as a fundamental aspect of their healthcare pratices, utilizing them to prevent and address various health conditions, includingasthma, diabetes, cardiovascular disease, cancer, gastrointestinal disorders, and skin disorders(Chrysostomou *et al*., 2024). However, most medicinal plants used in conventional healthcare have not undergone toxicological testing, which can lead to negative effects and interactions with drugs(Neergheen-Bhujun, 2013; Tamilselvan *et al*.2014). It is false to believe that medicinal herbs are safer and have fewer side effects(Amorha et al., 2018). Research has shown that using medicinal plants without determining their toxicity poses a risk to one's health(Joseph *et al*., 2024). To evaluate the safety and potential harm of chemicals, toxicological methods are employed(Moreira *et al*., 2014). These protocols evaluate the effects of single or repeated chemical doses on animals to control risks associated with the manufacture, handling, and use of chemicals(Bello*et al*. 2019). *Diaphananthe bidens* (D. bidens) is a climber and epiphyte member of the Orchidaceae family with strong wire stems(Aba *et al.*, 2019). The leaf decoction of this plant is used to treat asthma, inflammatory illnesses, and diabetes in the South Eastern States of Nigeria(Onyegbule *et al*., 2022). Traditional usage of the aqueous leaf extract has led to extensive research on its anti-diabetic and anti-hyperglycemic properties(Ottah *et al*., 2012). However, there is not enough scientific information available on the toxicity of leaf extracts especially when administered over prolonged periods. This study aims to investigate the plant's toxicological effects and document its safety profile in vivo.

Materials and Methods:-

Chemicals and Reagents:

Reagent kits and reagents were used to analyze various substances. Reagents used include cytokine, perchloric acid, sodium carbonate, Folin-Ciocalteu's reagent, hydrogen peroxide, and thiobarbituric acid. Other products included HCL, Potassium dichromate, Potassium ferricyanide, blood urea nitrogen, creatinine, alkaline phosphatase, aspartate aminotransferase, and Alanine aminotransferase reagents from Span Diagnostic Ltd, luteinizing hormone, and follicle-stimulating hormone kits from Mybiosource and distilled water.

Plant materials and collection:

In June 2023, *Diaphanathe bidens* leaves were harvested in Nsukka, Enugu State, Nigeria. Taxonomist Alfred Ozioko collected and authenticated the plants in the Department of Pharmacognosy and Traditional Medicine's herbarium. The leaves were washed and shade-dried for 28 days and then pulverized using a mechanical grinder for solvent extraction.

Animals

Adult rats (140–150 g) were used for the study. The animals were transported to the Animal House of Pharmacology and Toxicology Department, Nnamdi Azikiwe University, Awka. The rats were fed with pelletized feed from Vital Feeds, Nigeria,provided free water and kept in ordinary cages. The animals acclimatized for 7 days before the study. The study followed the NIH Guide and OECD 425 guidelines for the Care and the approval for the research was obtained from the Nnamdi Azikiwe University Animal Research and Ethics Committee (**NAU/AREC/2024/0088**).

Extraction, phytochemical analysis and estimation of total phenolic content

Extraction

Six kilograms (6 kg) of pulverized leaves of D. bidens were cold macerated in 30 L of aqueous ethanol (70%) for 72 h with intermittent shaking. The resulting solution was filtered, and the filtrate was pre-concentrated in vacuo using a rotary evaporator at 40°C and thereafter, dried to a constant weight using an open water bath at 50°C to obtain the ethanol extract.

Fractionation (Liquid-liquid Chromatography)

The ethanol extract (100 g) was dissolved in distilled water and subjected to liquid-liquid partition successively with 2.5 L of n-hexane, ethyl acetate, and then butanol using a separating funnel to obtain the soluble fractions. The remaining fraction after the partitioning was taken as the water fraction. The fractions were pre-concentrated using a rotary evaporator at 40^{o}C and dried using a water bath at 50^{o}C. The water fraction was freeze-dried at -50^{o}C using a Telstar LyoQuest freeze dryer(Brígido *et al*., 2021).

Phytochemical analysis and estimation of total phenolic content

The phytochemical analysis of the leaf extract and fractions was carried out using standard methods (Odebiyi and Sofowora, 1978; Trease and Evans, 1989; Harborne, 1998). The total phenolic content by Folin Coicetteu’s assay was determined using the method of Kim *et al* (2003)

Experimental design

Acute toxicity (LD_{50}) Study

Miller and Tainter's method of 1944(Randhawa, 2009) was used to conduct an acute toxicity test on female rats. Rats of about the same size (140-150 g), aged9-10 weeks, were randomly selected and left for 7 days before the acute toxicity study. The ethanol extract was administered as a single oral dose in varying doses (100-10,000 mg/kg) using stock solutions prepared shortly before administration. The animals were observed for toxic symptoms and mortality for the first 30 minutes, then at, 2 hours, 6 hours, 12 and 24 hours. After 14 days, the number of deceased rats was counted and the percentage of mortality was calculated. The percentage dead for 0 and 100 were corrected before determining probits.

Corrected % Formula for 0 and 100% mortality: For 0% dead: 100(0.25/n) for 100% dead: 100(n-0.25/n). Where n = number of animals in a group (Ogbuehi et al., 2015).

The percentage mortality in each group was transformed to probit using a probit table. Probit values were plotted against log doses and then the dose corresponding to probit 5, i.e., 50%, was extrapolated using the regression equation of the line graph.

The Standard Error (SE) of the LD50 was calculated from the following formula:

Approx. SE of LD50 = $\frac{(\text{Log LD}84 - \text{Log LD}16)}{\sqrt{2N}}$---------- equation 1

Where N is the number ofanimals in each group.

Quantification of Effective dose of *D. bidens* leave extract and fractionsin immunosuppressive animals

The dose-response curve of percentage immune stimulation against log dose was plotted and the half-maximal effect of the treatments was extrapolated from the regression line graph equation and the dose of ethyl acetate which was the most bioactive fraction with highest stimulation (186mg/kg)used for the experiments. The effective dose, 186mg/kg, double ED50 (372mg/kg)and 744mg/kg were used as low, medium and high doses respectively in the animal group dosing(McCollough and Schueler, 2000).

Sub-chronic toxicity studies

Fourth-two (42) healthy albino rats randomized into 7 groups of 6 animals each were used. Group 1 served as control and received the vehicle used in dissolving the extract and most active fraction (10 ml/kg 5% Tween 20). Groups 2, 3, and 4 received the ED50 dose (186 mg/kg), double the ED50 (372 mg/kg) and 4 x the ED50 (744 mg/kg) of the most active fraction (ethyl acetate fraction). Groups 5, 6, and 7, were administered the ethanol extract of D. bidens at the same doses as the most active fraction for comparative effect. Treatment was done orally daily every 9 am for 90 days. The animals were weighed before treatment and on the 31st, 61st and 91st day of the study. Blood samples were collected through the retro-orbital plexus before treatment and on Day 91 after the sub-chronic administration of the extract and ethyl acetate fraction. Heparinized blood samples were used for the estimation of haematological parameters - Haemoglobin concentration (Hb), Packed cell volume (PVC), Red blood cell (RBC) count, white blood cell (WBC) count and platelet count (Diallo *et al*., 2010). Serum samples were used for the analysis of biochemical parameters - Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), alkaline phosphatase (ALP), Blood Urea Nitrogen (BUN) and Creatinine (Bigoniya *et al*., 2015). Reproductive hormones LH and FSH analysis were also carried out on the serum sample using enzyme-linked immunosorbent assay kits.

Haematological Analysis

The refined retro-orbital bleeding method was used to obtain high-quality blood samples (Tiwari *et al*., 2016). This involves gently removing the eyelid and stretching the upper eyelid to expose the eye, allowing for careful retrieval using a Pasteur pipette. Blood samples were then deposited in an EDTA aliquot tube for haematological assay using an automated blood analyzer, sysmax KX-21, Japan to determine a comprehensive panel of haematological parameters, including total red blood cells (RBC), and haemoglobin level (Hb). Pack cell volume (PCV), white blood cells (WBC, and platelet counts (PT) (Orélien *et al*., 2016).

Assessmentof Biochemical parameters

Following blood sample collection, the samples were placed in a non-heparinized container, allowed to clot for thirty minutes, and then centrifuged for ten minutes at 300 rpm. Standard kits are used to measure vital liver, kidney, and lipid profile testing under a light spectrophotometer.

Liver function test
Alanineaminotransferase (ALT), aspartic aminotransferase (AST) and Alkaline phosphatase (ALP) were among the significant liver enzymes that were measured from the serum of the rats given the crude extracts and bioactive fractions ethyl acetate of Diaphanathe bidensutilizing the modified techniques of Reitman and Frankel (1957) as described by Rao(2011) and Sahoo *et al*(2015).

Kidney function test
Serum creatinine and blood urea nitrogen (BUN) were estimated by the method described by Tietz, (1976) and Heinegard and Tiderstrom, (1973) respectively using BUN and creatinine test kits (Teco Diagnostics, USA) as described bySomchit *et al*(2014) and Moro *et al* (2016) respectively.

Reproductive hormone (LH and FSH) function test
The Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH) tests were conducted using a Bybiosource test kit with their catalogue numbers as stated. The kits included micro ELISA plates pre-coated with Rat LH and FSH. The plates were incubated for 45 minutes, washed three times, and then washed five times. The plate was then incubated for 30 minutes and washed. The plate was then incubated for 20 minutes in the dark. The stop solution was added to stop the reaction. The colour shift at 450nm was measured immediately after adding the stop solution. The standard curve was used to interpolate FSH levels in each sample. The LH concentration was extrapolated from this standard curve. Both tests were conducted following the manufacturer's instructions(Abo-El Maaty and El-Shahat, 2012).

Histopathology analysis
After being gently removed, the liver and kidney weremeticulously rinsed in ice-cold saline solution and fixed in 10% formalin, processed, sectioned and stained for analysis.

Statistical analysis
SPSS version 18.0 was used for statistical analysis, with the 95% confidence interval calculated as mean +- crucial t-values x standard error. Independent statistical tests were used to identify differences between groups.
Where *P <0.05 is compared to basal/pretreatment, $^{\#}$P<0.05 is compared to vehicle control and $^{a, b}$P<0.05 is compared to extracts and fractions

Results:-

Phytochemical and total phenolic content Analysis.
The phytochemical and the total phenolic contents of the extract and fractions of Diaphanathe bidens, revealed the presence of different bioactive molecules with ethyl acetate fractions having the highest total phenolic content as shown in Table 1.

Table 1:- Phytochemical analysis and total phenolic content of the extract and fractions.

Phytocompounds	Extract	N-Hexane Fraction	Ethyl acetate Fraction	Butanol Fraction	Water Fraction
Flavonoids	+	-	+	+	+
Saponins	+	+	-	+	+
Tannins	+	+	+	+	+
Reducing sugar	+	-	+	+	+
Steroids	+	+	-	+	-
Terpenoids	+	-	-	+	-
Alkaloids	+	-	+	+	+
Glycosides	+	-	+	+	+
TPC (mgGAE/g)	175.3	4.9	328.2	148.6	94.1
Yield (%)	2.56[a]	10.28[b]	40.32[b]	31.07[b]	18.29[b]

+ Present, - absent. a - calculated from 6kg pulverized leaves, b – calculated from 100 g extract. TPC = Total phenolic content

Acute toxicity (LD50) testing

The limit dose of 5000mg/kg was orally administered to animals, the dose did not cause any mortality. Doses adjusted to 6,000, 7,000, 8,000, 9,000 and 10,000mg/kg and grouped as shown in Table 2.

Table 2:- Calculation of LD50 from the Millner and Tainter method.

Groups	Dose (mg/	Log dose	no of deat	% mortali	Corrected	Probit
1	6000	3.778151	0	0	2.5	3.04
2	7000	3.845098	1	10	10	3.72
3	8000	3.90309	2	20	20	4.16
4	9000	3.954243	4	40	40	4.75
5	10000	4	6	60	60	5.28

The LD_{50} of the extract is determined by probits value against the log dose as shown in figure 1.

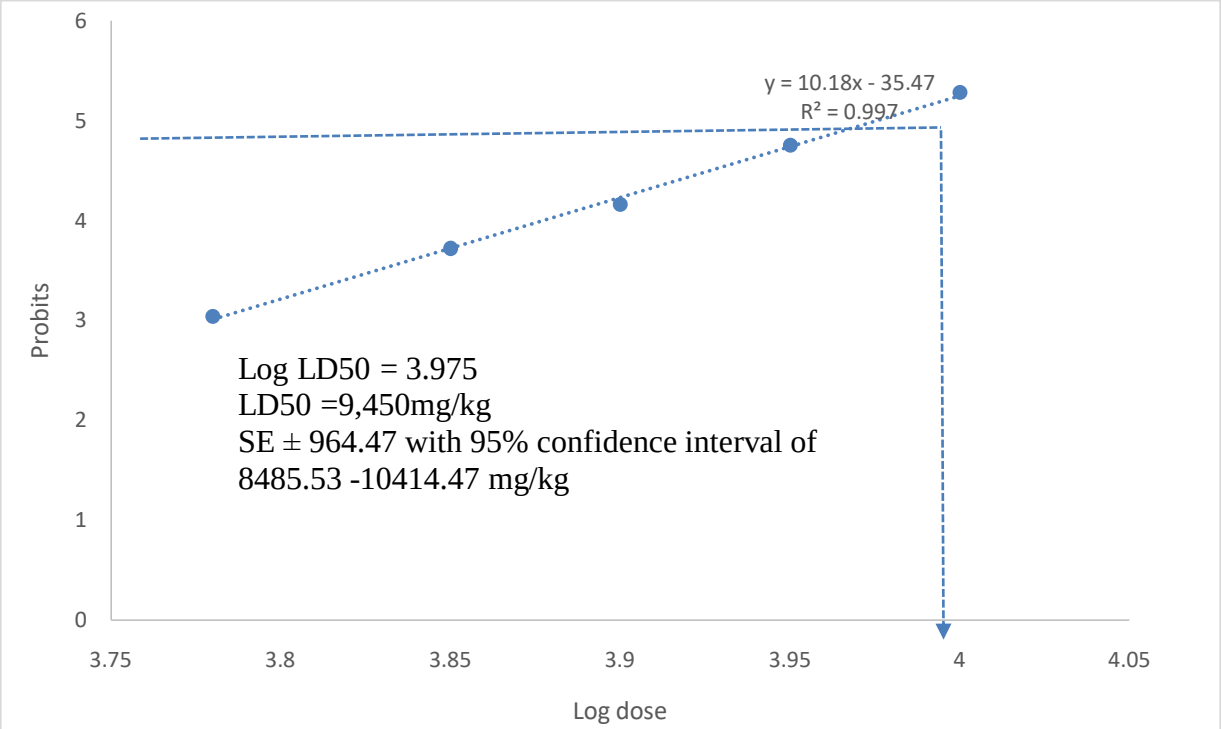
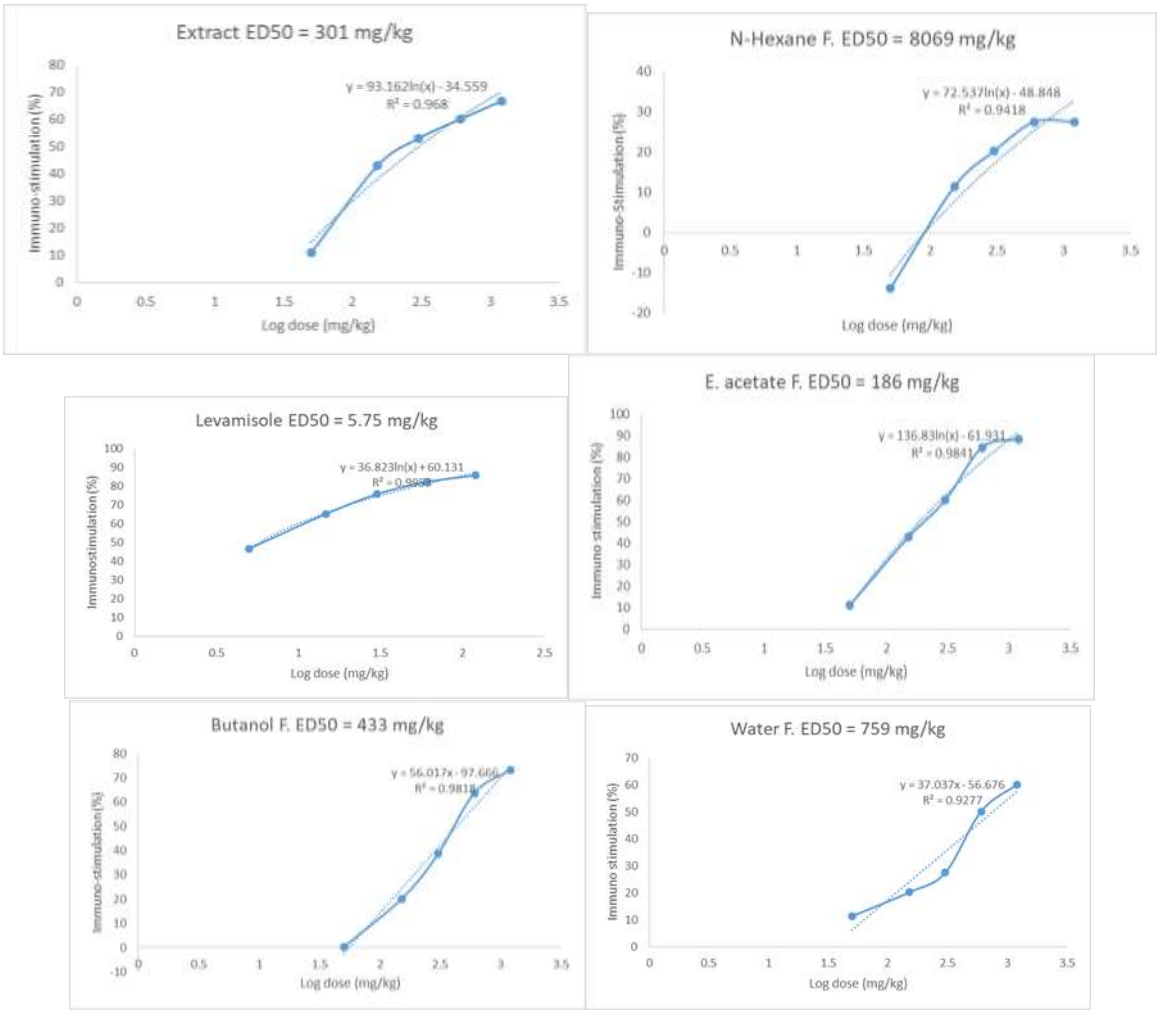


Fig. 2: LD50 Determination from Regression line graph

The result of the acute toxicity testing showed that *Diaphanathe bidens* leave extract is safe at 9,450mg/kg as shown in Table 2 and Figure 1, without any clinical signs of drowsiness, increased excitability, severe pain and necrosis after 7 days of oral administration, but signs of weakness, and loss of appetite were recorded at the 14-day oral treatment and death recorded in a dose-dependent manner (Table 2).

Determination of effective dose (ED_{50}) of D. bidens leaf extract and fractions

The extract and fractions showed dose-response activity at the tested doses (50-1200 mg/kg) just like the reference standard (5-120 mg/kg). Of all the fractions, the n-hexane fraction produced the least effects while the ethyl acetate fraction showed the highest. The extract's median effective dose (ED50) was calculated as 301 mg/kg while that of the n-hexane, ethyl acetate, butanol and water fractions are 8069, 186, 433 and 759 mg/kg respectively as shown in figure 3.



Fi**gure 3:-**Dose-response curve for the determination of the effective dose of the extract and fractions of D. bidens.

Effect of extract and ethyl acetate fraction on body weights

The extract and ethyl acetate fraction, like the vehicle control, showed a time-dependent increase in body weight. However, a dose-dependent decrease in body weight was recorded for both extract and ethyl acetate fraction treated groups. Compared to the vehicle control group, only the extract showed a significant ($P<0.05$) reduction in body weight on days 31st, 61st and 91st day. However, no significant ($P>0.05$) difference was recorded when compared between the extract and ethyl acetate fraction effect at the same doses as shown in Figure 4.

Effects of 90-day oral administration of D. bidens leaf extract and ethyl acetate fraction on liver function parameters (ALT, AST and ALP) in Swiss rats

The study examined the effects of extract and ethyl acetate fraction on serum ALT, AST, and ALP concentrations in D. bidens. The pre-treatment ALT concentrations showed similar values across groups, with no significant ($P> 0.05$) difference between the treated and control groups. Subchronic treatment with the extract and ethyl acetate showed significant ($P<0.05$) differences at various doses. The extract showed a significant ($P<0.05$) reduction in serum ALT at all doses, while the ethyl acetate fraction lost this reduction at lower doses (Figure 5a). The pre-treatment AST concentration showed no significant difference, but treatment with the extract led to an important ($P<0.05$) reduction in AST for the 372 mg/kg treated group. The extract and ethyl acetate fraction at 186 and 372 mg/kg doses did not show a significant ($P>0.05$) variable effect on serum AST. However, at a higher dose of 744 mg/kg, the ethyl acetate fraction produced significantly ($P<0.05$) lower serum AST compared to the extract-administered group at the same dose(Figure).Administration of the extract and ethyl acetate fraction of D. bidens decreased serum ALP concentration compared to the basal values. These reductions were significant ($P<0.05$) at 372 and 744 mg/kg of the extract and at all doses of the ethyl acetate fraction when compared to the vehicle control

group. Comparison between the extract and ethyl acetate at the same doses revealed significant variation in effect at the highest dose (744 mg/kg), with the ethyl acetate fraction having a more reductive effect on serum ALP compared to the extract (Figure 5c).

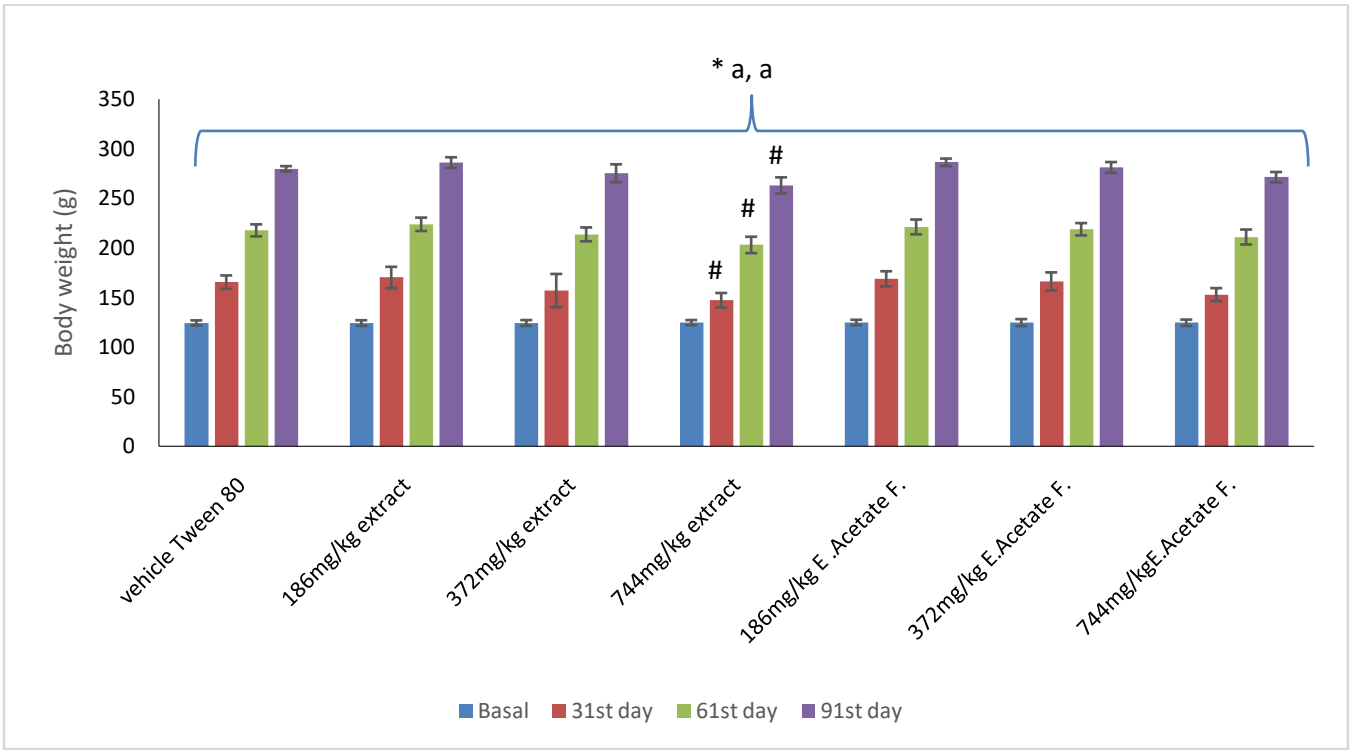


Figure 4:- Effect of extract and ethyl acetate fractions of D. bidens on body weight.

Where: * P<0.05 compared to basal/pre-treatment values; # P<0.05 compared to vehicle control group; different letters alphabet (a,b) P<0.05 compared to the extract and ethyl acetate fraction treatments

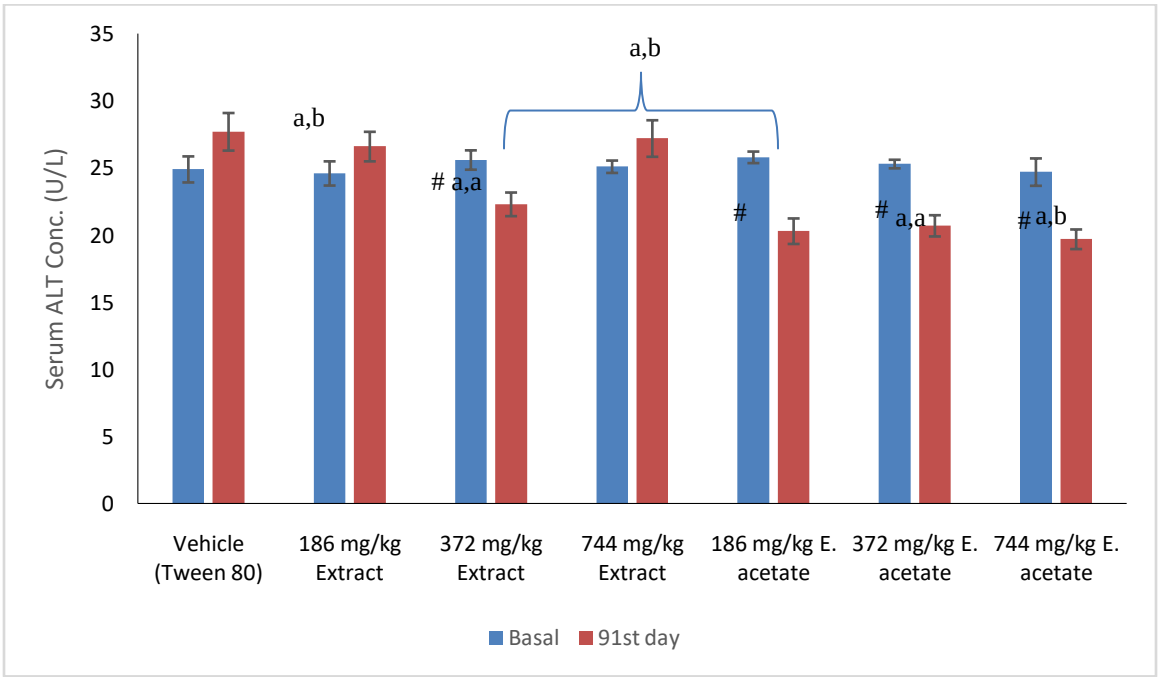


Figure 5a:- Effect of the extract and ethyl acetate fraction of D. bidens on serum Alanine aminotransferase.

Where: * P<0.05 compared to basal/pre-treatment values; # P<0.05 compared to vehicle control group; different letters alphabet (a,b) P<0.05 compared to the extract and ethyl acetate fraction treatments

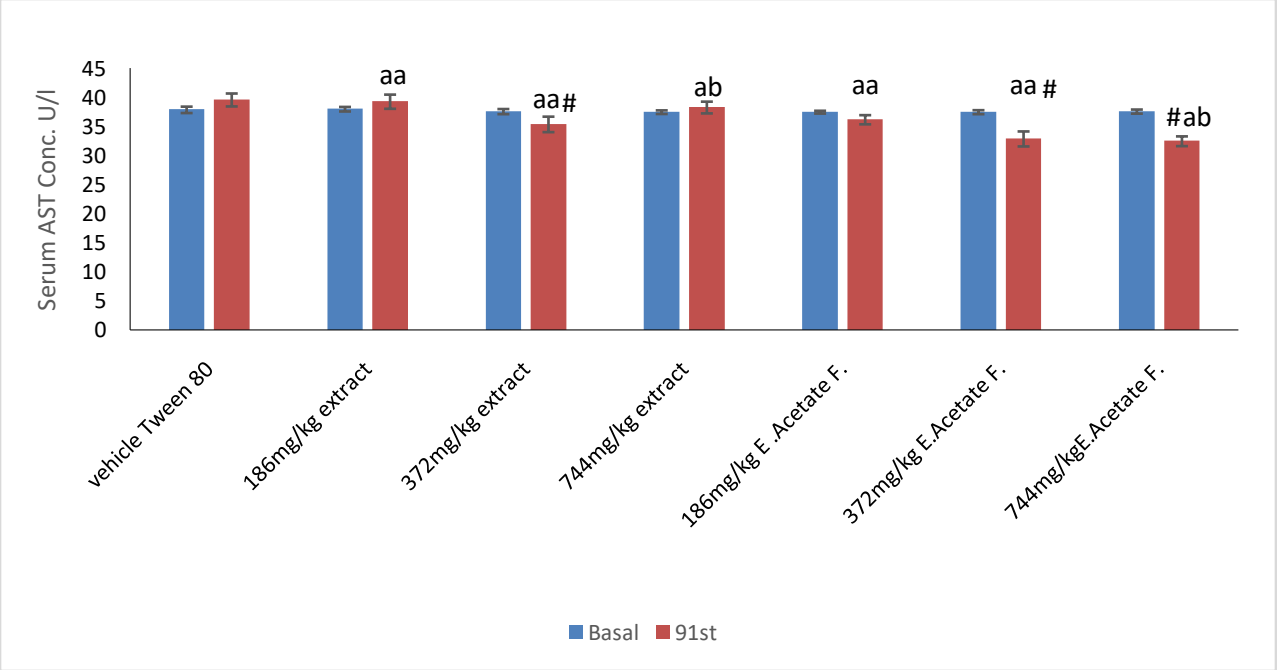


Figure 5b:- Effect of extract and ethyl acetate fractions of D. bidens on serum Aspartate aminotransferase (AST). Where: * P<0.05 compared to basal/pre-treatment values; # P<0.05 compared to vehicle control group; different letters alphabet (a,b) P<0.05 compared to the extract and ethyl acetate fraction treatments

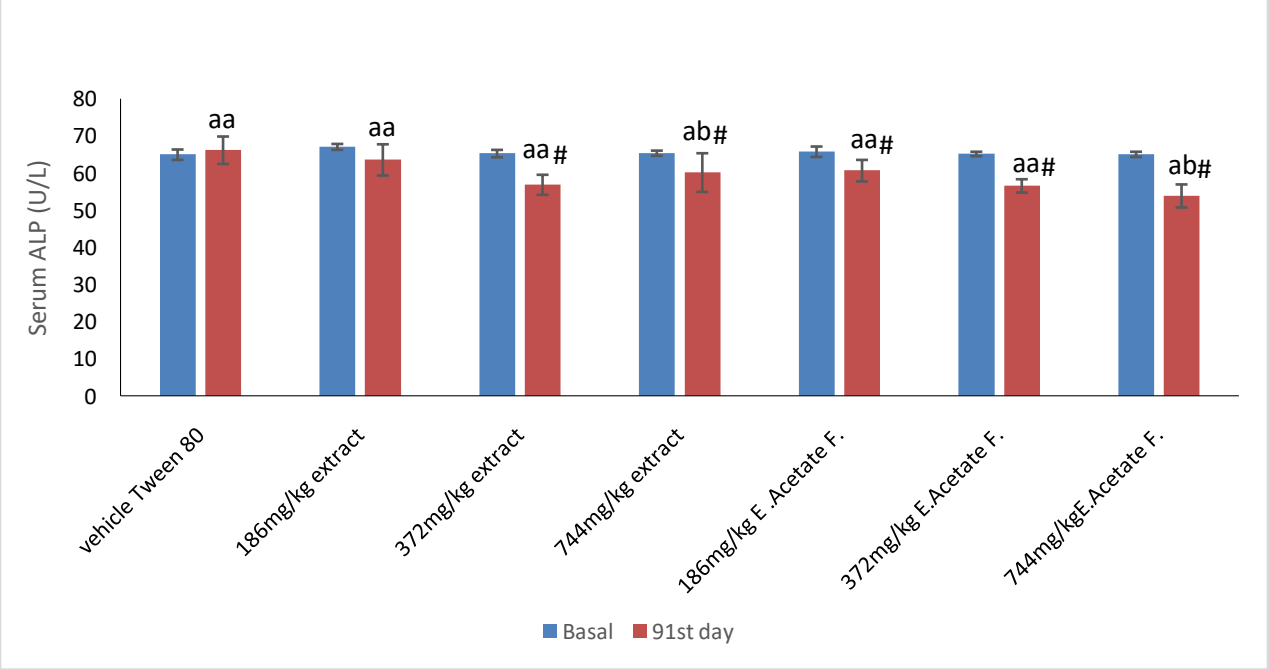


Figure 5c:- Effect of extract and ethyl acetate fractions of D. bidens on serum Alkaline Phosphatase (ALP). Where: * P<0.05 compared to basal/pre-treatment values; # P<0.05 compared to vehicle control group; different letter alphabet (a,b) P<0.05 compared to the extract and ethyl acetate fraction treatments.

Effects of 90- day oral administration of D. bidens leaf extract and fractions on kidney function parameters (BUN and CREA) in Swiss albino rats

The extract showed a variable pattern of effect on the blood urea nitrogen concentration (Figure 6a). At the low and high doses (186 mg/kg and 744 mg/kg respectively), there was a decrease in concentration while at 372 mg/kg, an increase was recorded just like in the vehicle control group. The ethyl acetate fraction maintained a similar trend of effect (reduction in BUN) across all the doses. These effects exhibited by both the extract and ethyl acetate fraction were not significantly ($P>0.05$) different from each other at various doses administered. Similarly, when compared to the vehicle control group, administration of the extract and ethyl acetate fraction did not significantly ($P>0.05$) alter the BUN concentration across the doses.

The result of the effect of the extract and ethyl acetate fraction on serum creatinine revealed that the extract showed a non-significant ($P>0.05$) increase in serum creatinine concentration across the doses while the ethyl acetate fraction showed dose-dependent reduction at 186 and 372 mg/kg which was significant ($P<0.05$) at the higher dose (372 mg/kg) compared to the vehicle control group (Figure 6b). At the highest dose of 744 mg/kg, the ethyl acetate fraction however, showed an increase in serum creatinine concentration compared to the recorded basal value, though this increase was not of significant ($P>0.05$) value.

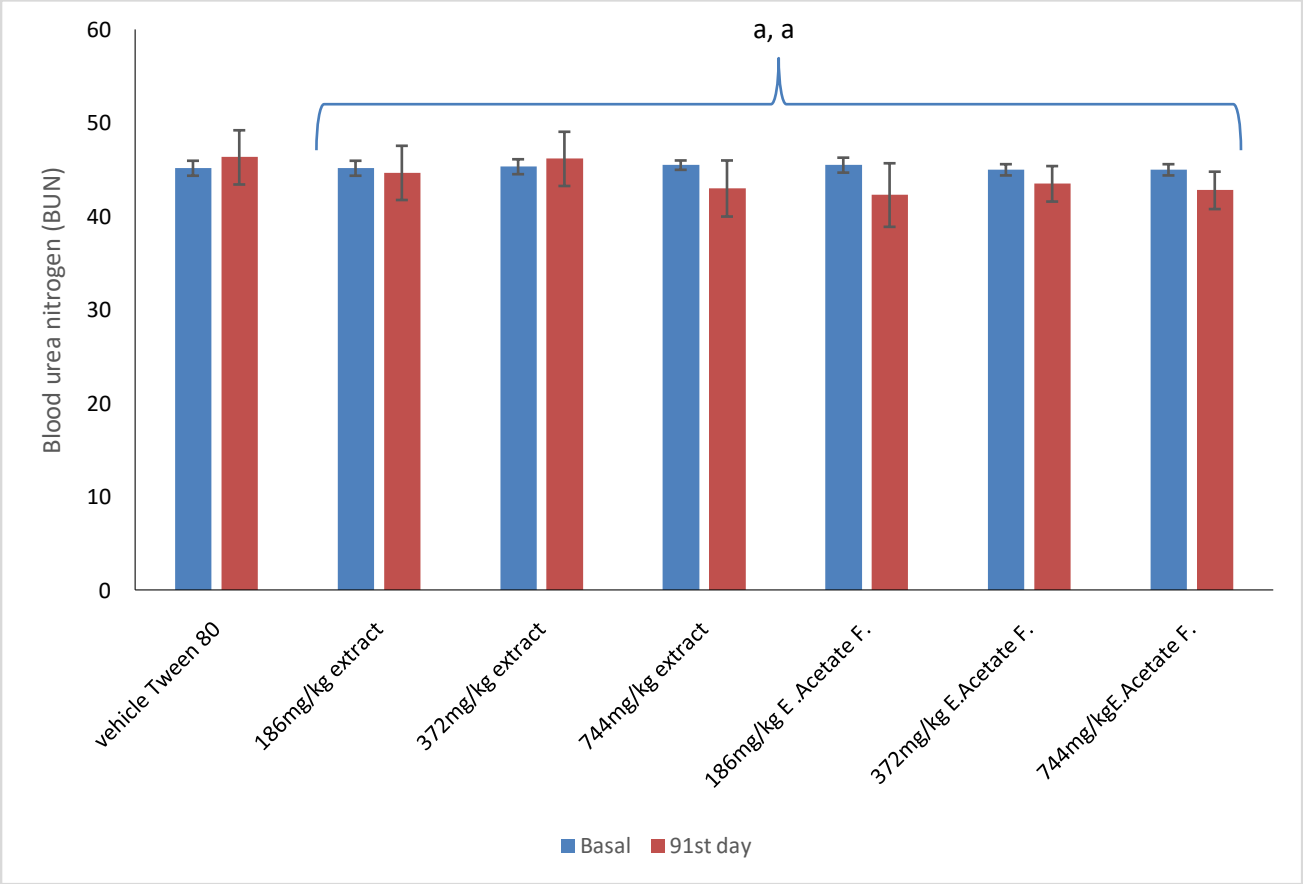


Figure 6a:- Effect of extract and ethyl acetate fractions of D. bidens on Blood urea nitrogen (BUN). Where: * $P<0.05$ compared to basal/pre-treatment values; # $P<0.05$ compared to vehicle control group; different letter alphabet (a,b) $P<0.05$ compared to the extract and ethyl acetate fraction treatments.

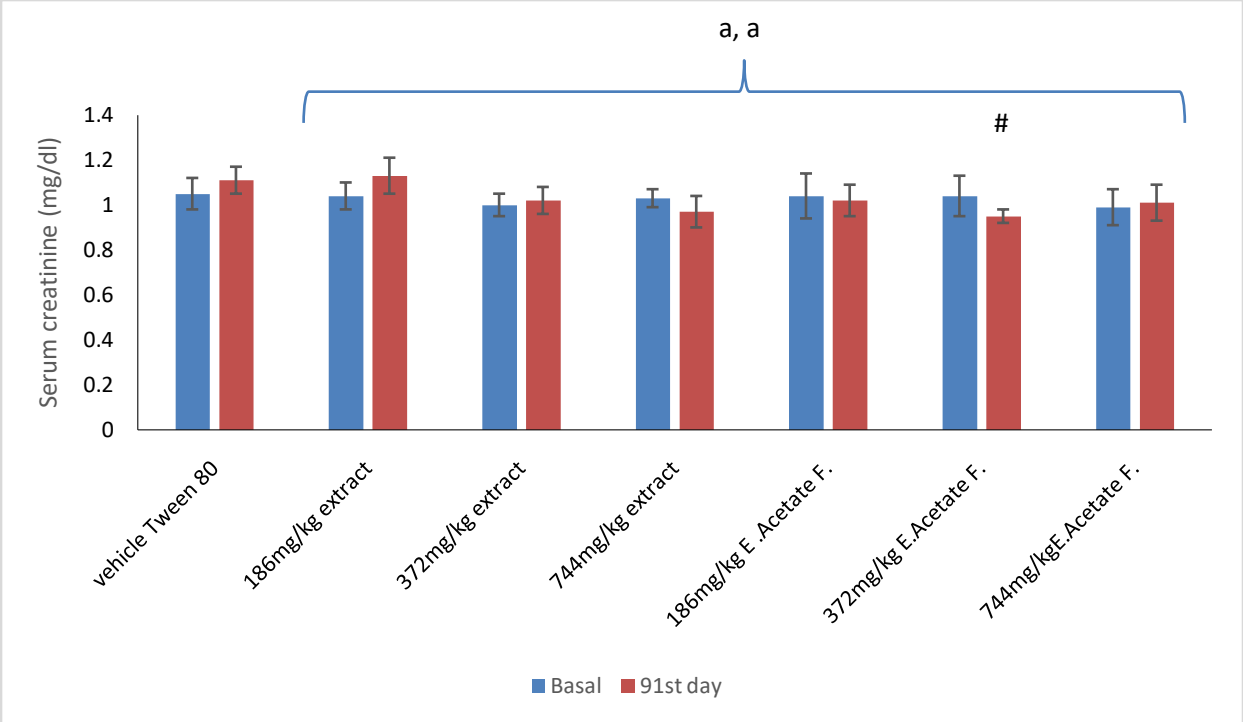


Figure 6b:- Effect of extract and ethyl acetate fractions of D. bidens on serum Creatinine.
Where: * P<0.05 compared to basal/pre-treatment values; # P<0.05 compared to vehicle control group; different letter alphabet (a,b) P<0.05 compared to the extract and ethyl acetate fraction treatments

Effects of oral 90-day administration of D. bidens leaf extract and fractions on haematological parameters in Swiss albino rats

Administration of the extract and ethyl acetate fraction produced a negligible effect on PCV and Hb concentration. Dose-dependent decrease was produced for the extract-treated animals while the ethyl acetate fraction showed a dose-dependent increase. Compared to the vehicle control group, these changes were not of significant (P>0.05) value. However, compared between both treatments (extract and ethyl acetate fraction), significant (P<0.05) differences in their effects on PCV and Hb were recorded at 744 mg/kg dose (Figures 7a and 7b).

For the RBC count, administration of the extract at 186 mg/kg produced a significant (P<0.05) increase compared to the vehicle control group while at 372 and 744 mg/kg, significant (P<0.05) decreases were recorded. Ethyl acetate fraction showed a dose-dependent increase in RBC count that was of significant (P<0.05) value at 372 and 744 mg/kg compared to the vehicle control group. At all tested doses, the extract and the ethyl acetate fraction showed significant (P<0.05) differences in their effects on RBC. The extract showed a higher value at 186 mg/kg while ethyl acetate fraction produced a higher increase at 372 and 744 mg/kg doses (Figure 7c).

Both extract and ethyl acetate fraction administration produced a dose-dependent increase in WBC count. However, only the extract at 372 and 744 mg/kg was able to produce a significant (P<0.05) increase compared to the vehicle control group. Also, the difference in the effect produced by the extract and ethyl acetate fraction was only significant (P<0.05) at 744 mg/kg when compared between both treatments (Figure 7d).

Unlike the control group which showed an increase in platelet count at the 91st day compared to the basal (0 day) value, the extract and ethyl acetate fraction maintained a reduction in platelet count at all administered doses. However, when compared to the vehicle control group, these effects were not of significant (P<0.05) value. Similarly, compared between the extract and ethyl acetate fraction, both treatments failed to produce significant (P>0.05) difference at all the dose levels.

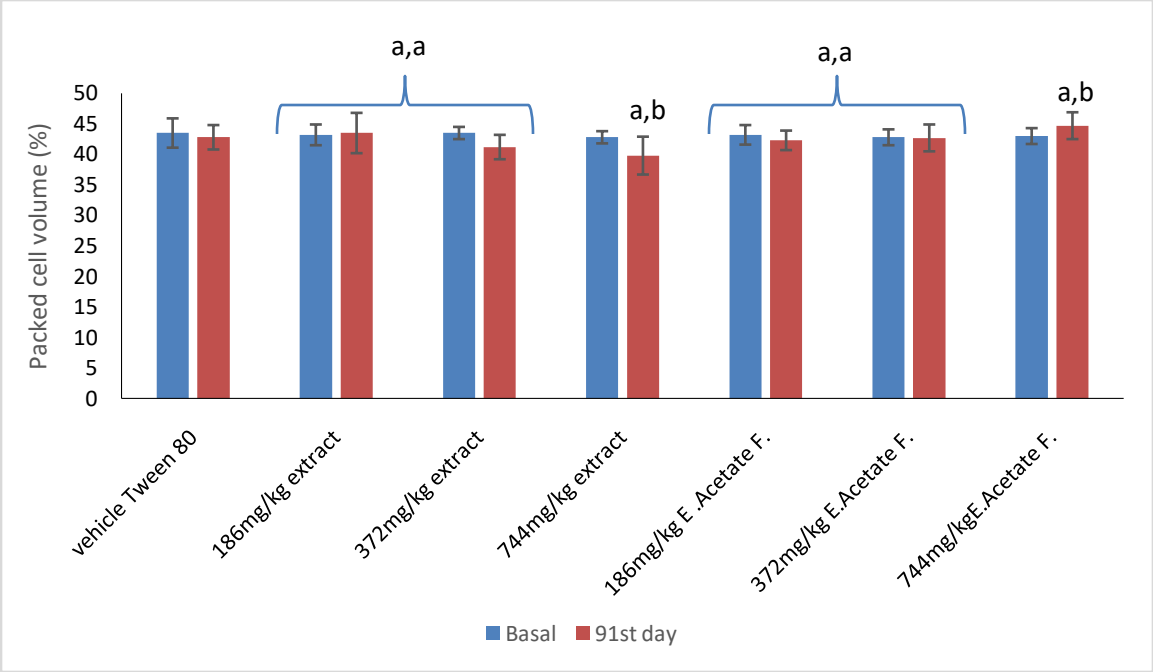


Figure 7a:- Effect of extract and ethyl acetate fractions of D. bidens on packed cell volume (PCV).
Where: * P<0.05 compared to basal/pre-treatment values; # P<0.05 compared to vehicle control group; different letter alphabet (a,b) P<0.05 compared to the extract and ethyl acetate fraction treatments.

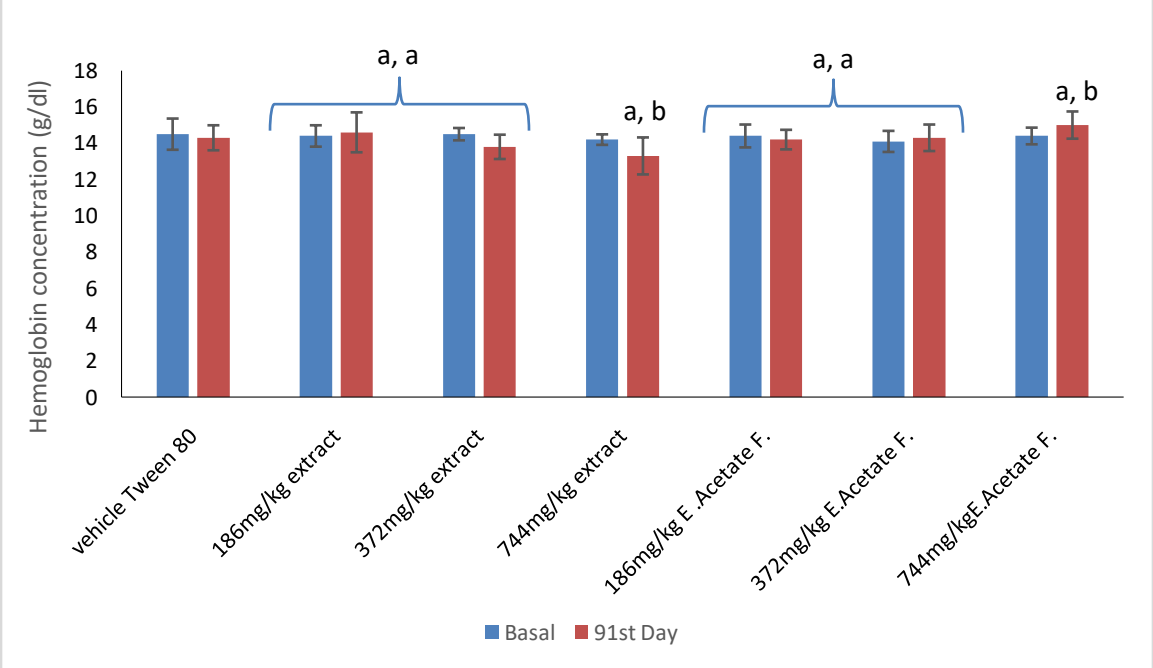


Figure 7b:- Effect of extract and ethyl acetate fractions of D. bidens on haemoglobin (Hb) concentration.
Where: * P<0.05 compared to basal/pre-treatment values; # P<0.05 compared to vehicle control group; different letter alphabet (a,b) P<0.05 compared to the extract and ethyl acetate fraction treatments.

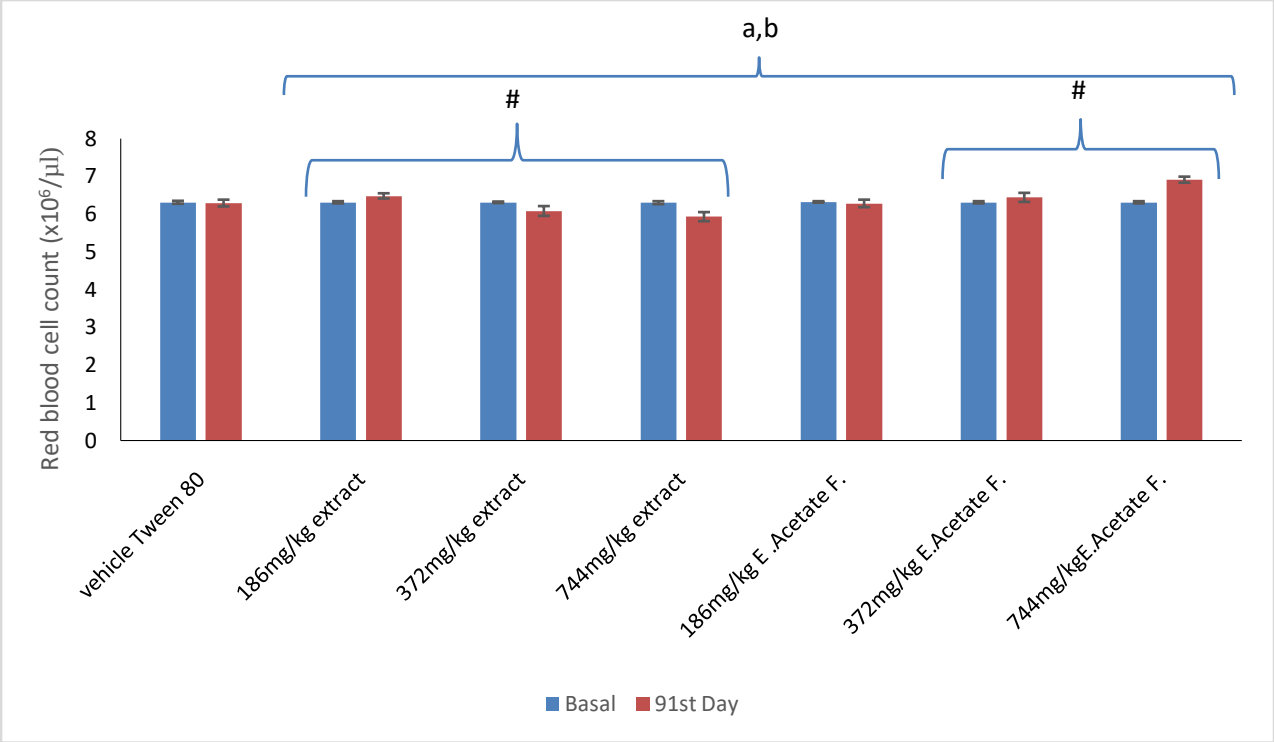


Figure 7c:- Effect of extract and ethyl acetate fractions of D. bidens on Red blood cell (RBC) count.
Where: * P<0.05 compared to basal/pre-treatment values; # P<0.05 compared to vehicle control group; different letter alphabet (a,b) P<0.05 compared to the extract and ethyl acetate fraction treatments.

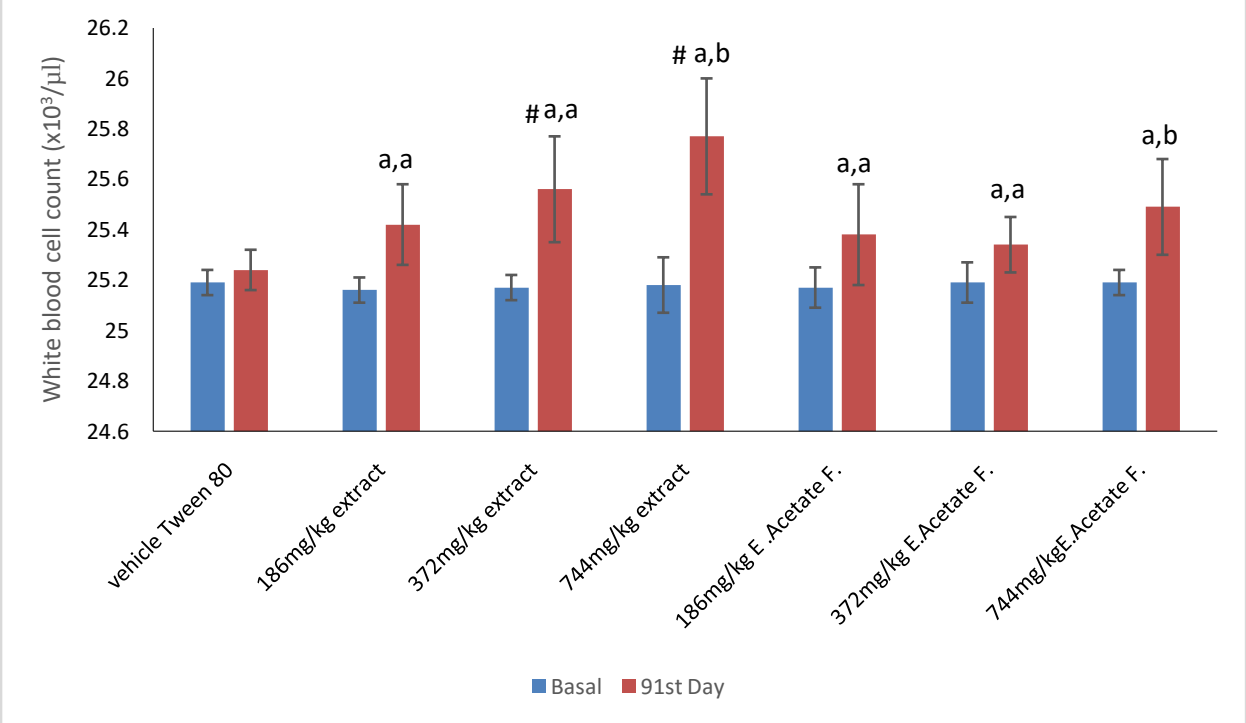


Figure 7d:- Effect of extract and ethyl acetate fractions of D. bidens on White blood cell (WBC) count.
Where: * P<0.05 compared to basal/pre-treatment values; # P<0.05 compared to vehicle control group; different letter alphabet (a,b) P<0.05 compared to the extract and ethyl acetate fraction treatments.

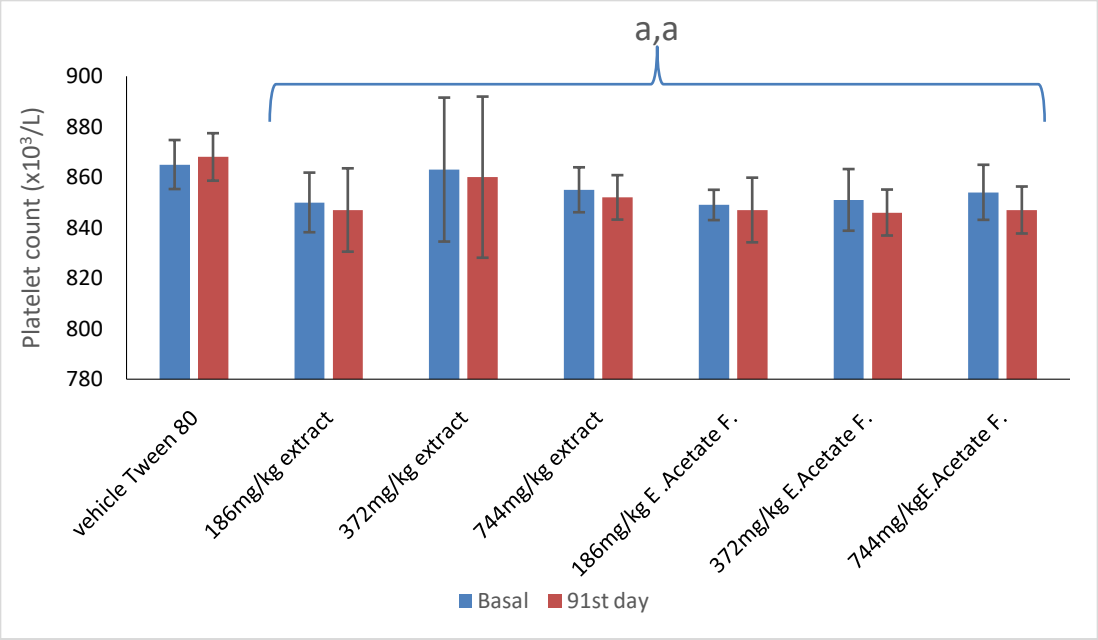


Figure 7e:- Effect of extract and ethyl acetate fractions of D. bidens on Platelet count.

Effects of oral 90-day administration of D. bidens leaf extract and fractions on reproductive hormone parameters (FSH and LH) in Swiss albino rats

Administration of the extract at different doses for 90 days showed a dose-dependent reduction in FSH. The same basal concentration was maintained for animals that received the least dose (186 mg/kg). As the doses increased to 372 and 744 mg/kg, there were 0.6 and 2.5% reductions respectively. These effects were however not up to the magnitude of reduction recorded for the control group (5% Tween 80). Dose-dependent reductions were also recorded following ethyl acetate fraction administration for 90 days at 186 and 372 mg/kg (Figure 8a).

For LH serum concentration, the extract produced a reduction at, 186 and 744 mg/kg doses and an increased concentration at 372 mg/kg. The ethyl acetate fraction also produced an increased concentration at all the tested doses (Figure 8b).

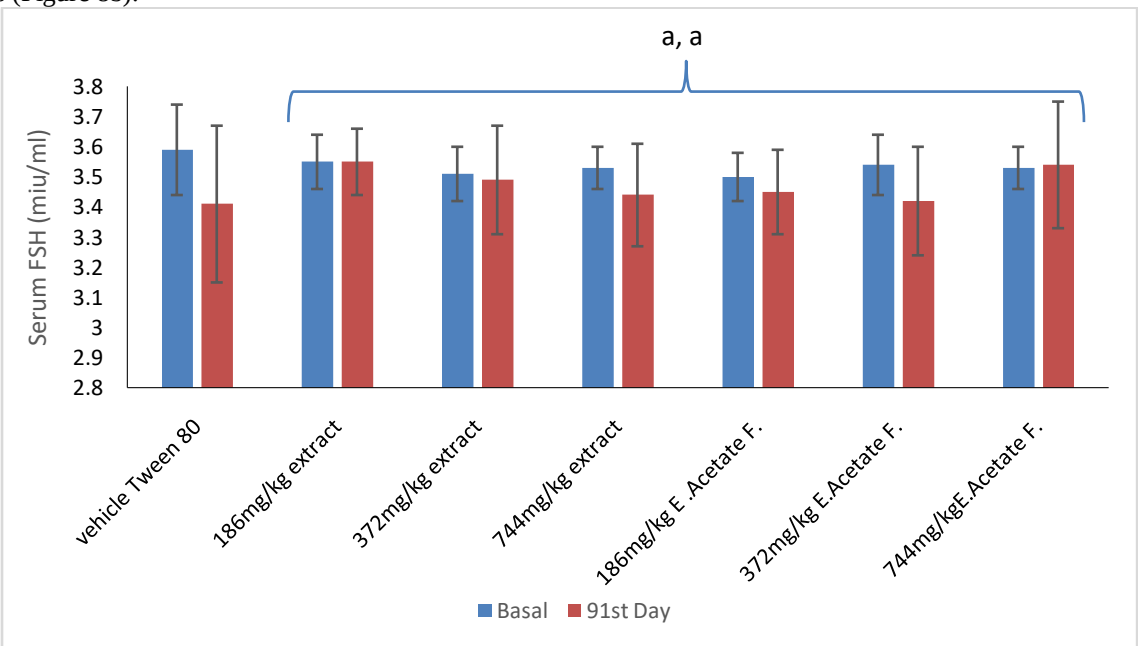


Figure 8a:- Effect of extract and ethyl acetate fractions of D. bidens on serum Follicle stimulating hormone (FSH).

Where: * P<0.05 compared to basal/pre-treatment values; # P<0.05 compared to vehicle control group; different letter alphabet (a,b) P<0.05 compared to the extract and ethyl acetate fraction treatments.

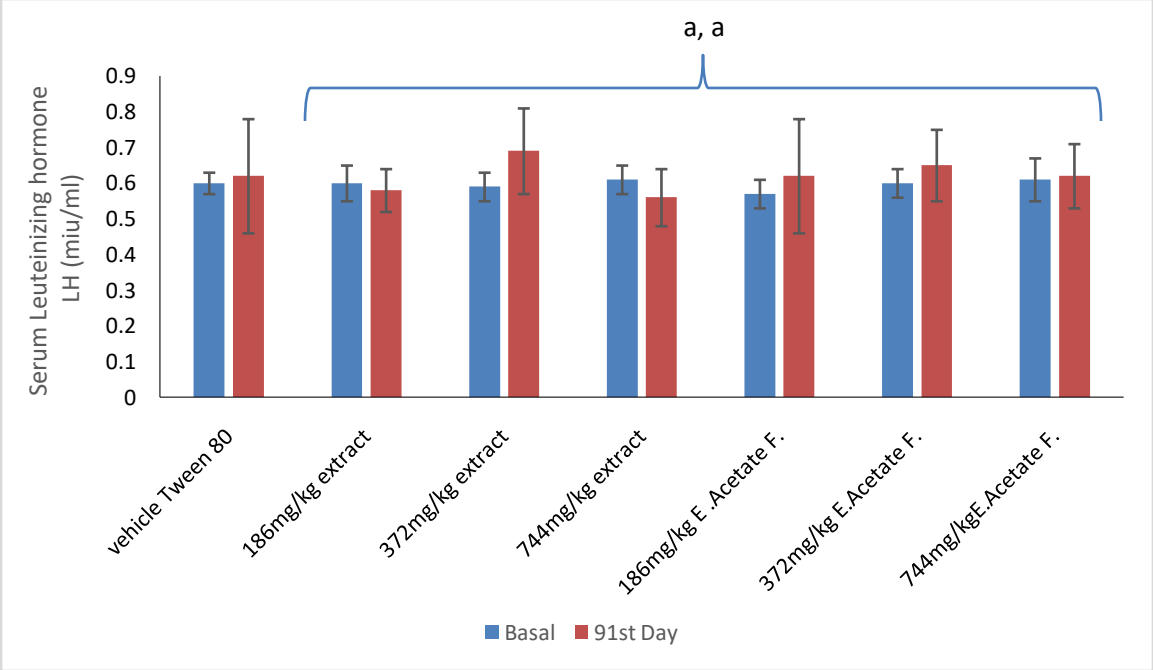


Figure 8b:- Effect of extract and ethyl acetate fractions of D. bidens on serum Luteinizing hormone (LH).
Where: * P<0.05 compared to basal/pre-treatment values; # P<0.05 compared to vehicle control group; different letter alphabet (a,b) P<0.05 compared to the extract and ethyl acetate fraction treatments.

Effects of oral 90-day administration of D. bidens leaf extract and fractions on liver and kidney histology of Swiss albino rats.

Slides A, B, and C represent photomicrographs of the extract-treated animals with doses of 186, 372 and 744 mg/kg respectively. Slide D represents the control or vehicle-treated group while slides E, F, and G represent the photomicrograph of the ethyl acetate-treated animals with the doses of 186, 372 and 744 mg/kg respectively. The H and E staining was evident from the purple coloration of the nuclei and pink coloration of the cytoplasm. The slides showed normal hepatic cords radiating from the central vein (arrow). The hepatocytes also showed normal histological features. The hepatic sinusoids with red blood cells in the capillaries stained bright red can also be seen from the slides. No pathological architecture was detected from the slides (Figure 9a)

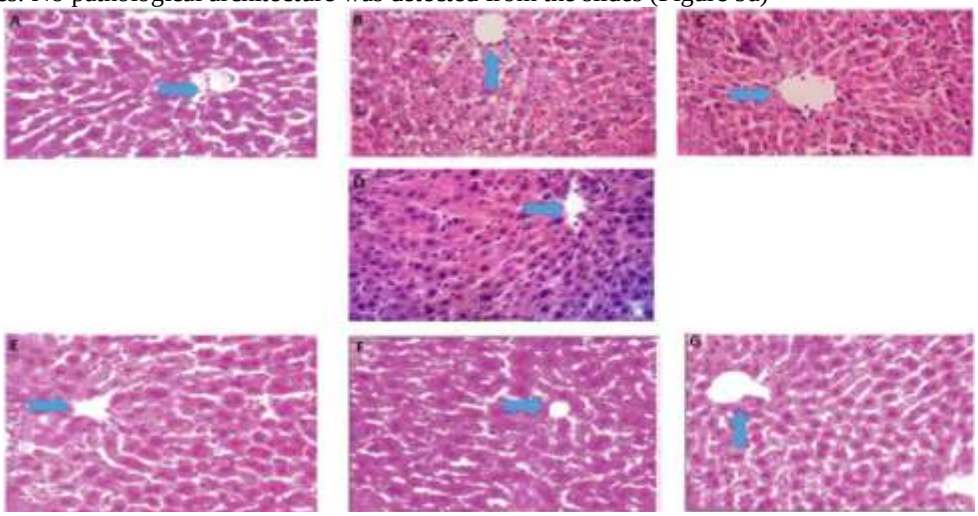


Figure 9a:- Effects of D. bidens leaf extracts and fractions on liver histology of Swiss albino rats.

Slides A, B, and C represent photomicrographs of the extract-treated animals with doses of 186, 372 and 744 mg/kg respectively. Slide D represents the control or vehicle-treated group while slides E, F, and G represent the photomicrograph of the ethyl acetate-treated animals with the doses of 186, 372 and 744 mg/kg respectively. The normal renal histological architecture was observed with a green arrow showing the glomerulus while the black and red arrows showed the bowman capsule and renal tubules respectively (Figure 9b).

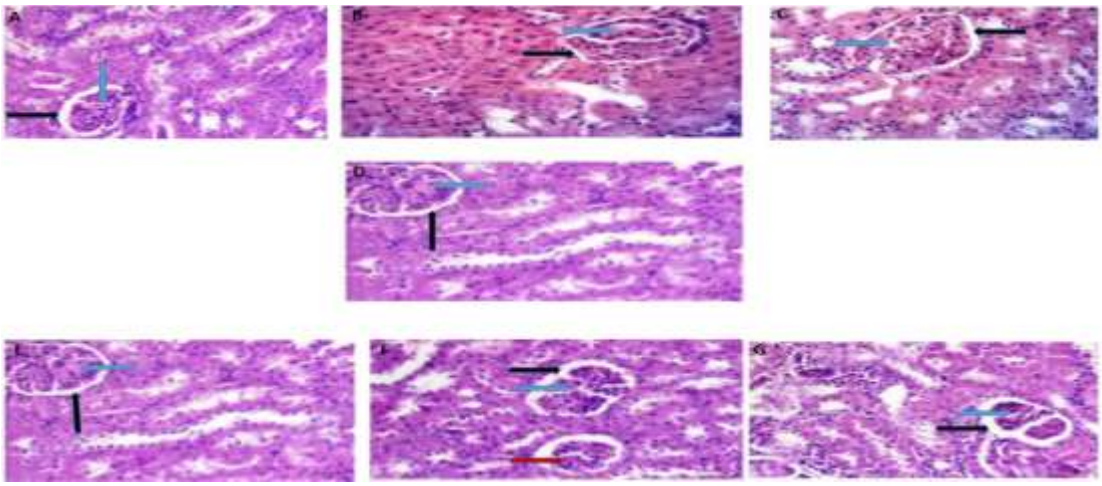


Figure 16:- Effect of D. bidens leaf extract and fraction on renal histology of Swiss albino rats.

Discussion:-

In this study, the toxicological assessment of *Diaphanathe bidens* (D. bidens) leaf extract and fractions in Swiss albino rats was evaluated by estimating the biochemical, haematological, reproductive hormone parameters, and liver and renal histopathologies after orally 90-day repeated treatments. The result of the acute toxicity of the *D.bidens* leaf extract is 9450 mg/kg after fourteen (14) days of oral treatment. Murtala *et al* (2024) indicated that therapeutic agents with oral values exceeding 2g/kg are regarded as safe, suggesting the LD_{50} of the extract is within the range of a non-toxic substance and tolerated by the animals. The effective dose (ED_{50}) of *Diaphanathe bidens* leaf extract and fractions was estimated using levamisole as a reference standard in immunosuppressive animals and the ED_{50} of the extract and fractions are recorded as; 301, 8069,186, 433 and 759 mg/kg for crude extract, n-hexane, ethyl acetate, butanol and aqueous fractions respectively. The LD_{50} recorded agrees with the one recorded by Onyegbule *et al*(2022) which stated that the LD_{50} of *D. bidens* is above 5000mg/kg.

The ethyl acetate fraction recorded the highest yield percentage 40.32% with a total phenolic content of 328.2, signifying the most bioactive fractions compared to the crude extract, n-hexane, butanol and aqueous fractions. This suggests that the phytochemical constituents in the plants are mostly non-polar compounds. The phytochemical constituents of *D. bidens*showed the presence of tannins, reducing sugars, flavonoids and alkaloids in all the extracts and fractions in varying degrees. The presence of these secondary metabolites agrees with Aba *et al*(2019). At the same time, steroids, terpenoids and saponins are absent in ethyl acetate fractions. The tannins present in all the extracts and fractions exhibit various pharmacological including the ability to scavenge free radicalsand provideantioxidant effects; antiviral activity; anti-diabetic, anticancer, anti-inflammatory and cardio-protective (Hossain *et al*., 2021). This finding revealed why the *D. bidens* extract decoction is used to treat and manage various disease conditions, including osteoarthritis and asthma, in South-Eastern Nigeria.

D. bidens leaf extracts and ethyl acetatefraction demonstrated a time-dependent increase in body weight, indicating a potential stimulation of appetite and a corresponding increase in feeding patterns. However, both the extract and ethyl acetate fraction-treated groups showed a dose-dependent reduction in body weight. The extract shows a significant effect ($P < 0.05$). On days 31, 61, and 91, the extract caused a significant decrease in body weights compared to the vehicle control groups. However, there were no notable differences observed between the extract and ethyl acetate at equivalent doses.

The liver is a vital organ in the body that has various functions. It functions primarily in detoxification, metabolism, digestion and immunity (Bemidinezhad *et al*., 2023). Diseases associated with the liver are alcohol cirrhosis, viral

hepatitis, and non-alcoholic fatty liver (Sahoo *et al*., 2015). The important liver biomarker enzymes which support its optimal function are alanine aminotransferase (ALT), Aspartic aminotransferase (AST) and alkaline phosphatase (ALP) (Owumi and Dim, 2019). Elevated serum levels of these enzymes are indications of hepatic injuries (Nouioura et al., 2023). Oral subchronic extract and ethyl acetate treatment for 90 days indicated significant ($P<0.05$) differences at varied dosages. The treatment with 372m/kg of the extract significantly decreased serum ALT ($P<0.05$) compared to vehicle control groups, as did all ethyl acetate fraction doses (186, 372, 744mg/kg). Significant ($P<0.05$) reduction in serum ALT with the extract was lost at lower dosages (186mg/kg) and higher doses (774mg/kg). Comparing the extract and ethyl acetate fraction at the same doses indicated similar and dose-dependent changes. Compared to the extract, the ethyl acetate fraction significantly reduced serum ALT at 186mg/kg ($P<0.05$). Similarly, at 774 mg/kg, significant ($P< 0.05$) differences were observed across treatments. At 372 mg/kg, both medications had similar effects with no significant differences ($P>0.05$) between groups. Similarly, none of the groups had significantly different pre-treatment AST levels ($P > 0.05$). After extract treatment, a significant ($P<0.05$) drop in AST levels was seen in the 372 mg/kg treated group. Serum AST significantly decreased ($P<0.05$) in ethyl acetate fraction groups at 372 and 744 mg/kg dosages compared to the vehicle control group. Neither the extract nor the ethyl acetate fraction at 186 and 372 mg/kg affected serum AST ($P > 0.05$). At a higher dose of 744 mg/kg, the ethyl acetate fraction significantly ($P<0.05$) reduced serum AST compared to the extract group. D.bidens extract and ethyl acetate fraction lowered serum ALP levels compared to baseline. Significant decreases ($P<0.05$) were observed at 372 and 744 mg/kg of the extract and all ethyl acetate fraction doses compared to the vehicle control group. At the highest dose (744 mg/kg), the ethyl acetate fraction had a greater reductive effect on serum ALP compared to the extract ($P<0.05$). The extract and the ethyl acetate fractions probably have membrane stabilizing effect to account for the decrease in the enzyme levels, and thus protective.

The result of liver histology showed normal hepatic cords, hepatocytes and no pathological architecture detected after sub chronic extract and ethyl acetate fractions treatments, thus supporting the assessment that the extract is harmless to the liver.

Blood urea nitrogen (BUN) and creatinine are critical indicators for evaluating kidney functions. BUN measures urea concentration, a byproduct of protein breakdown, and creatinine, a byproduct of muscle tissue degradation. Increased levels of BUN and creatinine indicate potential kidney dysfunction and toxicity.(Bemidinezhad et al., 2023). After sub-chronic administration, the extract and ethyl acetate fractions affected blood urea nitrogen differently. The concentration declined at low and high doses but increased at 372 mg/kg. At all doses (186, 372 and 744 mg/kg), ethyl acetate reduced BUN. At varied doses, these results were similar. Compared to the vehicle control group, the extract and ethyl acetate fraction did not change BUN concentration. The extract increased serum creatinine neutrally across dosages, while the ethyl acetate fraction significantly ($P<0.05$) decreased dose-dependently between 186 and 372 mg/kg. The ethyl acetate fraction increased serum creatinine levels compared to the basal at 744 mg/kg, but not significantly ($P>0.05$). The reduction caused by the extract and ethyl acetate could be due to plant secondary metabolites, flavonoids, tannins and glycosides. (Yang *et al*., 2019), Cao *et al*(2022) and Peng et al (2023) reported that the different secondary metabolites isolated from flavonoids reduced the levels of serum BUN and creatinine in mice, thus, alleviating kidney dysfunctions. Therefore, the leaf extract and fractions of diaphanathe bidens could possess nephroprotective effects based on this study which was found to significantly decrease the serum levels of blood urea nitrogen and creatinine. This is collaborated based on the result of the kidney histopathogies, which showed normal renal architecture (i.e., glomerulus, bowman capsules and tubulus) after subchronic oral treatment of rats.

Furthermore, elevated concentrations of serum follicle-stimulating hormone(FSH) and luteinizing hormone (LH) serve as indications of potential reproductive health disorders (Pratama *et al*., 2024). LH stimulates ovulation growth of corpus luteum and progesterone release, thus, acts to augment progesterone by granulose cells which stimulates FSH at midcycle (Farahbod and Soureshjani, 2018). The extracts and fractions of Diaphanathe bidens leaf caused non-significant ($P>0.05$) reductions in the serum levels of FSH and LH in all the tested groups. The non-significant reduction caused by the extract and fractions of different doses showed that D. bidens is non-toxic and safe for reproductive hormone parameters. This is in contrast to Baffoe *et al* (2021) who showed that hydroethanolic root extracts of three different herbs exerted detrimental effects on the reproductive hormones with prolonged usage. The findings from this research were supported by Udoh (2012) who showed that various doses of ethanolic extracts of G.africanum caused a dose-dependent reduction in levels of serum FSH and LH. The non-significant reductions in serum levels of FSH and LH could be due to the presence of secondary metabolite in D. bidens, which exerted a negative feedback mechanism with the elevated levels of the hormones.

The haematological parameters including pack cell volume (PCV), Haemoglobin (Hb), and the count of erythrocytes (RBC), white blood count (WBC) and platelets (Pt), offer insights into toxicity affecting blood components. The results of the haematological assessment after subchronic oral administration demonstrate that D. bidens leaf extract and fractions are safe in all doses for the blood of the animals. The findings indicate that D. bidens may possess immune-boosting activities, along with antiviral and anti-bacterial effects, as evidenced by the notable increase in white blood cells that help the body in combating foreign pathogens. The observed increase in RBC and PCV levels in the treated groups, suggests that D. bidens enhances erythrocyte production. These findings were supported by Aina *et al*(2023); Absalom*et al* (2013), and Omoirri and Harrison (2022).

Conclusion:-

The findings from the toxicological assessment of *D.bidens* leaf extract and fractions indicate that *D. bidens*exhibits no toxicity. The specific organ toxicity assessment also indicates there are not harmful effects on liver enzymes, renal parameters, blood and reproductive hormone biomarkers, and important organ architecture, composition and functions at treatment doses. Consequently, *D. bidens* demonstrates safety for long-term usage and could exhibit properties related to anti-viral effects due to the presence of secondary metabolites, especially tannins and saponins.

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Conflict of interest: no conflict of interest to be disclosed
Ethical approval: The toxicological profile of *Diaphananthe bidens* leaf extract and fractions in Swiss albino rats was approved by Nnamdi Azikiwe University Animal Research and Ethics Committee under the approval number, NAU/AERC/02024/0088

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