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Full Length Research Paper

Prolonged (28 days) toxicity study, phytochemical and proximate composition of the aqueous leaf extract of *Diosphyros mesipiliformis*

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The study aimed to determine the toxicity level of aqueous leaf extract of *Diospyros mespiliformis* on Wister albino rats and the phytochemical and proximate composition of *Diospyros mespiliformis*. The plant extract was found to contain Tannin, Saponin, flavonoid, glycoside, volatile fatty acid and Resin as constituent following phytochemical analysis. On further study using proximate analysis shown that the following constituents were found moisture (1%), Ash (7%), lipid (1.5%), fibre (2.5%), Nitrogen (1.89%), crude protein (11.81% and Carbohydrate (74.3%). Sub-chronic toxicity studies of aqueous leaf extract of *Diospyros mespiliformis* was conducted following 28 days administration of 1000, 2000 and 3000mg/kg to three group rats respectively, while the fourth group served as a control. Change in body weight, haematological and serum biochemical parameters as well as gross post changes used as indices of toxicity. The percentage yield of the extract was 16.0%. These were inappetence and weight loss in the first week of the study in the experimental groups but were no significant changes in body weight, haematology and serum biochemistry (P>0.05). It can therefore be inferred that this extract is safe at a dose of 3000mg/kg in rats following consecutive daily administration for 28 days.

Keywords: Plant extract, *Diospyros mespiliformis,* toxicity study, phytochemical analysis

INTRODUCTION

Herbalism is a traditional or folk medicine practice based on the use of plants and plant extracts. It is also known as Botanical Medicine, Medicinal Herbalism, Herbal Medicine, Herbology, and Phytotherapy (Archaya et al., 2008). Herbal medicine derived from plant extracts is being increasingly utilized to treat wide variety of clinical disease (Gupta et al., 2004). It therefore becomes necessary to identify phytochemical components of local medicinal plants usually employed by herbalist in the treatment of disease, especially with advocacy for the integration of traditional medicine in health care Programmed in Nigeria (Aderotimi and Samuel, 2006). Herbal remedies are seen by some as a treatment to be preferred to pure medicine compounds which have been industrially produced (Elvin-Lewis, 2001).

However, herbalists criticize mainstream studies on the ground that they make sufficient use of historical usage, which has been shown to be useful in drug discovery and development in both the past and the present (Fabricant and Farsworth, 2001). In 2001, researchers identified 122 compounds used in mainstream medicine which were derived from “ethno medical” plant sources; 80% of these compounds were used in the same or related manner as the traditional ethno medical use (Fabricant and Farsworth, 2001). In the 13th century, Bin al Baitaor described more than 1,400 different plants, foods, and drugs, over 300 of which were his own original discoveries (Diane, 2002). A number of herbs have been though to likely cause adverse effects (Talalay and Talalay, 2001). Furthermore, adulteration, inappropriate

formulations or lack of understanding of plant and drug interaction have led to adverse reactions that is sometimes life threatening or lethal. The extensive use of natural plants as primary health remedies due to their pharmacological properties is quite common (Conco, 1991). The investigation of the efficacy of plant – based drugs has been paid great attention because of their few side effects, cheapness and easy availability (Kumara *et al,* 2001). According to the World Health Organization, 80% of the world population still relies mainly on plant drugs (WHO, 1978). However, the same is not true of Veterinary Medicine, even though the integration will be easier with the later (Nwude, 1997). In Nigeria, the traditional concept of animal diseases and their treatment and other traditional concept of animal diseases and their treatment and other traditional practices have been documented (Arowolo and Awoyele, 1992; Nwude, 1997; Abdu et al., 2000). The extensive use of natural plants as primary health remedies due to their pharmacological properties is quite common (Conco, 1991). *Diospyros mespiliformis* locally called "Kaiwa" "Kanya" in Hausa language; it is a known tree belonging to the family Ebenaceae. It is used in the treatment of wounds and analgesia in North Western part of Nigeria, but its safety has been evaluated. Therefore, this research was conducted for the purpose of establishing the toxicity profile of the plant.

The objectives of this work were to; Evaluate behavioral changes like dullness and alterations in body weight in rats treatment with extract of *Diospyros mespiliformis.* To assess the hematological parameters such as packed cell volume, hemoglobin concentration, white and red blood cells count as well as platelets counts, in rat's treatment with the aqueous extract of the plant. To evaluate the biochemical changes such as serum enzymes (Alanine aminotransderanse, aspartate aminotransferase and alkaline phosphatese) and the electrolytes level such as (sodium, potassium, chloride and biocarbonate ions) in rats treated with the extract.

MATERIALS AND MTHODS

Thirty five (35) Wister albino rats of only one sex weighing between 100 and 200g obtained from Faculty of Pharmacy, Ahmadu Bello University, Zaria were used for the experiment. They were acclimatized for 2 weeks in the experimental animal house/room of the Veterinary Physiology and Pharmacology Department of Usmanu Danfodiyo University, Sokoto. The plant material was collected at Dundaye in Sokoto State with the help of a traditional healer. The collected plant material was authenticated to be *Diospyros mespiliformis* by a taxonomist in the Botany Unit, Faculty of Science, Usmanu Danfodiyo University, Sokoto as recommended by Kumar *et al*, (2001). A sample with voucher no. 29 has been deposited in the Botany Unit for reference.

Extraction of plant materials

The air dried plant material was reduced to coarse powder by pounding in a mortar by a wooden pestle. The dissolution was 100 g of the powder per 500 ml of distilled water, therefore 134 g of the powder was weighed using Melter® PK balance and placed in a two litre beaker containing 670 ml of distilled water. The mixture was then vigorously and manually shaken for the first 6 h, alternatively shaking for 10 min and resting for 15 min. It was then allowed to stand for the next 18 h. After this 24 h period, it was shaken again for the 10 min after which it was filtered into a clean weighed beaker using size 1 Whitman filter paper. The volume of the filtrate was noted and then placed in an electric drier and evaporated slowly at 50°C as was earlier described by Farnsworth and Soejarto, 1998). After the filtrate had dried to paste, it's weight was noted and the percentage yield was calculated as follows:

$$\%\text{Yield} = \frac{\text{Weight of dried filtrate} \times 100}{\text{Weight of the pulverized material used}}$$

Acute toxicity study

Three rats out of 35 were used to conduct the limit dose test on plant using revised up and down procedure at 72 h interval. The remaining rats were grouped into four (4) groups of six rats each and kept in separate cages a week to the commencement of the specific experiments. They were fed on pellets of grower's mash produced by Vital feeds, Jos, Nigeria and tap water was provided *adlibitum.*

Sub-chronic toxicity study

The grouped rats were labeled groups A, B, C, and D with 6 rats each. Groups A – C were administered 1000, 2000 and 3000 mg/kg of the 30% preparation of the extract of *Diospyros mespiliforms* daily by oral route with the aid of sterile 2 ml syringe for 28 days consecutively, after their weight were determined. Rats in group D served as control and received distilled water at 5 ml/kg by the same route and the same number of days consecutively. Weight taking was done weekly. On the 29^{th} day, the animals were weight, anaesthetized individually using chloroform and two blood samples were taken from each. One in EDTA sample bottle and the other in plain sample bottle, for haematological and serum biochemical analysis respectively. The citrated blood samples were analyzed for haematological parameters using auto haemo analyzer (hp, Japan) and blood samples in plain sample bottles for serum biochemistry.

Biochemical analysis

Determination of alanine transaminase

ALT was determined using the method of Reitmen and Frankel, 1957)

Principle

$$\alpha \text{ - Oxogluterate} + \text{L – alanine} \xrightarrow{\text{GPT}} \text{L – glutamate} + \text{pyruvate}$$

Glutamic – pyruvic transaminase is measured by monitoring the concentration of pyruvic, hydrazone with 2, 4 – dinitrophenyl hydrazine (Reitman and Frankel, 1957).

Materials

Serum, pipette, test tubes, cuvette, incubator colorimeter cotton wool and stop watch.

A. Reagent composition

Contents	Initial Concentration of solutions
Buffer	
Phosphate buffer	100mmol/l, pH 7.4
L – alanine	200mmol/l
α – oxoglutarate	2mmol/l
2, 4 – clinitrophenyl hydrazine	2mmol/l
Sodium hydroxide	0.4mol/l

The reagents were packed in serum glutamic pyruvic transaminase assy kit with cat No. AL 100, 2x100ml Ms 641.

Procedure:

The following are pipette into test tubes. The test tube were mixed and incubated for exactly 30 min at 37°C
The test tube was mixed, and the absorbance of the samples (A sample) against the sample blank was red after 5 min.

	Sample Blank	Sample
Sample	-	0.1ml
Solution 1	0.5ml	0.5ml
Sodium Hydroxide	5.0ml	5.0ml

Determination of aspartate transaminase

Reitman and Frankel, (1957) was used for this study.

Principle

$$\alpha \text{ - Oxogluterate} + \text{L –Aspartate} \xrightarrow{\text{GOT}} \text{L – glutamate}$$

+ oxaloacetate of oxaloacetate Glutanmic –oxoaloacetic transaminase is measured by monitoring the concentration hydrazone formed with 2, 4 – dinitropheyl-hydrazine.

Materials

Serum, pipette, test-tubes, cuvette, incubator, colorimeter, cotton wool and stop watch. The reagents were packaged in serum GOT assay kit with cat No. AS101, 2 x 200. This enzyme reagent and buffer had been prepared and ready for use.

Reagents composition

Contents	Initial Concentration
Buffer	
Phosphate buffer	100 mmol/1,pH 7.4
L-aspartate	100 mmol/l
A-oxoglutarate	2 mmol/l
2,4-dlinitrophenyl hydrazine	2 mmol/l
Sodium hydroxide	0.4 mmol/l

Procedure

The following were pipette into the test-tubes. The test tubes were mixed and allowed to stand for 20 min at 20°C C to 25°C. The test tubes were mixed and the absorbance of the sample (A) sample) against the Sample Bank was red after 5 min.

	Sample Blank	Sample
Sample	-	0.1ml
Solution 1	0.5ml	0.5ml

The test tubes were mixed and incubated for exactly 30 min at 37°C

Solution 2	0.5ml	0.5ml
Sample	0.1ml	
Sodium Hydroxide	0.5ml	0.5ml

	Sample Blank	Sample
Sample	-	0.1ml
Solution 1	0.5ml	0.5ml

The test tubes were mixed and incubated for exactly 30 min at 37°C

Solution 2	0.5ml	0.5ml
Sample	0.1ml	
Sodium Hydroxide	0.5ml	0.5ml

Determination of alkaline phospatase

The Alkaline Phosphatase level in the samples was undertaken using standard methods (Rec, 1972).

Principle

p-nitropheylphophate + H_2O phosphate +p-netrophenol

Materials

Serum, pipette, test-tubes, cuvette, (1 cm light path incubator, cotton wool and stop watch).

Reagent composition

Buffer
Diethanolamine buffer = 1mol/l, 7.8
$MgCl_{2\ =}$ 0.5mmol/l
Substrate
p-nitrophenylphosphate = 10mml/l

The reagents were packed in alkaline phosphatase assay kit with cat.No. AP. 501, 5 x 20 ml.

1. Buffer 1 x 505 ml
2. Substrate 5 x 2 ml

The enzyme reagent was prepared

Procedure:

Two test tubes were labeled sampled and blank. The semi-micro pipette was used to pipette 0.02 ml of the serum into the sample test-tube and 1.00ml of the reagents was pipette each into the sample tube and blank tube. The test-tubes were mixed thoroughly and absorbance was red at 405 nm simultaneously for 1, 2, and 3 min respectively.

Wavelength = Hg 40nm
Cuvette = 1 cm light path
Temperature = 25, 30, 37°C
Measurement: Against air

The following were pipette into cuvette

Sample 0.02ml
Reagent 1.00ml

The sample was mixed, the initial absorbance was red, and the absorbance was red again after 1, 2 and 3 min simultaneously.

Calculation

The activity if ALT was calculated using the following formula.

Analysis of Variance (ANOVA)

The results were presented as mean + SD and subjected to Difference between means compared were considered significant at ($p<0.05$) (Steel and Torrie, 1980).

phytochemical analysis of the aqueous extract of

RESULTS

Percentage yield

The percentage yield of the extract is calculated below;

$$\% \text{ yield} = \frac{\text{weight of evaporated extract}}{\text{Weight of pulverized material}} = \frac{21.5 \times 100}{134} = 0.160 \times 100 = 16.0\%$$

Acute toxicity

Three rats were used to determine the limit dose test on the plant which was found to be $\geq$ 5000mg/kg. all the tested rats survived the limit dose test.

Sub-Chronic toxicity studies

Effect of the extract on physical activities

The rats used in this study showed various dose dependent signs of toxicity within the first week of the experiment. The signs observed include rough hair coat, depression and inappetance.

Effect of the extract on weight

There were no significant differences in the weight of the rat in the experimental groups when compared to the control following 4 weeks treatment with the extract although there was a slight loss of weight in the first week in the experimental groups. The results are presented on (Table 1).

Effect of the aqueous extract of D. mesipiliformis on haematological parameters and indices of rats

The effect of the extracts on hematological parameters and indices is presented in (Tables 2 and 3). there were no significant ($p>0.05$) changes in the haematological parameters and indices (Hob) concentration, Rbc count, MCV, MCH, MCHC, and PLT) of the rats in all the groups when compared with the control, except for the packed cell volume (PCV).The rats that received 2000 mg/kg had significant ($p<0.05$) lower PCV values than those in the control and other treatment groups. Table 4 shows the results of the effects of the extracts on the liver enzyme while (Table 5) shows that of electrolytes. The results indicated that the extract has no significant ($p>0.05$) effect on the serum enzymes and electrolyte when compared to the control. Table 4 shows the results of the

Table 1. Effect of administration of various doses of the aqueous extract of *D. mesipiliformis* on body weight of rats.

Dose (mg/kg)	Initial	1st week	2nd week	3rd week	4th week
1000	256.45 ±23.17	248.13± 10.55	260.87± 13.375	263.98 ± 11.8	269.50 ± 14.15
2000	222.63± 53.17	218.00 ± 45.04	230.45± 45.86	232.63± 63.62	233.30 ± 64.00
3000	242.555±27.73	239.60± 27.23	239.88 ± 29.58	252.60± 28.92	252.15 ± 32.10
Control	216.35± 26.62	206.28± 16.84	219.38 ± 23.17	224.78± 24.71	225.68 ± 26.90

Table 2. Effect of administration of various dose of the aqueous extract of D. mesipiliformis on haematologial parameters and indices of the rats.

Groups	Dose (mg/kg)	PCV%	Hb (g/dl)	Rbc (x10^{12}L)	MCV (fl)	MCHC (g/d)	MCH (p/g	PLT (x10^{9}/L)
A	1000	52.34 ± 11.37						
B	2000							
C	3000							
D	Control							

Key:-Significance ($p \leq 0.05$); PCV = Packed cell volume; Hb = Haemoglobin; Rbc = Red blood cells; MCV = Mean Corpuscular volume; MCHC = Mean Corpuscular volume; MCH = Mean Corpuscular haemoglobin; PLT = Platelet

Table 3. Effect of the aqueous of the aqueous extract *D. mesipiliformis* on white blood cells and differential counts of rats.

Groups	Dose (mg/kg)	WBC (x10^{9}/L	GRA (%)	LYM (x10^{9}/L)
A	1000	6.20 ±4.281	89.13± 4..950	5.68 ± 3.20
B	2000	11.72 ± 5.186	95.20 ± 0. 928	10.37 ± 4.30
C	3000	7.21 ± 1.707	86.22 ± 4.021	7.22 ± 2.29
D	Control	7.80 ± 1.631	87.12 ± 4.021	6.98 ± 3.131

Key:-WBC = White blood cell; GRA = Granulocytes; LYM = Lymphocytes.

Table 4. Effect of the aqueous extract of *D. mesipiliformis* on liver enzymes

Groups	Dose (mg/kg)	WBC (x10^{9}/L	GRA (%)	LYM (x10^{9}/L)
A	1000	6.20 ±4.281	89.13± 4..950	5.68 ± 3.20
B	2000	11.72 ± 5.186	95.20 ± 0. 928	10.37 ± 4.30
C	3000	7.21 ± 1.707	86.22 ± 4.021	7.22 ± 2.29
D	Control	7.80 ± 1.631	87.12 ± 4.021	6.98 ± 3.131

Key- AST = Aspartate Aminotranferase; ALP = Alkaline phosphatase; ALT = Alanine Aminotranferase

Table 5. Effect of the aqueous extract of *D. mesipiliformis* on liver enzymes electrolyte.

Groups	Dose (mg/kg)	Na^+ (MEq/L)	K^+ (MEq/L)	CT	HCO_3^-
A	1000	130.00 ± 16.250	4.501 ±0.862	99.20 ± 2.365	24.00 ± 0.601
B	2000	136.00 ± 14.369	5.30 ± 0.737	89.50 ± 0.929	22.00 ± 1.542
C	3000	138.00 ± 10.069	4.6 ± 0.531	95.1 ± 1.172	23.00 ± 0.612
D	Control	131.00 ± 9.512	4. 4 ± 0.498	99.0 ± 2.118	25.00 ± 1.130

Key: Na^+ = Sodium ion; K^+ = Potassium ion; Cl^+ = Chloride ion; $HC0_3^-$ = Bicarbonate

effects of the extracts on the liver enzyme while (Table 5) shows that of electrolytes. The results indicated that the extract has no significant ($p>0.05$) effect on the serum enzymes and electrolyte when compared to the control. Tables 6 and 7 show the results of phytochemical screening and proximate analysis respectively.

DISCUSSION

The percentage yield of 16.0% suggests that the plant *Diospyros nespiliformis* contains a lot of fibres that will form out to be residues after extraction, although the result of proximate analysis indicates that the plant has

Table 6. Phytochemical screening of the aqueous leaf extract of *D. mesipiliformi.*

Qualitative test	Result
Alkaloids	
Meyer's	+
Hagaer's	++
Wagner's	N.D
Tannin	++
Saponin	++
Saponin glycosides	++
Flavonoid	++
Cardiac glycoside	N.D
Steroid	N.D
Glococide	++
Volatile fatty acid	+
Anthraquinone	N.D
Resin	+

Keys N.D = not detected; + = detected

low fibre content (2.5%) and high ash (7%). The manifestation of weight loss and inappetance observed in the rat in the study may be due to reduced feed and water intake mainly in the first seven days of the experiment which could be attributed to the presence of antinutritional substance like tannin and saponin contained in the extract (Nayak *et al,* 2007). Acute toxicity test using the limit dose of the up and down procedure 5000mg/kg on the plant extract produced no mortality after 48 hours of observation. There were no significant ($p>0.05$) changes in the weight of the rats that received doses of the leaf extracts compared with the initial weight of the animals, this could be due to high content of protein and carbohydrate (11.81%) and (74.3%) respectively. Changes in haematological parameters were not observed in the extracts treated groups when compared with the control ($p>0.05$) except in PCV of rats that received 200 mg/kg. this may be an indication that the plant is relatively safe to haemopoetic system at the dose used. The slight decrease in packed cell volume of the rats received 2000 mg/kg dose of the extract of *D. Mespiliformis* may not be associated with the extract but other endogenous problems that were not apparent more so that there was no effect on those that received the extract at 300mg/kg. No significant change was produced by the extract on the white blood cells and differential counts of the treated rats ($p>0.05$). This might be an indication that the doses used in this experiment have no adverse effects on the haemapoetic system. In this study, there were no significant changes in both cationic and anionic electrolyte concentrations, which may indicate no remarkable effect of the extract on these ions. Sodium and potassium are cations of the extracellular and intracellular fluid respectively and are closely related to water balance and lack of kidney pathology respectively (Coles, 1986; Morag, 1989). With respect to serum enzymes (ALT, AST, ALP). There were no significant changes observed in the rats treated with the extracts in this study. The liver enzymes, aspartate and alanine amino transferases (AST and ALT) are involved in amino acid metabolism, large amount of ALT are present in the liver, kidneys, cardiac muscle and skeletal muscles. This could be as a result of presence of flavonoid as a constitutent of enzyme system involved in intermediary metabolism. Alanine Aminotransferase is known to be found principally in the liver Cheesbrough, (1991). Serum ALT and AST levels were always found to increase in liver cell damage and the greater the degree of the liver damage, the higher the activities of both enzymes (Cheesbrough, 1991). Alkaline phosphatase is widely distributed within different tissues possessing one or more of the isoenzymes (Whetby *et al.,* 1989). In bone liver and intestine, Alkaline phosphatase is a marker enzyme for the plasma membrane and endoplasmic (Wright and Plummer, 1974). It is often used to assess the integrity of plasma membrane (Akanji *et al.,* 1993). Significant elevation of serum alkaline phosphatase is an indication of cholestasis (Van Hoof and De Broe, 1994), with no effective control of ALP activity towards improvement in the secretory function of the hepatic cell.

Conclusion

It can be concluded from this study that the aqucous leaf extract of *Diospyros mespiliformis* is safe for use as a medicinal plant by both OECD and WHO standards.

Recommendations

This study was carried out using cold water extraction procedure as practiced by traditional healers. Therefore, it is recommended that further studies should be carried

out to further verify its safety by using other extraction method and solvents. This plant is traditionally used as an analgesic but this work only established the toxicity profile of the plant.

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