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ACUTE TOXICITY STUDIES OF ETHYL ACETATE METABOLIC EXTRACT OF ENDOPHYTIC FUNGI ISOLATE OF *MITRCARPUS SCABER*

IFEOMA S. ASOGWA[1,2], ANTHONY C AGBO[3]*, UDAUK P. ISE[4], CHINEDU E. IBEZIM [1], ANTHONY A. ATTAMA[1]*

[1]Department of Pharmaceutics, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka 410001, Enugu State, Nigeria.
[2]Department of Pharmaceutical Microbiology, Faculty of Pharmacy, Bingham University, Karu, Abuja, Nigeria. [3]Department of Pharmaceutical Microbiology & Biotechnology, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka, Nigeria. [4]Department of Pharmacology and Toxicology, Faculty of Pharmacy, Bingham University, Karu, Abuja, Nigeria.

ABSTRACT

Safety or toxicity assessment of newly discovered endophytes with potential therapeutic applications is very crucial prior to their approval for human use. This is to ascertain the possible undesirable outcomes of the drug agents within a short-term of 24 h of administration (acute toxicity). This study was

Introduction

Nature is sufficiently equipped with varieties of plants in which virtually all their parts, including the leaves, stem,

aimed at determining the adverse effects that could be observed within a short-term of 24 h of oral administration of multiply doses of metabolic ethyl acetate extracts of leaf parts of *Mitracarpus scaber* to rodents. In determining the median lethal dose (LD_{50}) of the crude extract, a method described by Lork was followed, which involves two phases of administration of test drug to experiment animals. In the first phase, oral gavage needle in a single dose disposable syringes were used to administer three dose levels (10, 100, and 1000 mg/kg) of ethyl acetate extract to mice of three in a group. Under critical observation, behavioral changes and other possible signs of death/adverse effects associated with the test extract were documented. The second phase was a deduction of first phase, eight mice were divided into four groups of two mice each. Each group was treated orally with graded doses of 1200, 1600, 2900, 5000 mg/kg respectively. Signs of adverse drug reactions and other side effects within 24 hours were observed and recorded and from the results gotten, final LD_{50} was evaluated. Results from the treatment showed that the animals were not subjected to any immediate risks of drug reaction or adverse effects on 24 h exposure and the LD_{50} for extract was > 5000 mg/kg. Also, on completion of the treatment some parameters evaluated showed significant differences when compared with standards. Generally, the result of this study shows that the metabolic crude extract of endophytic fungi isolated from *Mitracarpus scaber* showed no oral acute toxicity in mice. However, further evaluation of sub-acute and long-term toxicity studies using oral and other routes of administration is recommended.

Keywords: Endophytic fungi, *Mitrcarpus scaber*, Acute toxicity, Metabolitic extract, Median-lethal dose,

roots, fruits, seeds, barks as well as the flowers could be useful as both traditional and allopathic medicines (Abiole,2022). *Mitracarpus scaber*

is one among these numerous plants that are of vast ethno medicinal use. It belongs to the plant family of Rubiaceae, which is abundant in the tropics and Orients. *M. scaber* grows on degraded soils in Africa and Asia (Shinakafi, 2014), but it was also found in Latin America (Quadja *et al, 2018*). In Togo and many tropical country of African M. scaber can be harvested from June to November it is a yearly herbaceous plant with erect stems of about 53 cm high and branches that are about 8 cm long. The plant has lanceolate leaves that are simple, alternate, oblong about 4.5 cm long with an upper scabrous surface. Its inflorescence consists of clusters of small whitish flowers arising above the leaf axils which turn brownish yellow as the plant matures. At maturity, this plant makes white flowers at the level of each armpit of the leaves. Traditionally, it is commonly used in West African in the treatment of diseases, such as, infectious dermatitis, eczema, ringworm, toothaches, amenorhoea, dyspepsin, hepatic diseases, scabies, leprosy (Aboh, *et al*, 2014, Germano *et al,* 2000, Moussa *et al, 2015*). In the southern part of Nigeria (Delta State), the freshly harvested leaves of *M. scaber* are macerated and mixed with palm kernel oil to be used for skin diseases and tinea infections, and also a good relief for itching, lice infestation (Yameeogo, 1982). The young leaves are squeezed and rubbed on a fresh cut, wounds and ulcers. It has also been reported (Karou *et al, 2000*) that the plant can be used for infantile toothache, anti-parasitic and venereal infections. In Togolese traditional medicine, the plant was used to treat infected wounds, skin (kondoro *et al, 2017*). It was also used orally in combination with sesame to treat liver problems. With reoccurring cases of organ damages, which impetuous consumption of traditional medicines may have contributed to, toxicity testing of any agent that has shown some pharmacological effects has become very important prior to its application on humans. Meanwhile, toxicity studies could

be Acute, sub-acute or chronic and they are all standard methods employed by industrial pharmaceuticals for their routine testing for possible toxic effects of a new drugs. Acute oral toxicity test is a procedure that involves the estimation of oral median lethal dose (LD_{50}) which can kill 50% of the experimental animals either from a single exposure or multiply exposures of within or less than 24 h. Endophyte are microbes that are found inhabiting the intercellular in the tissues of plants without causing any infections, however, having gained a lot of attention as they have been found to possess high bioactive compounds with a very high medicinal values, there arises the need to checkmate the safety level of those compounds. Furthermore, there is no report yet on acute oral toxicity studies of endophytes found in *Mitracarpus scaber,* therefore, this research aimed at evaluating the acute oral toxicity of ethyl acetate metabolic extract of fungal endophytes isolates from leaf part of the plant.

MATERIALS AND METHOD.

Materials

The materials used for the study include: weighing balance, disposable syringe and gavage needle, petri dishes, endophytic fungi extract, dimethyl sulfoxide (DMSO) and ethyl acetate (Sigma Aldrich, U.K), Sodium hypochlorite, 70% alcohol, sterile distilled water (Lion's Table Water, UNN), local rice, ketamine and normal saline (Juhel Pharmaceutical, Awka).Other reagents were of analytical grade and were used without further purification.

Sample collection and authentication

The plant of *Mitracarpus scaber* is predominately found in Obukpa in Nsukka Local Government Area of Enugu State Nigeria. The leaves part of healthy and fully matured of this plant (5 kg) were collected and

duly identified by a taxonomist in the Department of Plant Science and Biotechnology, University of Nigeria Nsukka. It was further authenticated, by a botanist in the Department of Plant Science and Biotechnology and was assigned a voucher number UNH NO 10a and deposited at the herbarium collection center of the Department. The ethical clearance approval with the following reference number DOR/UNN/21/00032 from the Ethics Committee on Research in University of Nigeria, Nsukka for this research was sought and obtained.

Sterilization of sample materials

The whole processes of sterilization, cultivation, isolation and purification followed procedure described by Okezie *et al*,2017 with some modifications. Firstly the samples were subjected to pre-treatment step which involved washing the leaves separately under the tap water followed by sterile distilled water. This helped to remove adhering soil particles at the surfaces of the leaves. Afterwards, the second step involved the surface sterilization of the plant material to eliminate surface epiphytes. To achieve this, the samples were put through four step surface sterilization which entailed soaking the freshly collected plant parts in slow running tap water for 15 min, after which they were further immersed in 70% ethanol for 3 min and washed twice with distilled water. In addition, the already washed samples were again immersed in sodium hypochlorite solution (4%) for 5 min and washed thoroughly thice in distilled water and then rinsed in 70% ethanol for 3 min, before a final rinse in sterilized distilled water.

Cultivation and isolation of endophtes (fungi)

The washed samples were dried in the laminar flow cabinet on a sterile filter paper. To exposes the tissues in the mid-ribs and the leave vein,

a sterile knife was used to cut the samples to approximately 1 cm in length exposing the endophytes more. Having a total of 30 segments, three to six segments were planted per Petri dishes containing malt extract agar incorporated with chloramphenicol (500 mg/L) which were previously sterilized. The Petri dishes were properly sealed with parafilms, then incubated at 25 °C and were checked on alternate days. After 7 days hyphal tips of the actively growing fungi from the plant material, were inoculated onto a sterile malt extract agar (MEA) plates for sub culturing and were incubated for another 7 days to enable the organism grow and mature fully to expected optimal purity of the endophytes. This was repeated at an interval of two weeks to maintain purity and the maximum growths of the fungi were also observed on MEA. For identification, the morphological characteristics were observed visually such as the colony color, elevation and texture (Asogwa, *et al*, 2023). The process of optimization was followed for the production of metabolites, and the starting materials were taken from freshly sub cultured plates.

Purification of endophytic fungi isolates

The hyphal tips of a stock culture were sub-cultured by picking the hyphal tip, placing on a fresh MEA and incubating at 25 °C for 7 days. This was done to obtain to get rid of contaminates present in old culture. All transfers were done aseptically, to maintain pure isolates. Upon incubation, it was discovered that the mid-rid, gave two isolates as well as the leave blade making a total of four fungi isolated from the culture.

Fermentation processes of the isolates

From previous research, it has been documented that fungi endophyte thrives more in local rice medium than when it another substrate

(Asogwa, *et al, 2023*).). Hence, Local rice was used as the fermentation medium. The local rice (100 g) was weighed into a sterile conical flask, 200 mL of sterile water added onto it, and the content sterilized appropriately at 121 °C for 30 min, and then allowed to cool properly. Thereafter, segments were aseptically cut from the actively growing pure isolates on MEA and inoculated into the already sterilized local rice fermentation medium contained in a 500-mL Erlenmeyer flask. This was properly sealed with sterile cotton and kept on the shelf. The fermentation process was allowed for 21 days at 30 °C under static conditions.

Extraction of Fungal metabolites

The fermentation process was terminated by the addition of the extraction solvent (ethyl acetate) and each of the fermented medium in the sterile Erlenmeyer flasks was made homogeneous. Here, fungal biomass including the medium were cut into small lumps using a sterile glass rod and the mixture was homogenized with 500 mL of ethyl acetate in one litre Erlenmeyer flasks, and shook vigorously intermittently for 2 days and then filtered using Whatman filter paper (size: 188 mm). The filtrate was concentrated at 50 °C under reduced pressure using a rotary evaporator. The concentrated extract was further left to evaporate to dryness in a desiccator containing sodium hydroxide. The corresponding extracts were weighed and their respective percentage yields recorded in milligram.

Experimental animals

Seventeen adult male mice (*Mus musculus*) were used for the study. The animals procured from TwinVet Resource in Nsukka, Enugu State, Nigeria, were taken to the research facilities of the Department of Veterinary Anatomy, Faculty of Veterinary Medicine, University of

Nigeria, Nsukka. They were kept there and provided with adequate feed and water for a period of seven days before the commencement of the studies.

Ethics statement

The protocol was conducted in compliance with the Guide for the Care and Use of Laboratory Animals, 8th Ed, 2011. Animal sacrifice was performed by euthanasia, according to the University of Nigeria guidelines on Euthanasia, and efforts were made to minimize animal suffering (National research council., 2011).

Experimental design

Seventeen healthy adult male mice were weighed and their sexes determined. The acute oral toxicity (LD_{50}) was estimated following the standard method (Lorke, 1983). Dose levels of 10, 100, and 1000 mg/kg were administered using oral gavage needle in a single dose disposable syringe to three mice each for the first phase. The animals were observed for possible deaths or other side effects associated with the test substance in each group within 24 h of the treatment. In the second phase, which was deduced from the first phase, eight mice were grouped into four groups of two rats each and were treated with doses of 1200 mg/kg, 1600 mg/kg, 2900 mg/kg, and 5000 mg/kg orally respectively. They were also observed for 24 hours as in the first phase, and final LD_{50} value, determined.

Euthanasia

Each mouse was sacrificed by euthanasia. Euthanasia was achieved by injection of 1 ml of ketamine by intramuscular administration, using a syringe and hypodermic needle.

Histological preparation

Following euthanasia, the liver from each mouse was harvested and fixed/preserved in 10% neutral-buffered formalin before subjection to tissue processing procedures for the preparation of permanent mount of each tissue (Sofowora, 1993). The tissues were dehydrated through various grades of alcohol (60, 70, 80, and 90%) with a final bath in 100% ethanol (twice) to ensure total elimination of moisture. Clearing was performed in toluene in order to raise its refractive index to that of glass (1.5) to enable transparency of the cellular inclusions. The processes of infiltration and embedding were performed using liquid paraffin and molten paraffin wax using L-shaped mold, respectively. Sections were made using Rotary Microtome and the Hot plate method was used for mounting specimens onto slides. Staining of tissues was performed using hematoxylin and eosin stains. Canada balsam was used in mounting the tissues.

Light microscope examination

Leica binocular light microscope was used to examine the histomorphological characteristics of the liver, kidney and the spleen of the treated mice. Photomicrographs were captured using a Moticam Images Plus 2.0 digital camera (Motic China Group Ltd) attached to the Leica binocular light Microscope

Effect of graded doses of the fungal metabolites on some biochemical parameters.

Determination of alkaline phosphatase (ALP)

This was done using colorimetric method as described by Randox Laboratory Limited. In summary, 0.01ml of sample was pipetted into a cuvette containing 0.5ml of reagent (diethanolamine buffer, $MgCl_2$ and p-nitrophenylphosphate), mixed and the initial absorbance read

at 405nm and 30 °C. The timer was restarted simultaneously and readings were taken after 1, 2 and 3 min.

Calculation of the ALP in U/l at 405 nm/min was done as follows:

ALP (in U/l) =2760 X ΔA …… Eqn 1

Where

ΔA = change in absorbance; initial absorbance- absorbance at time T.

Determination of catalase (CAT)

Catalase activity may be measured quantitatively by used standard method (von Euler and Josephson, 1927) .The necessary dilutions of a preparation of catalase are made and placed in an ice-water bath. In this work, a 1: 2500 solution of catalase-Sarett was used. To 50 ml of 0.01 hydrogen peroxide containing 0.007 M phosphate buffer at a pH of 6.8 (which has been allowed to sit in a beaker of ice water for 5 minutes) was added 1 ml of properly diluted enzyme. Mix, and immediately pipette 5 ml. of this solution into a reaction tube containing 2 ml. of the 5 N sulfuric acid. Mix, and add 10 ml. of the permanganate solution. Mix, and read in the calorimeter against the reference tube with a 515 rnp filter. Repeat again after 3, 6, 9, and 12 min. You have to report the experiment i.e. put your sentences in reported speech.

Then calculate the K0 value and the Kat. F. value (2).

A blank reading is made with a mixture containing 2 ml. of acid, 5 ml. of distilled water, and 10 ml. of the permanganate solution.

The volume of permanganate remaining after the reaction with hydrogen peroxide is calculated according to the following formula:

$$\frac{D_{bl.} - D_S}{\text{Slope}} = \text{ml} \quad \text{……..Eqn. 2}$$

Where,

D_{bl} is the optical density of the blank reading; D is the optical density of the sample reading and Slope is the average slope of the standard curve.

Determination of aspartate aminotransferase (AST)

This was carried out by measurement against sample blank as described by Randox Laboratories Ltd. Two test tubes containing 5.0 ml of reagent 1 (phosphate buffer 100mmol/l at pH 7.4, L-aspartate 100mmol/l and α- oxoglutarate 2mmol/l) were labelled sample and sample blank respectively. Further, 0.1 ml of serum (sample) was pipetted into the sample test tube. The content of each tube were mixed and incubated for 30.0 min at 37 °C. Again, 5.0ml of reagent 2 (2, 4- dinitrophenyl hydrazine 2.0 mmol/l) was added to each test tube while 0.1ml of serum was added to sample blank test tube. The above tubes were stirred and allowed to stand for exactly 20 min at 25°C. Five milliliter of 10 % sodium hydroxide solution was added to each tube and allowed to mix thoroughly and then the absorbance of the sample (A sample) at 546nm against the sample blank was read after 5.0 min.

Determination of alanine aminotransferase (ALT)

This was carried out by measurement against sample blank as described by Randox Laboratories Ltd. Two test tubes containing 5.0 ml of reagent 1 (phosphate buffer 100mmol/l at pH 7.4, L-alanine 200mmol/l and α- oxoglutarate 2mmol/l) were labelled as sample and sample blank respectively. Further, 0.1 ml of serum (sample) was pipetted into the sample test tube. The content of each tube were mixed and incubated for 30.0 min at 37 °C. Again, 5.0ml of reagent 2 (2, 4- dinitrophenyl hydrazine 2.0 mmol/l) was added to each test tube while 0.1ml of serum was added to sample blank test tube. The above tubes were stirred and allowed to stand for exactly 20 min at 25 °C.

Five milliliter of 10 % sodium hydroxide solution was added to each tube and allowed to mix thoroughly and then the absorbance of the sample (A sample) at 546 nm against the sample blank was read after 5.0 min.

Determination of superoxide dimutase (SOD)

Superoxide dismutase activity was measured according to Zhang's method (Zhang,*et al,*2017) with some modifications. One milliliter of assay mixture contained 100 mM sodium phosphate buffer (pH 7.8), 57 µM nitroblue tetrazolium (NBT), 0.025% of Triton-X-100, 0.11 mM of EDTA, 0.01 M methionine, 1.3 µM riboflavin and 50 µg of total protein. The reaction was initiated by illuminating the assay mixture with fluorescent lights. After 7 min, the reaction was stopped by removing the light source. A control tube with assay mixture and enzyme was kept in the dark, while another tube without enzyme was kept in light to serve as control for the reduction of NBT by light. The absorbance of the reaction was read at 560 nm. Activity of SOD is reported as NBT reduction in light without enzyme minus NBT reduction with enzyme. One unit of enzyme activity is defined as the amount of enzyme required to inhibit the NBT reduction by 50% under the assay conditions. SOD data are presented as units of activity per mg protein.

Determination of gluathion (GSH-PX)

Analyzing of Glutathione Peroxidase (GSH-Px) activity was evaluated according to the standard procedure with a little modification (Paglia and Valentine,1967). Activity of GSH peroxidase was determined in spectrophotometric ally in absorbance at 340 nm. The results reported as kinetically.

Determination of malondiahyde (MAD)

Malondialdehyde (MDA) measurement was done using standard method (Janero, 1990). The principle of this method is based on the

measurement of the absorbance at 535nm as spectrophotometrically of the color that MDA forms with thiobarbituric acid (TBA) in acidic media. Standards were prepared using the 1,1,3,3, tetramethoxypropane. The results were calculated through standard graph

Effect of graded doses of the fungal metabolites on haematological parameters.

Determination of packed cell volume (PCV)

This was carried out using standard haematological procedure (Ochei and Kolhatkan, 2007). Well mixed anti coagulated blood was aspirated into a capillary tube with one end sealed with plasticine. The tube was spun in a haematocrit centrifuge for 5 min and then read off a PCV reader.

Determination of red blood cell (RBC)

This was done using standard haematological procedure (Ochei and Kolhatkan, 2007). Well mixed anticoagulated blood was diluted 1:20 with 10% Na_2CO_3 solution. The mixture was loaded into an improved Neubauer counting chamber. Appropriate squares were added up to determine the total red cell count.

Determination of white blood cell (WBC)

This was carry out using standard hematological procedure (Ochei and Kolhatkan, 2007). Well mixed anticoagulated blood was diluted 1:20 with Turks solution (2% glacial acetic acid) in a test tube. This was loaded into an improved Neubauer counting chamber. Appropriate squares were counted and added up to determine the WBC.

Determination of platelet count (PLT)

standare procedure by Riaz'S book was used (Riaz and Bhutta,2006) using a platelet counting Neubauer chamber**.** Taken 2 micromillitres of blood using micropipette and added 1.8 ml of 1% ammonium oxalate. Left to stand for15 min for complete lysis of RBCs. After which a Neubauer chamber was mounted and left to stay for 15 min in a high humidity before counting the large central square.

Haemoglobin (Hb) estimation

This was carried out using standard procedure (Ochei and Kolhatkan, 2007). Well mixed venous blood (20µl) was added to 5ml of Drabkins solution in a test tube to give a dilution of 1: 250. This was mixed and allowed to stand for 10 min at room temperature. The absorbance was calorimetrically determined at 540 nm using Drabkin's solution as blank. The absorbance reading was multiplied by a factor of 36.8 to give the actual hemoglobin value

Effect of graded doses of the fungal metabolites on white blood differentials.

This was carried out according to a premeditated method (Cheesbrough, 2000). A drop of anticoagulated venous blood was placed on the end of a clean dry slide. A clean smooth edged spreader held at an angle of 30 was used to spread the drop of blood, making a film of about 40 –50 mm. The film was immediately air dried by waving it back and forth and when completely dried, the film was fixed by placing 1 to 2 drops of moisture free methyl alcohol on the film and allowing it to dry on a staining rack. The dry blood film was covered with 3drops of undiluted Leishmans reagent (no 48) and 6 drops of pH 6.8 buffered water (reagent no 20) was equally added and mixed thoroughly using plastic bulb pipette. The mixed stain was allowed to stay for 10 min and then washed off with tap water. The back of the slide was wiped clean and made to stand on staining rack for the smear to dry. A drop of immersion oil was placed on the lower third of the

blood film and covered with a clean cover glass. The film was examined microscopically by focusing on the cells using 10x objective with the condenser iris closed sufficiently to see the cells clearly. Moving to a part of the film where the red cells overlap, the 40x objective was brought in and the iris diaphragm was opened more. Systematically, the blood film was examined and the different white cells in each field were counted using automatic differential cell counter. The absolute number of each white cell type was calculated by multiplying the number of each cell counted (expressed as decimal fraction) by the total WBC count.

Data and statistical analysis

Results obtained in the study were presented as mean ± standard error of mean (SEM) of sample replicates (n = 3). Significant differences between control and treatment groups were compared using one way analysis of variance (ANOVA) followed by post hoc Dunnett (2-sided) test. $P<0.05$ was considered to be statistically significant, while $p>0.05$ was considered to be statistically non-significant. Statistical Package for Social Science (SPSS-20, for windows) was the software used for data analyses. Graphical presentations of parameters, calculation of fifty percent inhibitory concentration (IC_{50}) for antioxidant activity and fifty percent effective concentration (EC_{50}) of the extract were done with logarithmic equation using Microsoft Excel, 2010.

RESULTS AND DISCUSSION

Observation of the reaction of mice

Table 1: **Acute oral toxicity studies of various doses of selected endophytic fungal metabolites on the mice**

Group	Dose (mg/kg)	No of death	% Mortality	Observation Period (h)	Symptoms of toxicity
(a) EXTRACT FROM LB1					
1	10	NIL	0	24	Stable
2	100	NIL	0	24	No symptom

3	1000	NIL	0	24	Dullness
4	1600	NIL	0	24	Occasional dullness
5	2900	NIL	0	24	Sluggish movement
6	5000	NIL	0	24	Initial restlessness
7	Control	NIL	0	24	
(b) ETRACT FROM LB2					
1	10	NIL	0	24	Stable
2	100	NIL	0	24	No sign of toxicity observed
3	1000	NIL	0	24	No toxicity observed
4	1600	NIL	0	24	No symptoms
5	2900	NIL	0	24	Occasional dullness
6	5000	NIL	0	24	Initiate staggered movement
7	Control	NIL	0	24	
(c) EXTRACT FROM MR1					
1	10	NIL	0	24	No toxicity observed
2	100	NIL	0	24	No sign of toxicity
3	1000	NIL	0	24	Initial dullness
4	1600	NIL	0	24	Calm
5	2900	NIL	0	24	Stable
6	5000	NIL	0	24	Sluggish movement
7	Control	NIL	0	24	
(d) EXTRACT FROM MR2					
1	10	NIL	0	24	Stable
2	100	NIL	0	24	No symptoms observed
3	1000	NIL	0	24	Little change in movement
4	1600	NIL	0	24	Very active
5	2900	NIL	0	24	No changes
6	5000	NIL	0	24	Dullness of animal
7	Control	NIL	0	24	

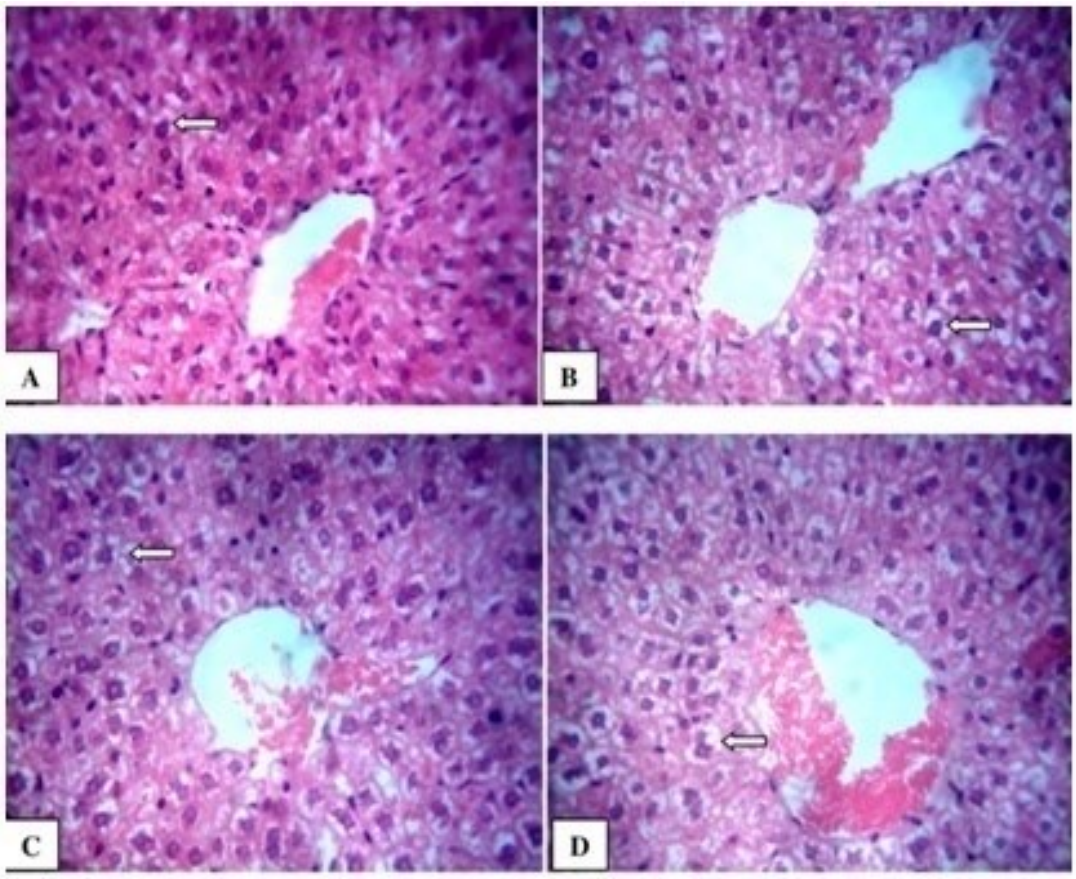
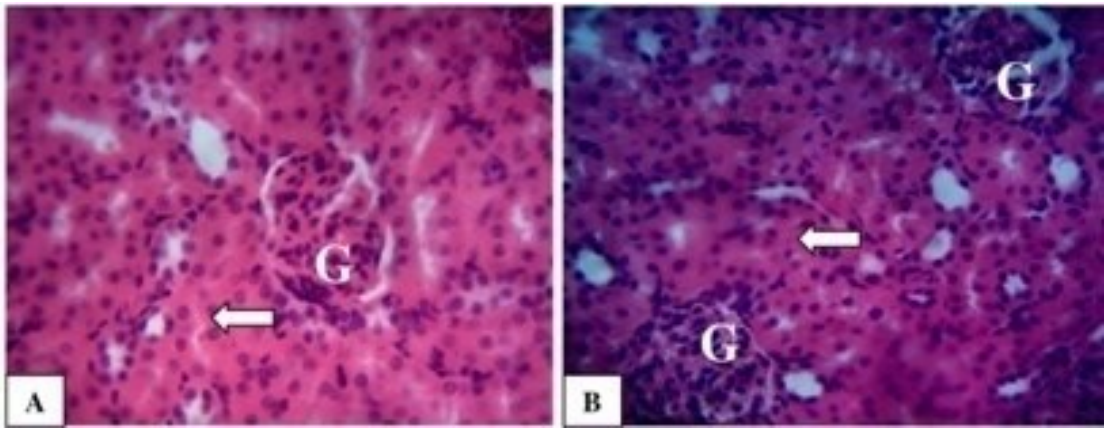


Figure 1: The photomicrograph of the liver showing varying degrees of fatty degeneration of hepatocytes indicated by vacuolated cells (arrows) in both the control group A and groups B, C and D (this is physiological and may be due to overfeeding). H and E × 400



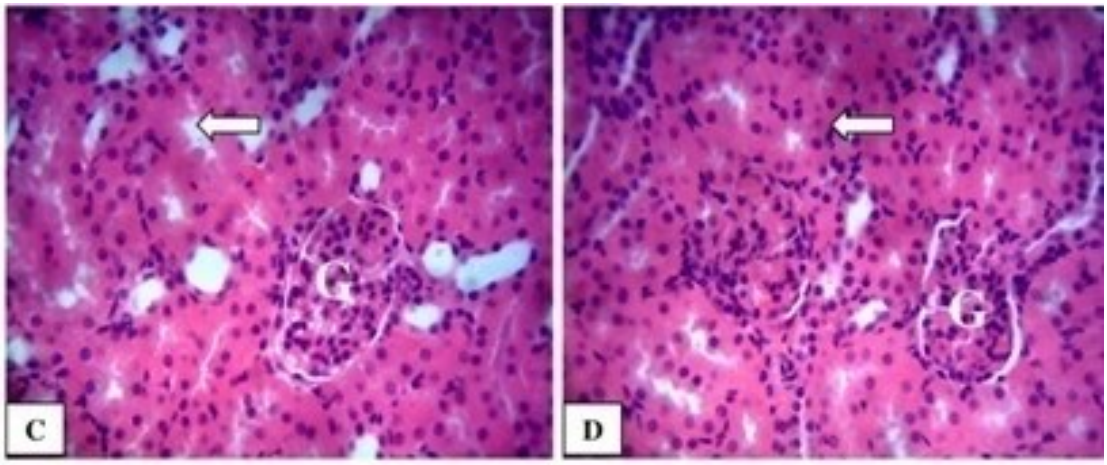


Figure 2: The photomicrograph of the kidney showing normal histological features of the glomerulus (G) and renal tubules (arrows) in groups A, B, C and D. H and E × 400

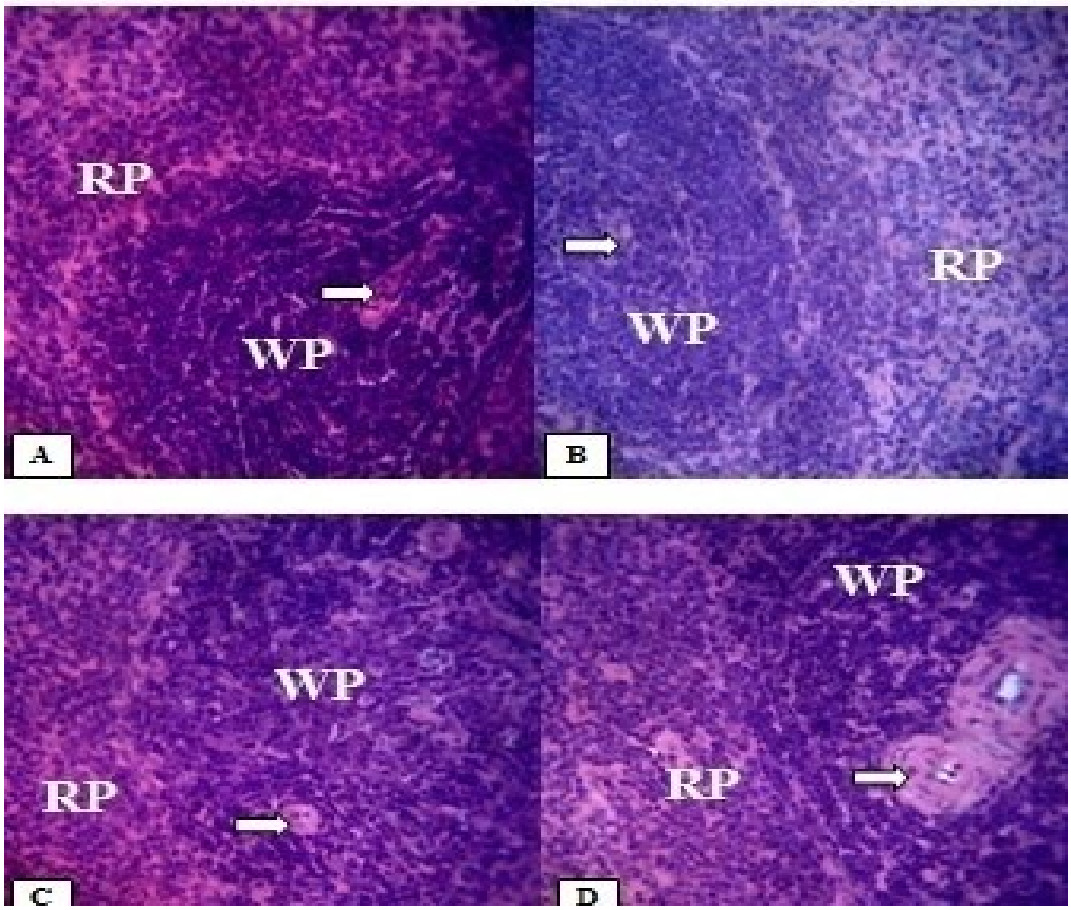


Figure 3: The photomicrograph of the spleen showing normal histological features of the white pulp (WP), red pulp (RP) and central arteriole in groups A, B, C and D. H and E × 400

Table 2: Effects of graded doses of the fungal metabolites on biochemical parameters of mice

Treatment	Dose (mg/kg)	GSH (g/dl)	SOD (mg/dl)	AST (IU/L)	ALT (IU/L)	ALP (IU/L)	Catalase (g/dl)	MDA(g/dl)
LB1	Control	23.12 ± 0.89	9.15 ± 0.18**	23.60 ± 0.98*	22.00 ± 0.71**	24.63 ± 0.60*	37.33 ± 1.26**	5.99 ± 0.14
	Low Dose	19.91 ± 0.47*	7.03 ± 0.22	29.80 ± 1.32*	27.20 ± 1.02	28.01 ± 0.42	28.65 ± 0.78**	8.02 ± 0.19
	Medium Dose	21.13 ± 0.61	8.45 ± 0.62*	27.00 ± 0.63*	23.60 ± 1.29**	26.07 ± 0.43*	30.26 ± 1.14*	7.61 ± 0.15
	High Dose	21.72 ± 0.47	7.84 ± 0.38	27.80 ± 1.56	24.60 ± 0.68	26.03 ± 0.58	32.35 ± 0.85	7.29 ± 0.10**
LB2	Control	21.72 ± 0.61	8.54 ± 0.38	28.40 ± 1.50	22.00 ± 0.68	24.63 ± 0.58	32.68 ± 0.01	8.12 ± 0.16
	Low Dose	21.13 ± 0.89	9.15 ± 0.22	29.20 ± 0.58	23.60 ± 0.71	26.00 ± 0.48	30.90 ± 0.76	5.99 ± 0.14
	Medium Dose	23.12 ± 0.47	7.03 ± 0.62	27.80 ± 0.98	27.20 ± 1.02	26.08 ± 0.42	37.08 ± 1.28	7.62 ± 0.19
	High Dose	19.91 ± 0.61	8.54 ± 0.18	24.90 ± 1.68	24.60 ± 1.29	28.00 ± 0.43	28.65 ± 1.14	5.98 ± 0.15
MR1	Control	24.12 ± 0.57	7.13 ± 0.58	23.80 ± 0.35	27.20 ± 0.67	24.68 ± 0.43	30.26 ± 0.85	8.21 ± 0.15
	Low Dose	23.10 ± 0.45	8.45 ± 0.34	27.00 ± 0.58	22.20 ± 1.29	26.07 ± 0.42	32.20 ± 0.76	7.29 ± 0.10
	Medium Dose	21.12 ± 0.68	7.84 ± 0.60	29.48 ± 0.56	23.60 ± 1.02	26.03 ± 0.60	34.87 ± 0.78	8.02 ± 0.19

	High Dose	19.80 ± 0.89	7.13 ± 0.16	23.13 ± 0.59	24.20 ± 0.68	28.01 ± 0.43	32.30 ± 0.85	5.99 ± 0.15
MR2	Control	20.54 ± 0.70	7.71 ± 0.48	27.68 ± 0.48	27.00 ± 0.58	24.80 ± 0.68	32.98 ± 1.28	7.29 ± 0.10
	Low Dose	23.40 ± 0.48	8.53 ± 0.37	27.00 ± 1.56	24.60 ± 1.18	28.00 ± 0.58	28.26 ± 0.76	8.02 ± 0.16
	Medium Dose	21.80 ± 0.76	8.00 ± 0.46	28.00 ± 0.58	22.60 ± 0.65	24.07 ± 0.42	28.79 ± 0.04	5.99 ± 0.16
	High Dose	22.08 ± 0.58	7.90 ± 0.56	23.80 ± 1.56	23.00 ± 1.02	26.07 ± 0.43	37.23 ± 1.14	8.21 ± 0.14

Values are presented as Mean ± Standard error of mean, n = 3. *p<0.05: Significantly different from control. **p<0.01: Significantly different from control. ***p<0.0005: Significantly different from control.

Key:
AST: Aspartate aminotransferase.
ALT: Alanine aminotransferase
ALP: Alkaline phosphate.
GSH; Glutathion
SOD: Superoxide dismutase
MDA: Malondiahyde

Table 3: Effects of graded doses of the fungal metabolites on hematological parameters of mice

Treatment	Dose (mg/kg)	PCV (%)	RBC($x10^{12}$/L)	WBC($x10^{9}$/L)	PLT ($x10^{9}$/L)	HB (g/dl)
LB1	Control	42.40 ± 0.68	7.64 ± 0.19	6800.00 ± 109.54	64.40 ± 1.72*	13.86 ± 0.24
	Low Dose	41.00 ± 0.44	7.74 ± 0.15	6960.00 ± 203.96	71.80 ± 3.26*	13.58 ± 0.86

	Medium Dose	39.60 ± 0.75*	6.90 ± 0.31	7440.00 ± 299.33	69.00 ± 1.10*	13.20 ± 0.11
	High Dose	42.40 ± 0.68	7.98 ± 0.91	6760.00 ± 203.96	72.40 ± 1.81	13.80 ± 0.11
LB2	Control	42.40 ± 0.68	6.90 ± 0.31	7360.00 ± 293.80	72.40 ± 1.81	13.86 ± 0.58
	Low Dose	39.60 ± 0.75	7.74 ± 0.15	6760.00 ± 203.97	71.80 ± 3.26	13.80 ± 0.86
	Medium Dose	41.00 ± 0.44	7.64 ± 0.19	6800.00 ± 109.55	69.80 ± 1.10	13.20 ± 0.12
	High Dose	42.40 ± 0.68	7.98 ± 0.19	7440.00 ± 206.93	64.40 ± 1.61	13.86 ± 0.24
MR1	Control	41.70 ± 0.43	6.90 ± 0.15	6800.00 ± 109.55	71.80 ± 1.72	12.56 ± 0.58
	Low Dose	42.40 ± 0.68	7.74 ± 0.31	7440.00 ± 299.58	69.40 ± 3.26	13.00 ± 0.11
	Medium Dose	39.90 ± 0.46	7.80 ± 0.91	6960.00 ± 203.96	72.40 ± 1.61	13.20 ± 0.15
	High Dose	39.60 ± 0.75	6.70 ± 0.19	7360.00 ± 109.59	72.40 ± 1.81	11.20 ± 0.86
MR2	Control	37.70 ± 0.46	6.70 ± 0.34	7450.00 ± 206.44	72.40 ± 1.81	12.56 ± 0.45
	Low Dose	41.40 ± 0.68	7.80 ± 0.91	7340.00 ± 209.48	69.40 ± 3.26	13.86 ± 0.24
	Medium Dose	42.40 ± 0.43	7.74 ± 0.15	6470.00 ± 109.98	71.80 ± 1.58	13.20 ± 0.11
	High Dose	39.90 ± 0.75	6.90 ± 0.19	6800.00 ± 109.96	64.40 ± 1.61	11.86 ± 0.58

Values are presented as Mean ± Standard error of mean, n = 3. *p<0.05: Significantly different from control. **p<0.01: Significantly different from control. ***p<0.0005: Significantly different from control.

Table 4: Effects of graded doses of fungal metabolites on white blood cell differentials of mice

Treatment	Dose (mg/kg)	Neutrophils (%)	Lymphocytes (%)	Esinophils (%)	Monocytes (%)	Basophils (%)
LB1	Control	57.00 ± 1.00	38.80 ± 1.02	1.80 ± 0.37	2.40 ± 0.24	0.00 ± 0.00
	Low Dose	58.00 ± 0.80	36.40 ± 0.75	2.00 ± 0.32	2.80 ± 0.37	0.00 ± 0.00
	Medium Dose	60.00 ± 0.89*	35.60 ± 0.51*	1.60 ± 0.24	2.80 ± 0.37	0.00 ± 0.00
	High Dose	58.40 ± 1.17	37.60 ± 1.21	2.00 ± 0.32	2.00 ± 0.32	0.00 ± 0.00
LB2	Control	57.00 ± 0.80	38.80 ± 1.00	2.00 ± 0.35	2.00 ± 0.32	0.00 ± 0.00
	Low Dose	56.40 ± 0.00	35.00 ± 0.58	2.80 ± 0.35	2.40 ± 1.73	0.00 ± 0.00
	Medium Dose	59.48 ± 0.80	35.60 ± 0.12	1.80 ± 1.00	2.00 ± 0.37	0.00 ± 0.00
	High Dose	58.00 ± 1.20	37.60 ± 0.75	1.60 ± 0.34	2.00 ± 0.32	0.00 ± 0.00
MR1	Control	58.08 ± 0.08	38.80 ± 1.17	1.80 ± 0.24	2.40 ± 0.24	0.00 ± 0.00
	Low dose	57.10 ± 0.08	37.80 ± 0.09	2.00 ± 0.32	1.80 ± 0.32	0.00 ± 0.00
	Medium Dose	56.10 ± 1.80	35.60 ± 0.12	2.00 ± 0.32	2.00 ± 0.32	0.00 ± 0.00
	High Dose	58.40 ± 0.80	36.40 ± 0.75	1.80 ± 0.35	2.80 ± 0.37	0.00 ± 0.00
MR2	Control	57.00 ± 0.98	38.00 ± 1.00	2.00 ± 0.00	2.00 ± 0.80	0.00 ± 0.00
	Low Dose	58.00 ± 0.80	36.80 ± 1.00	1.80 ± 0.37	2.40 ± 0.33	0.00 ± 0.00

	Medium Dose	57.40 ± 1.00	36.40 ± 0.75	1.80 ± 0.32	2.00 ± 0.24	0.00 ± 0.00
	High Dose	58.00 ± 0.80	38.80 ± 0.58	2.00 ± 0.24	2.80 ± 0.37	0.00 ± 0.00

Values are presented as Mean ± Standard error of mean, n = 3. *p<0.05: Significantly different from control. **p<0.01: Significantly different from control. ***p<0.0005: Significantly different from control.

DISCUSSION

Toxicity evaluation is critical in the production of new molecules for human application. Safety assessment of new endophytes was carried out in this study using animal model. From the results, the median lethal dose of the endophytes was estimated to be >5000 mg/kg using the oral route, indicating a high safety margin of the synthesized endophytes. This is in agreement with similar studies carried out on toxicological evaluations (Builders *et al,*2012, Awodele *et al*, l2016). There were no observable signs of toxicity such as writhing, salivation, drowsiness, fur bristling or paw licking following oral administration of the extract. Loss of weight has been indicated by researchers to be a positive sign of toxicity (Awodele *et al*, l2016, Yemitan etal,*2016,* Udom,*et al,* 2022). In this study, weekly changes in weight of treatment groups were no different from the control indicating that the extract was not harmful to the animals' growth and development (Yemitan *et al, 2016*).

Biochemical analyses from treated animals with endophytic extract revealed decreased levels of MDA at a dose of 100 mg/kg suggestive of free ROS scavenging ability even at a lower dose. However, subsequent increase in dose to the tune of 1000 mg/kg did not decrease MDA levels significantly. It therefore implies that the ROS

scavenging ability of the endophytic extract was not dose dependent. SOD and GSH levels were seen to be increased also, albeit not dose dependent. This outcome is in line with green plants and green fungi due to their richness in supply of secondary metabolites such as polyphenols and alkaloids (Yemitan etal,*2016,* Udom,*et al)..*).

The results from histopathology studies show that on the administration of the graded doses 10 -5000 mg/kg to the test animals, there was no toxicity recorded on the liver (Fig.1), kidney (Fig. 2) and spleen (Fig.3) of those animals. There were no lesion or inflammation noticed on those organs, their histological features were reported to be normal. However, the varying degrees of fatty degeneration of hepatocytes indicated by vacuolated cells in the liver were recorded to have been physiological and may be due to overfeeding, as they were also observed in control group.

Analysis of graded doses of endophytic bioactive extract on biochemical, haematological parameter and white blood differentials after the acute oral toxicity testing.

On analysis the white blood differentials, biochemicals and haematological parameters of the mice, it was ascertained that these parameters were increased significantly ($p < 0.05$). The white blood differentials parameters studied are shown in Table 3 below. State and discuss the implication of this result.

For all the metabolic extracts from the endophytic fungal isolates, it was observed that there was about 2-3% decrease of white blood cell parameters at low and medium doses, when compared to the control. However, these changes still fail within the normal ranges of these parameters. This implies that the metabolites have no negative effects on the white blood cell differentials.

Table 2 below shows the Effects of graded doses of the fungal metabolic extracts on hematological parameters of mice. MR1and

MR2 at high dose shows a marked Hb reduction while at low dose a little increase was observed. This could imply that at high doses this extract may result to anemic conditions. Never the less, at low and medium doses the rest parameters are within the standard rangers. Also it has been reported in literature that the observed elevation in PCV and RBC is an indication that the endophytic metabolites may have the tendency of inducing erythropoiesis/hematopoiesis (Ochei and Kolhatkan, 2007).

As presented in Table 2, are results of evaluation of the metabolites on biochemical parameters such as ASP, ALP, ALT, SOD and GSH in the liver. LB1 extract showed a remarkable rise in AST, ALT and ALP levels with LBI extract while that of SOD and GSH had no pattern.

Generally, the effects of the fungal extracts on biochemical parameters were not dependent on dose. This is because to many the values obtained at higher doses are smaller than both the low and medium doses.

Secondly, the elevated levels of liver enzymes observed with the endophtic fungal extracts do not pose any threat to the liver as the figures obtained are still within the normal ranges.

CONCLUSION

The acute oral toxicity test carried out on the endophytic fungal extract showed that the extracts are relatively safe in humans with LD50 >5000 mg/kg. The observed increase in PCV, HB, RBC and other haematological parameters at graded doses when compared with the control implies that the metabolite has haematopoeitic properties. The immunological parameters tested showed significant increase in neutrophils and lymphocytes relative to the control whereas eosinophils remained at 2% for both control and crude extracts in all

the fungal isolates, an indication of the immune modulatory potential of the ethyl acetate extracts.

DECLARATION OF CONFLICT OF INTEREST/ FUNDING STATEMENT

All the authors of this article have no conflict of interest to declare and this research did not receive any specific grant from any funding agencies in the public, commercial, or not-for- profit sectors.

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