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Effects of Methanol Leaf Extracts of *Calotropis Procera* on the Histopathology Index of Wistar Rats

Precious Bukola Alabi, Joan Mayowa Oniemola, Enojo Onoja

Department of Science Laboratory Technology, Kogi State Polytechnic, Lokoja, Kogi State, Nigeria

Abstract: - Calotropis procera Linn commonly known as 'sodom apple', is a tropical plant growing of about 1050 meters (family name). The phytochemical analysis of Calotropis procera methanol leaf extract administered to wistar rats were evaluated for histopathology study of the wistar rat liver. The qualitative and quantitative phytochemical composition of Calotropis procera determined contained Cardiac glycoside (+), Tanins (+), Alkaloids (++), Flavonoid (+), Saponin (+) and Total phenol (+++). The quantitative constituent includes: Cardiac glycoside (5.93±0.01 Mg DE/g), Tanins (8.70±0.02 GAE/g), Alkaloid (64.31±0.06 %), Flavonoid (6.10±0.05 Mg DE/g), Saponin (2.71±0.04 Mg DE/g), Total phenol (277.25±0.25 GAE/g). Twenty (20) Wistar rats with the average weight of 120 ±0.5 g were used in this study with five groups of four animals each (n=4). Group A (Positive: control) was on commercial feed, Group B (Standard 1 mg dexamethasone/kg b.wt), Group C to E were administered the graded doses of extracts (100 mg, 150 mg and 200 mg/kg/b.wt) for 14 days. The histopathology results revealed an increase in the production of kupffer cells in the liver and mild dilation of the sinusoidal space and central vein in the group administered with graded doses of the leaf extract.It was observed that methanol leaf extract of Calotropis procera is safe for consumption at recommended dose.

Keywords: Calotropis procera, Plant Extract, Wistar Rats, Phytochemical Analysis, histopathology

I. Introduction

Calotropis procera is a serious weed in pastures, overgrazed rangelands and poorly managed hay fields. It successfully competes with desirable pasture species and is capable in forming dense thickets that interfere with stock management, particularly mustering activities. This plant was used first time as a medicinal plant by *VedSushruta*, which is about 800–900 AD. *Calotropisprocera* is used from very ancient period in folk beliefs as well as a drug of choice for different ailments.

Calotropis procera is used by various tribes of the world as a curative agent for ailments such as skin disease, elephantiasis, toothache, asthma, leprosy, and rheumatism [11].According to [4]**,** different parts such as leaves, roots and bark, flower, fruits, stem, and latex of the plant have been reported to possess various phytochemicals which might possess various pharmacological activities.

The leaves contain ascorbic acid, calactin, calotoxin, calatropagenin, calotropin, polysaccharide containing D-arabinose, D-glucose, D-glucosamine and L-rhamnose, calotropagenin, and 3-proteinase.

Traditionally, *Calotropis. procera* is used to treat common house hold diseases like fever and diarrhea [9], [13] reports that the root bark treats leprosy, menorrhagia and snake bite. The leaves also have been boiled together with fresh milk to obtain cheese. Varying toxic effects of *Calotropisprocera* in animals occurs through air borne allergies, touch and consumption. The wide spread loss of livestock and low animal production are attribute to its existence in the arid Northern regions of Nigeria, West Tropical of Africa [2].[3] explored the immunologicalactivities of the root bark of *Calotropis procesra* in the mice models.

Root bark extract of *Calotropisprocera* was investigated at doses of 50, 100 and 200 mg/kg for immunomodulatory activity using immunological tests in mice, delayed type hypersensitivity, humoral mediated antibody titer, vascular permeability, hematological profile and cyclophosphamide-induced myelosuppression. The extract was active and stimulated the defense system by modulating several immunological parameters. The latex protein extract was found to protect against *Listeria monocytogenes* in experimental infections [6], [9].

Histological section has been placed onto glass slides. In contrast, cytopathology examines free cells or tissue micro-fragment (as "cell blocks") [10]. Histopathological examination of tissues starts with surgery, biopsy or autopsy. The tissue is removed from the body or plant, and then often following expert dissection in the fresh state, placed in a fixative which stabilizes the tissue to prevent decay. The most common fixative is formalin (10% neutral buffered formaldehyde in water).

The tissue is then prepared for viewing under a microscope using either chemical fixation or frozen section. If a large sample is provided, e.g. from a surgical procedure, then a pathologist looks at the tissue and select the part most likely to yield a useful and accurate diagnosis-this part is removed for examination in a process commonly known as grossing or cut up.Larger

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samples are cut to correctly situate their anatomical structures in the cassette. Certain specimens (especially biopsies) can undergo agar pre-embedding to assure correct tissue orientation in cassette and then in the block and then on the microscopic slide. This is then placed into a plastic cassette for most of the rest of the process [12].

The present study was therefore undertaken to extract and determine the *in vivo* effect of *Calotropis procera* leaf on the liver histopathology of *Wistar* rats.

II. Experimental

Chemicals, drugs and equipment used

Chemicals

Methanol, Chloroform, sodium hydroxide, hydrochloric acid, sulphuric acid, potassium hydroxide, acetic anhydride, ferric chloride, ammonia, fehlings A and B, Meyers, dragendoff's and hagers reagents (BDH) England.

Drugs

Cyclophosphamide and dexamethasone were obtained from Sigma-Aldrich, UK.

Enzyme assay kits

The enzyme assay kits used and their sources/manufacturers include:

Alanine phosphatase kit - Randox Laboratory Ltd, crumlin CO. UK.

Alanine aminotransferase kit - Randox Laboratory Ltd, crumlin CO. UK.

Aspartate aminotransferase kit- Randox Laboratory Ltd, crumlin CO. UK.

Creatinine reagents kit - TECO diagnostics, USA.

Urea reagent kit – TECO diagnostics, USA

Uric acid reagent kit- TECO diagnostic, USA.

MAC-ELISA kit - TECO diagnostic, USA.

Equipment

The equipment used and their brand include:

Centrifuge - Hermle Laboratories, Wehingen, Germany.

Weighing balance - Mettler Toledo

Spectrophotometer - Analytik Jena, Germany

Microscope - Olympus, China

Enzyme – Linked Immunosorbent Assay (ELISA) Machine, Germany.

Collectionof Plant Material

A fresh matured plant part of *Calotropis procera* leaves were collected from Anyigba town, Kogi State, Nigeria.

Plant Preparation

and processed as follows; the leaves were washed with tap water to remove the dirt and rinsed with distilled water, air dried at room temperature for three weeks then pulverized to powdered form using an electronic blender. 400g of the powdered sample was soaked in methanol (98%v/v) by cold maceration with occasionally shaken for 48hrs.

Plant Extraction

For the extraction of its constituent in the ratio 100g: 300ml for 72 hours, the mixture was filtered using whatman (No1) filter paper and the filtrate was pooled, concentrated and evaporated to dryness using a rotary evaporator. The crude methanol extract was stored in airtight container and refrigerated. The percentage yield of the extract was calculated using the relation [14].

Phytochemical Test

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Phytochemical tests were carried out on the extract using standard procedures to identify the constituent as described by [13, 14].

Qualitative Test

Test for Saponin: 0.4 ml of the extract was added to 4ml of distilled water in a test tube and the solution was shaken vigorously and observed for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously after which it was observed for the formation of emulsion.

Test for Tannins: Two methods were used for the determination of tannins:

a. To 10 ml of a freshly prepared 10 % KOH in a beaker, 0.5 ml of the extract was added to dissolve. A dirty precipitate observed indicated the presence of tannins.
b. 0.5 ml of the extract was boiled in 10ml of water in a test tube and then filtered. A few drops of 0.1 % ferric chloride was added and the solution observed for brownish green or a blue-black colouration.

Test for Cardiac Glycosides (Keller-Killeen's Test): 0.5 ml of the plant extract was dissolved, 5 ml of water was added and 2ml of glacial acetic acid solution containing one drop of ferric chloride solution. This was under played with 1ml of concentrated H_2SO_4. A brown ring at the interface indicated the presence of a deoxysugar characteristics of cardenolides. A violet ring may appear below a brown ring while in the acetic acid layer a greenish ring may form just above the brown ring and gradually spread throughout this layer.

Test for Flavonoid: Two methods were used for this analysis

a. 3g of the extract was heated with 10 ml of ethyl acetate over a steam bath for 3minutes, the mixture was filtered and 4 ml of the filtrate was shaken with 1ml of dilute ammonia solution. A yellow colouration indicated the presence of flavonoid
b. 5ml of dilute ammonia was added to a portion of the extract, then 1ml of concentrated Sulphuric acid was added. A yellow colouration indicated the presence of flavonoid

Test for Alkaloids: The extract was added to HCl and filtered and divided in two equal portion for the following analysis

a. The first filtrate was treated with Meyer's reagent (potassium mercuric iodide). The formation of a yellow coloured precipitate indicated the presence of alkaloids.
b. The second filtrate was treated with dragendoff's reagent (solution of potassium bismuth iodide). The formation of red precipitate indicated the presence of alkaloids. Filtrare was treated with Hagers reagent (saturated picric acid solution). Presence of alkaloid was confirmed by the formation of yellow coloured precipitate.

Test for Total Phenol:

a. Ferric chloride test: 1 ml of the extract was added to 1 ml of 5% FeCl3; dark green colour depicts the presence of the phenols
b. Lead acetate test: 1 ml of the extract was added to 0.5ml lead acetate; while precipitation reveals the presence of the phenols.

Quantitative Test

Determination of Tannins

One gram of the powdered (sample) in a conical flask was added to 100 cm3 of distilled water. This was boiled gently for 1 hour on an electric hot plate and filtered using number 42 (125 mm) Whatman filter paper in a 100 cm3 volumetric flask. Addition of 5.0 cm3 Folin-Denis reagent and 10 cm3 of saturated Na2CO3 solution into 50 cm3 of distilled water and 10 cm3 of diluted extract (aliquot volume) was carried out after being pipetted into a 100 cm3 conical flask for colour development. The solution was allowed to stand for 30 minutes in a water bath at a temperature of 25°C after thorough agitation. With the aid of a Spectrum Lab 23A spectrophotometer optical density was measured at 700 nm and compared on a standard tannic acid curve. Dissolution of 0.20 g of tannic acid in distilled water and dilution to 200 cm3 mark (1 mg/cm3) were used to obtain tannic standard curve. Varying concentrations (0.2–1.0 mg/cm3) of the standard tannic acid solution were pipetted into five different test tubes to which Folin-Denis reagent (5 cm3) and saturated Na2CO3 (10 cm3) solution were added and made up to the 100 cm3 mark with distilled water. The solution was left to stand for 30 minutes in a water bath at 25°C. Optical density was ascertained at 700 nm with the aid of a Spectrum Lab 23A spectrophotometer. Optical density (absorbance) versus tannic acid concentration was plotted.

The following formula was used in the calculation:

$$\text{Tannic acid (mg/100g)} = \frac{C \times \text{extract volume}}{} \times 100$$

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aliquot volume x weight of sample

where C is concentration of tannic acid read off the graph.

Determination of for Alkaloid

200 cm3 of 10% acetic acid in ethanol was added to the powdered sample (2.50 g) in a 250 cm3 beaker and allowed to stand for 4 hours. The extract was concentrated on a water bath to one-quarter of the original volume followed by addition of 15 drops of concentrated ammonium hydroxide dropwise to the extract until the precipitation was complete immediately after filtration. After 3 hours of mixture sedimentation, the supernatant was discarded and the precipitates were washed with 20 cm3 of 0.1 M of ammonium hydroxide and then filtered using Gem filter paper (12.5 cm). Using electronic weighing balance Model B-218, the residue was dried in an oven and the percentage of alkaloid is expressed mathematically as

$$\% \text{ Alkaloid} = \frac{\text{Weight of Alkaloid}}{\text{Weight of sample}} \times 100$$

Determination of Cyanogenic glycoside

200 cm3 of distilled water was added to one gram of the powdered sample and allowed to stand for 2 hours for autolysis to occur. Full distillation was carried out in a 250 cm3 conical flask containing 20 cm3 of 2.5% NaOH (sodium hydroxide) in the sample after adding an antifoaming agent (tannic acid). Cyanogenic glycoside (100 cm3), 8 cm3 of 6 M NH4OH (ammonium hydroxide), and 2 cm3 of 5% KI (potassium iodide) were added to the distillate(s), mixed, and titrated with 0.02 M AgNO3 (silver nitrate) using a microburette against a black background. Turbidity which was continuous indicates the end point.

Content of cyanogenic glycoside in the sample was calculated as:

$$\text{CG (mg/100g)} = \frac{\text{Titre value (cm}^3\text{) x 1.08 x exact volume}}{\text{Aliquote volume (cm}^3\text{) x sample weight (g)}} \times 100$$

Where CG is Cyanogenic Glycoside

Determination of Phenols

Defatting of 2 g powdered sample was carried out for 2 hours in 100 cm3 of ether using a soxhlet apparatus. The defatted sample (0.50 g) was boiled for 15 minutes with 50 cm3 of ether for the extraction of the phenolic components. Exactly 10 cm3 of distilled water, 2 cm3 of 0.1 N ammonium hydroxide solution, and 5 cm3 of concentrated amyl alcohol were also added to 5 cm3 of the extract and left to react for 30 minutes for colour development. The optical density was measured at 505 nm. 0.20 g of tannic acid was dissolving in distilled water and diluted to 200 mL mark (1 mg/cm3) in preparation for phenol standard curve. Varying concentrations (0.2–1.0 mg/cm3) of the standard tannic acid solution were pipetted into five different test tubes to which 2 cm3 of NH3OH, 5 cm3 of amyl alcohol, and 10 cm3 of water were added. The solution was made up to 100 cm3 volume and left to react for 30 minutes for colour development. The optical density was determined at 505 nm.

Determination of Saponin

100 cm3 of 20% aqueous ethanol was added to 5 grams of each wood powder sample in a 250 cm3 conical flask. The mixture was heated over a hot water bath for 4 hours with continuous stirring at a temperature of 55°C. The residue of the mixture was reextracted with another 100 cm3 of 20% aqueous ethanol after filtration and heated for 4 hours at a constant temperature of 55°C with constant stirring. The combined extract was evaporated to 40 cm3 over water bath at 90°C. 20 cm3 of diethyl ether was added to the concentrate in a 250 cm3 separator funnel and vigorously agitated from which the aqueous layer was recovered while the ether layer was discarded. This purification process was repeated twice. 60 cm3 of n-butanol was added and extracted twice with 10 cm3 of 5% sodium chloride. After discarding the sodium chloride layer the remaining solution was heated in a water bath for 30 minutes, after which the solution was transferred into a crucible and was dried in an oven to a constant weight. The saponin content was calculated as a percentage:

$$\% \text{ Saponin} = \frac{\text{Weight of saponin}}{\text{Weight of sample}} \times 100$$

Determination of Flavonoid

50 cm3 of 80% aqueous methanol added was added to 2.50 g of sample in a 250 cm3 beaker, covered, and allowed to stand for 24 hours at room temperature. After discarding the supernatant, the residue was reextracted (three times) with the same

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volume of ethanol. Whatman filter paper number 42 (125 mm) was used to filter whole solution of each wood sample. Each sample filtrate was later transferred into a crucible and evaporated to dryness over a water bath. The content in the crucible was cooled in a desiccator and weighed until constant weight was obtained. The percentage of flavonoid was calculated as

$$\% \text{ Flavonoid} = \frac{\text{Weight of Flavonoid}}{\text{Weight of sample}} \times 100$$

Animal Grouping and Administration of Plant Extract

Rats were divided into five (5) Groups (A to D) of four (4) animals. The plant extract was administered to the rats in the test groups orally using an oral cannula with rubber tubing, while the rats in the control group received distilled water. The extract was administered once daily within the hours of 08:00 am and 09:00 am. All the rats were allowed to feed on normal animal feed and drink water *ad libitum* under a strict compliance with the guide for

animal research, as detailed in [7], Guidelines for the care and use of Laboratory Animals. Rats in group A were administered 1ml distilled water, a graded dose of 100 mg/kg, 150 mg/kg and 200 mg/kg of the plant extract was administered to the animals in group B to D for 14 days [3].

Animal Sacrifice and Collection of Organ

After appropriate doses and the completion of the experiment, the rats were anaesthesised in large glass jar containing cotton wool soaked in ether. The rats at subconscious stage were brought out of the jar, quickly dissected and a section of the liver was collected and stored in the test bottle containing 10% formalin solution for histopathology.

Histopathological examination

One rat was randomly selected from each of the study groups for histopathological examinations. The liver organ was collected and fixed in 10% formalin, embedded in paraffin and sectioned to obtain 4 µm thickness paraffin sections by a slide microtome. The obtained tissue sections were collected on a glass slide, deparaffinized in xylene, hydrated in descending series of ethyl alcohol, stained by hematoxylin and eosin stains (H&E) hydrated in ascending series of ethyl alcohol, cleared in two changes of xylene, mounted with DPX and examined by light microscope.

III. Result Analysis and Discussion

Result Analysis

Table 1: Phytochemical Screening of *Calotropis procera*

Phytochemical parameters	Qualitative analysis	Quantitative analysis (nm)
Tannins	+	8.70±0.02 GAE/g
Total phenols	+++	277.25±0.25 GAE/g
Alkaloids	++	64.39±0.06 %
Saponins	+	2.71±0.04 Mg DE/g
Flavonoids	+	6.10±0.05 MG DE/g
Cardiac glycosides	+	5.93±0.01 Mg SS/g

Results are expressed as mean ± SEM.

Key: + = slightly present

++ = more present

RESULTS ON HISTOPATHOLOGY OF THE LIVER

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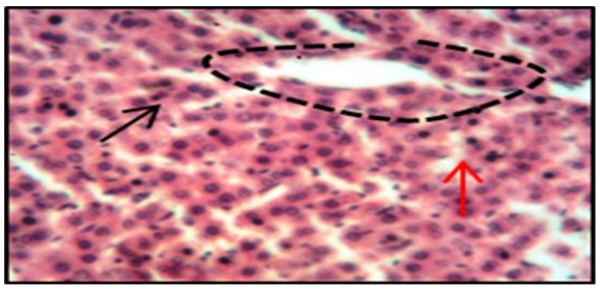

Figure 1:A histopathological analysis of rat liver administered with distilled water *(ad libitum)*. Magnification X200

Group I (Control)

Photomicrograph of the liver organ of experimental animal at day 14 with a normal histomorphological appearance of the liver displaying a central halo of typical size and spacing.Portal vein encircled by widely spaced hepatocytes. (Dotted circle: Central portal vein, Red arrow: Sinusoidal space, Black arrow: Hepatocyte, Green arrow: Kupffer cell).

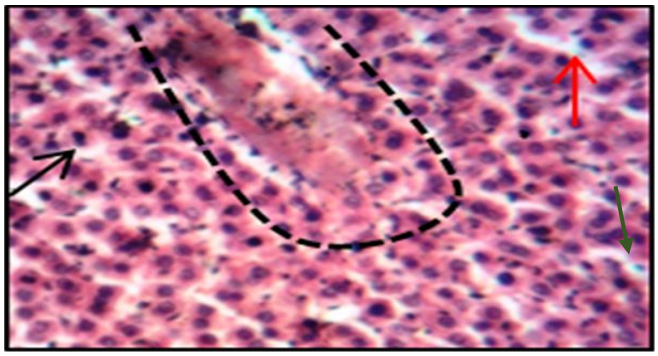

Figure 2:A histopathological analysis of rat liver administered with dexamethasone drug at day 14. Magnification X 200.

Group II (Dexamethasone 1 mg /kg/b.wt)

Photomicrograph of the liver organ of experimental animal at day 14 treatment with oral administration of 1 mg/kg b.wt Dexamethasone shows that hepatocytes are widely dispersed around the primary portal vein, which is generally large and spaced apart in a normal histomorphological appearance of the liver. The central portal vein has atrophy, which is visible. (Dotted circle: Central portal vein, Red arrow: Sinusoidal space, Black arrow: Hepatocyte and Green arrow: Kupffer cells).

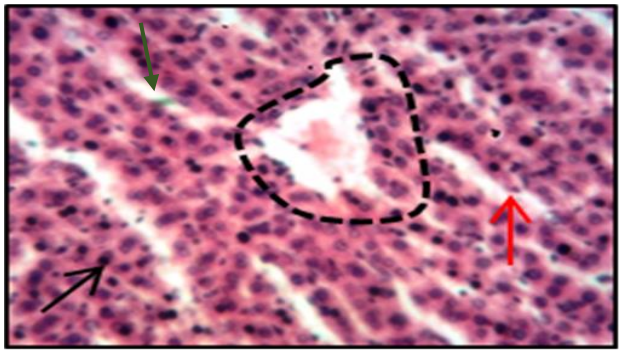

Figure 3: A histopathological analysis of rat liveradministered with methanol leaf extract of *Calotropis procera*at day 14. Magnification X 200

Group III (CPLE at 100 mg/kg b.wt)

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Photomicrograph of the liver organ of experimental animal with 100 mg/kg b.wt*Calotropis procera* leaves extract post treatment at day 14 administered orally displaying the liver's normal histomorphological appearance andhepatocytes are widely scattered around a central vein that is normal in size. Mild central portal vein atrophy and sinusoidal space dilation are both visible (Dotted circle: Central portal vein, Red arrow: Sinusoidal space, Black arrow: Hepatocyte)

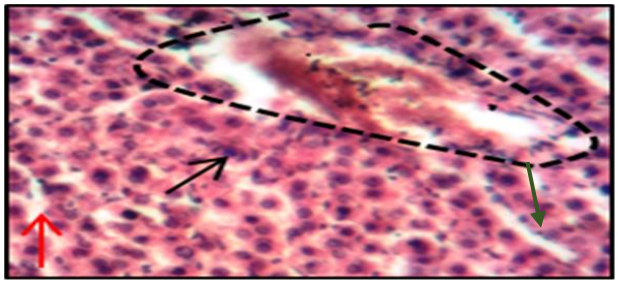

Figure 4: A histopathological analysis of rat liveradministered with methanol leaf extract of *Calotropis procera* day 14. Magnification X200

Group IV (CPLE at 150 mg/kg b.wt)

Photomicrograph of the liver organ of experimental animal with oral administration of 150 mg/kg b.wt of *Calotropis procera*leaves extract at day 14 post treatment displaying a typical liver histomorphological presentation with a central vein that is spaced in a halo and is surrounded by tightly packed hepatocytes. There is visible mild central portal vein atrophy. (Dotted circle: Central portal vein, Red arrow: Sinusoidal space, Black arrow: Hepatocyte and Green arrow: Kupffer cell).

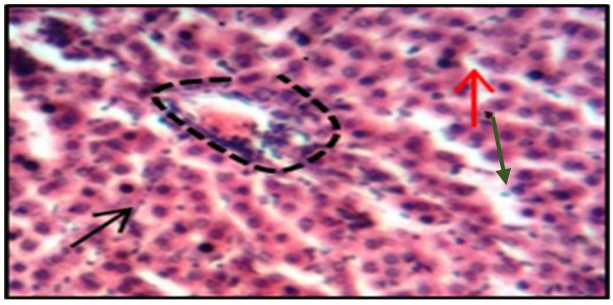

Figure 5: A histopathological analysis of rat liveradministered with methanol leaf extract of *Calotropis procera* at day 14. Magnification X200

Group V (CPLE at 200 mg/kgb.wt)

At day 14 following treatment, a photomicrograph of the experimental animal's liver taken after oral administration of 200 mg/kg/b.wt of *Calotropis procera* leaf extract reveals a typical-sized central vein with a halo of space around it, encircled by a dense population of hepatocytes. There is a slight widening of the sinusoidal gap and a slight narrowing of the central portal vein. (Dotted circle: Central portal vein, Red arrow: Sinusoidal space, Black arrow: Hepatocyte and green arrow: Kupffer cell)**.**

Discussion

From the experiment carried out in this research on the *in vitro* study of *Calotropisprocera* leaf extract, the presence of phytochemical components including, alkaloids, cardiac glycosides, tannins, saponins, flavonoids, and phenol that are known to have medicinal and pesticide activities were present. The extract was high in phenol content (277.25±0.25 GAE/g), which has an anti-inflammatory effect by regulating innate and adaptive immunities through the modulation of different cytokines. Phenols also help to promote immunity against foreign pathogens, prevent immune diseases and help in modulation of the immune system by either being protective or harmful to the intestinal immune of the host **[8]**. Phenols give plant antioxidant activity, making it possess the capacity to scavenge free radicals and reduce lipid oxidation [5]. The presence of alkaloid, which is abundant in the leaves of *Calotropisprocera*, may be the cause of the plant's toxicity. Alkaloids are naturally nitrogenous plant compounds. They are heterocyclic substances that strongly affect animal nervous systems and have the potential to cause animal mortality [1].

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Histopathology of the liver revealed no notable alterations to the structural architecture were found in the control group's liver histopathology. A densely dispersed hepatocyte with a modest sinusoidal space dilation was seen in the group receiving dexamethasone treatment, along with what appeared to be central portal vein atrophy.

A minor atrophy of the central portal vein, dense hepatocyte distribution, a mild sinusoidal space dilation, and formation of kupffer cells along the sinusoidal space wall were all observed in all groups treated with *Calotropis*procera leaf extract.

IV. Conclusion

The investigation on the histopathological parameters on *Wistar* rats administered with methanol leaf extract of *Calotropis procera* revealed no significant toxicity at 100, 150 and 200mg/kg b.wt, there was no death recorded, no behavioural changes seen in all the groups, the body weight of all groups of animals increased throughout the week of administration. Hence, it may be concluded that methanol leaf extract of *Calotropis procera* is safe for consumption as herbal product at recommended dose.

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