Histological and Stereological investigation of Ethanol Leaf Extract of Vernonia amygdalina (ELVA) on Cerebral Cortex of Murine Malaria Model (MMM).

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ABSTRACT

Over a period of time histopathological approaches in study of disorder or drug interaction has been reported to face applicable conclusion i.e limitations involving assumptions. Stereology is an optimal tool for quantitative assessment of mass or tissue morphology. Malaria constitute of the devastating pandemics of African countries and the world at large where the risk group are mostly pregnant women and children under five years thus this was conducted as curative study to investigate the histological changes of ELVA in cerebral cortex of young mice inoculated with *Plasmodium berghei (Pb)* NK- 65 strain using some stereological tools. Twenty-five (25) young mice, mixed sex, aged 2-3 weeks, weighing between 5-7g, were acquired from the Animal Facility, Faculty of Pharmaceutical Sciences of Ahmadu Bello University Zaria, Nigeria and divided into group of five with five mice per group. Group 1 was administered distilled water, Group 2 was inoculated with *P. berghei* parasite, Group 3 (*Pb* + ELVA 250mg/kg), Group 4 (*Pb* + ELVA 125mg/kg) and Group 5 *Pb* + 10mg/kg of Chloroquine. Parasitemia level was examined 48 hours and followed by appropriate design protocol. 72 hours (D2) after innoculation parasite quantification was carried follow by treatment which lasted five days (D4). On the 7th day animals were sacrificed, brain tissues removed and fixed in Bouin's fluid, processed for histopathology observation using Haematoxyline and Eosin (H and E), and Cresyl Echt Violet stains. Stereological estimation includes neuronal count and volume estimate using physical fractionator and cavalier principle. Results shows that ELVA is dose dependent on the parasite quantification. We observed histo-pathological changes in treated group in a dose related pattern, obtained estimates of affected cell numbers, volume and correlate the relationship with observable indicators. The affected cells justify the potency of the extract and highlight the recovery stage of the treated group which could be a measure for the integrity of the cell. In conclusion Stereological tool has shown some level of proficiency in drawing a correlation between the variables and minimize assumptions in estimating level of disorder or interactions.

Keywords: *Vernonia amygdalina,* ELVA, *P. berghei,* Chloroquine, Stereology.

INTRODUCTION

Malaria has devastated major civilizations since the dawn of humanity and remains a considerable burden to society; it is responsible for more than 200 million cases and nearly half a million deaths and about 300-500 million people being infected each year (WHO 2017). Malaria is the most common primary health problem in tropical and developing countries of sub-saharan Africa and South East Asia. The fatal rates for this disease are extremely high in children and pregnant women due to compromised immunity

The first and most common antimalarial drug, that is still used today, is quinine, isolated from the bark of Cinchona species in 1820 (Beckmann 1958). Another antimalarial drug chloroquine was synthesized in 1940 and was the only drug used for the treatment of malaria (Bharel *et al.*, 1996). There has been very little improvement in the control of malaria, in spite of various control programs. Control of malaria is complex as the Anopheles mosquito have

developed resistance to many insecticides & the appearance of drug resistance strains of Plasmodium (Choubey and Parmita 2015). Therefore, new drugs or drug combinations are urgently needed that should have novel modes of action or be chemically different from the drugs in current use as such the use of this stereological tool to quantify drug host interaction is important.

Stereology provides efficient tools for estimation of geometric quantities such as volume, surface area, length, or number of objects for 3-D tissue structures, contained within an organ from measurements made on a set of 2-D sections (West, 1993; Gundersen, 1999). Stereological analyses involve a two-step process (West, 2002). Statistical sampling principles are used to obtain statistically valid histological sections from an organ to reduce the amount of tissue for analysis without reducing the precision of the estimate (Gundersen, 1999; Nyengaard, 1999). Design-based stereology provides estimates of total volume, surface area, length, or cell number in an organ, making no assumptions about the structures in an organ. Strict adherence to the principles of stereology guarantees accuracy; that is, the mean of the estimates comes closer and closer to the true value with replication. Accurate estimation of particle numbers from tissue sections became possible only in 1984 with the invention of the physical dissector (Sterio, 1984; Boyce, 2010).

MATERIALS AND METHODS

Materials

The following are some of the materials: Digital Microscope (Celestron and Olympus), Distilled water, Laptop Computer, Computer software, Bouin's fluid, Beakers, Specimen bottles, Digital weighing balance, 1ml and 5ml syringes and injecting needles, Dissecting tray, Chloroform and Dissecting kit.

Plant Collection and Extraction

The fresh leaves of *Vernonia amygdalina* plant were collected based on Ethnobotanical description and with the help of local traditional healers around Mada Area Development Council in Gusau local government area of Zamfara State, Nigeria, in May 2017. The plant was identified and authenticated at Botany Department, Faculty of Life Science, Ahmadu Bello University Zaria, and given a voucher number (12063). The Fresh leaves of *Vernonia amygdalina* were cleaned from extraneous materials, air-dried under shade at room temperature then pounded into powder in Biochemistry Laboratory, of Umaru Musa Yaradua University Katsina state, Nigeria. The powdered plant material was weighed (500g) using SCIENTECH Mode No SL 3100D Rev-c accuracy class (II) (Debella, 2002).

Ethanol Extract Preparation

Powdered *Vernonia amygdalina* (500g) was macerated with 5000ml, 80 % of ethanol for 72 hours with intermittent agitation by Orbital shaker at 120 revolutions per minute. The supernatant part of agitated material filtered with 15 cm Whatman grade1 filter paper two times. The filtrate of *Vernonia amygdalina* was then concentrated using Rotary evaporator (BUCHI R250,Switzerland) at 40^{o}C to remove methanol and further dried using in a lyophilizer (CHRIST, 3660 Osterode/harz/, France) to remove water and the extract were kept at −20°C until used (Debella, 2002). A total of 235g (53%) yield of the extract was obtained from the 500g of the powder.

Table 1. Percentage yield of ethanol extract of *Vernonia amygdalina*

Plant Species	Solvent	Weight of powder	Vol. of solvent	Yield (g)	% of yield
Vernonia amygdalina	Ethanol	500g	5000ml	235	53

In Vivo Acute Toxicity Tests

Acute oral toxicity of ethanol leaf extract of *Vernonia amygdalinaa* was evaluated. Healthy adult Swiss albino mice maintained under standard laboratory conditions were used for acute toxicity test according to (LORKE, 1983). The varying dose was orally administered in accordance to the guideline of lorke's and no mortality observed in all mice. According to the up and down guideline of the lorke's method the mice were observed for 24 hours for any toxicity signs like changes in physical appearance, behavioral change, motor and feeding activities, hair erection, rigidity, lacrimation, reduction in motor.

Experimental Animals Preparation

The animals employed for this study were premature young male and female 2-5 weeks old Albino Swiss mice (5-7g). The mice were obtained from the Animal Facility, Faculty of Pharmaceutical Sciences of Ahmadu Bello University Zaria, Nigeria. The mice were put to full experimental condition because the experiment took place in the same venue of purchase by first screening the mice for any possible parasitic infection. Blood was collected from the tail with anticoagulant (EDTA) of each mouse. A thin blood smear was made from each blood sample and stained. These were used to screen the mice for haemo-parasites. They were then housed in cages and given standard pellet diet and water *ad libitum*. The animals were maintained under the natural light-dark cycle throughout the duration of the study. For in-vivo anti-malarial assays of plant extracts, the mouse-infective chloroquine sensitive strain of *P. berghei* NK-65 obtained from National Institute of Medical Research Lagos State was kept alive by continuous intraperitoneal (i.p.) serial blood passage from mice to mice on research demand by the laboratory staff of the animal facility of Faculty of Pharmaceutical science, Ahmadu Bello University Zaria.

Seven-day curative test (Rane's test)

Evaluation of curative anti-malarial potential of the extract was done using a method described by Ryley and Peters, (1970) and modified by Carvalho *et al*., (1990). To assess the curative potential of the ethanol extracts on established *P. berghei* infection, 25 young mice were divided into five group's five mice each. These were inoculated with *P. berghei* intra peritoneally on the first day and infection was allowed to be established for 72 hours (Table 2.).
Thin blood films stained with Giemsa were prepared from tail blood of each mouse on day 3 and 7 to monitor the parasitaemia level. The mice were observed till the 7th day.

Table 2. Showing grouping and treatment

Groups	Treatments
Grp1	Distilled water (Positive control)
Grp2	Pb (Negative control)
Grp3	Pb + ELVA 250mg/kg
Grp4	Pb + ELVA 125mg/kg
Grp5	Pb + CQ 10mg/kg

Parasite and its quantification

Blood sample were collected from tail snip of each mouse according to methods of Kalra *et al*. (2006). The smears were made on clean microscope slides, fixed with absolute methanol for 15 min and stained with 15% Geimsa stain at pH 7.2 for 15 min. The stained slides were then washed gently using distilled water and air dried at room temperature.

Each stained slide was examined under Olympus microscope (CHK2-F-GS, Taiwan) with an oil immersion objective of 100x magnification power to evaluate the percent suppression of each extract with respect to the control groups. The microscope eye piece shows about 100 red blood cells per field (Dikasso *et al*., 2006).

The parasitemia level was determined by counting minimum of five fields per slide with about 100 RBC in random field of the microscope.

Percent parasitemia and percentage of suppression was calculated using formula described in all model (Adhroey *et al*., 2011).

$$\% \text{ Parasitaemia} = \frac{\text{Number of Parasitized RBC}}{\text{Total RBC counted}} \text{ X } 100\ \%$$

$$\% \text{ Suppression} = \frac{(\text{Parasitaemia in normal control}) - (\text{parasitaemia in treated group})}{\text{Parasitaemia in normal control}} \text{X } 100\ \%$$

Animal Sacrifice and Tissue Samples

The mice were sacrificed through cervical dislocation at completion of each test according to the test battery. Blood sample was collected for parasitaemia quantification and the brain tissue was then dissected out and fixed immediately in Bouin's fluid (Bancroft and Gamble, 2008).

Tissue Process

Haematoxylin and eosin (H and E) staining method

The haematoxylin and eosin staining methods were carried out by de-waxing the tissue in two changes of xylene for three minutes each, hydrated by passing them through descending grades of alcohol (100%, 95%, 90%, 70%) for three minutes each, then stain in Harris haematoxylin for ten minutes and then washed in tap water to remove excess stain. The slides were then flooded with acid alcohol for few seconds for differentiation and then washed in tap again. The slides were then blued in Scott's tap water for five minutes and then counter stain in Eosin for three minutes. Sections were rinsed in tap water and then dehydrated in ascending grades of alcohol and cleared in xylene. Afterwards they were then cover slipped using a media (Frieda, 2007). Photomicrographs were taken after examination of the slides using digital Am scope (MD 900) microscope.

Tissue and Stereological Process

Mice were perfused with paraformaldehyde and whole brains were removed and fixed in 10% neutral-buffered formalin (NBF). After fixation, cerebrum was section into two hemisphere and a section dissected out, the thickness was measured with a Vernier caliper, and it was processed and embedded in paraffin wax. Frozen sections were deparaffinized and hydrated to distilled water and then stain for 3 to 5 minutes in Cresyl echt violet solution. The stained sections were rinsed in two changes of distilled water and placed in 95% alcohol for 30 seconds. Sections were transferred to absolute alcohol for 30 seconds and then placed in xylene and balsam-xylene mixture for 1minute and 2 minutes respectively. The sections were taken through several changes of xylene and mounted with synthetic resin. All procedures were conducted in accordance with the Ahmadu Bello University Ethical committee on Animal Use and Care.

The region of interest was layer I and II of the cerebral cortices. The cerebral cortex was sectioned at microtome using the physical Fractionator (physical dissectors collected via SURS, with the sampling fraction tracked) with a goal of obtaining 4 to 6 physical dissectors per animal. Utilizing physical dissector protocol consecutive thin (30 mm thick) sections were captured at each sampling interval (in this study, the interval was 120 mm) and were placed side by side on the microscope slide, thus yielding a section sampling fraction (ssf) of 1/40 (every 40th section was taken for stereological analysis). A random start between 1 and 40 was chosen for each animal using a random number table, and section pairs were collected uniformly across the tissue thereafter. Sections were stained with Cresyl echt Violet to

identify Nissle substance of cells as a general nuclear marker. Stained slides were scanned and virtual slides were imported into the Visiopharm stereology software. An image analysis algorithm was created by the user to identify cell that pick up much stain expressed in the nucleus to be the target cell as an indication to parasitic activity (Positive stain) and guide Proportionate sampling, which uses image analysis to assign a "weight" to each sampling field (corresponding to the amount of positive staining as detected by the algorithm) this was then use in calculating the probability of a positive count within that sampling field. The software then samples according to this probability

The volume of the cerebral cortex was estimated using point counting method, i.e Cavalieri's principle Howard and Reed (1998); Mouton (2002) as reported by Javad *et al.,* (2016) Briefly a grid of points was laid over the section on the computer and the points falling on the layers I and II were counted. After applying the point counting grid on the sampled sections in a systematic-random fashion, we counted the number of points hitting on each layer of the cerebral cortices. Volume of the cerebrum cortex layers was determined by applying the following formula:

$$\text{Volume} = \sum P \times d \times t \times a\ (p)$$

Where $\sum P$ represents the total number of points hitting the layer I and II respectively, d is the average distance between sections (0.12 mm), t is the mean section thickness 0.030mm and a (p) is equal to the area associated with one point in the grid Abusaad *et al.,* 1999.

Statistical Analyses

Data collected were presented as Mean ± SEM and were statistically analyzed using Statistical Package for Social Science (SPSS), Version 20 (IBM, Incorp, NY). ANOVA was used to compare the levels of parasitaemia across the groups and also establish a relationship between cell counts and volume estimated. The results were presented as the Mean ± SEM (Standard Error of the Mean) and statistical significance was considered at a 95% confidence interval ($P<0.05$).

RESULTS

Acute exposure of mice to ELVA.

The acute exposure of mice to ethanol extract of *Vernonia amygdalina* (ELVA) at the doses of 2900 and 5000mg/kg body weight given during *in vivo* study showed no abnormalities. There was no death or any sign of toxicity in any of the mice.

Basic indicator evaluation

The mice inoculated with *P. berghei* had features of fever and these include decrease in food intake with consequent loss of weight. The rectal temperatures were significantly higher in the infected mice. Hair coats in these mice were rough. Signs of the infection subsided after the treatment with ELVA and Chloroquine. There was no death recorded and every data collected from this indicator shows no statistical significance.

Seven-Day Curative effect of ELVA.

Treatment of *Plasmodium berghei* infected mice with ethanol extract of *Vernonia amygdalina* at 250 and 125 mg/kg had a curative effect during the seven days period (Table 3.1). There was a high mean parasitemia level in group 2 (4.34 ± 0.57). After treatment, mice in group 3 (treated with 2500 mg/kg of the extract) had a significantly lower ($p< 0.05$) mean parasitemia level (2.48 ± 0.22). This was also significantly lower ($p< 0.05$) than the value of mean parasitemia in group 4 (3.76 ± 0.35) and group 5 (2.64 ± 0.10). Earlier day 3, the differences in mean parasitemia among the groups were not statistically significant ($p< 0.05$).

The extract exhibited a dose dependent curative effect with a slight difference between doses. The ethanol extract given at doses 250 and 125 mg/kg and CQ at 10mg/kg had shown statistically significant ($p<0.015$) parasitemia suppression of 59.5%, 45.6% and 50.6%, respectively, as compared to group 2.

3.4 Histopathology of cerebral cortex.

The cerebral cortex of mice in group 1 during the curative study showed normal pyramidal and few stellate cells in layer I and II. In overall observation of the cellular ultrastructure there is an establish necrosis in the treated group compared to the positive control group. Majority of the cells in the cerebral cortex of the mice in group 2 were degenerated. Similar degeneration of cells within the cerebral cortex were observed in group 3 and group 4. The mice in group 5 of this category also had degenerated cells but not compared to the group 2.

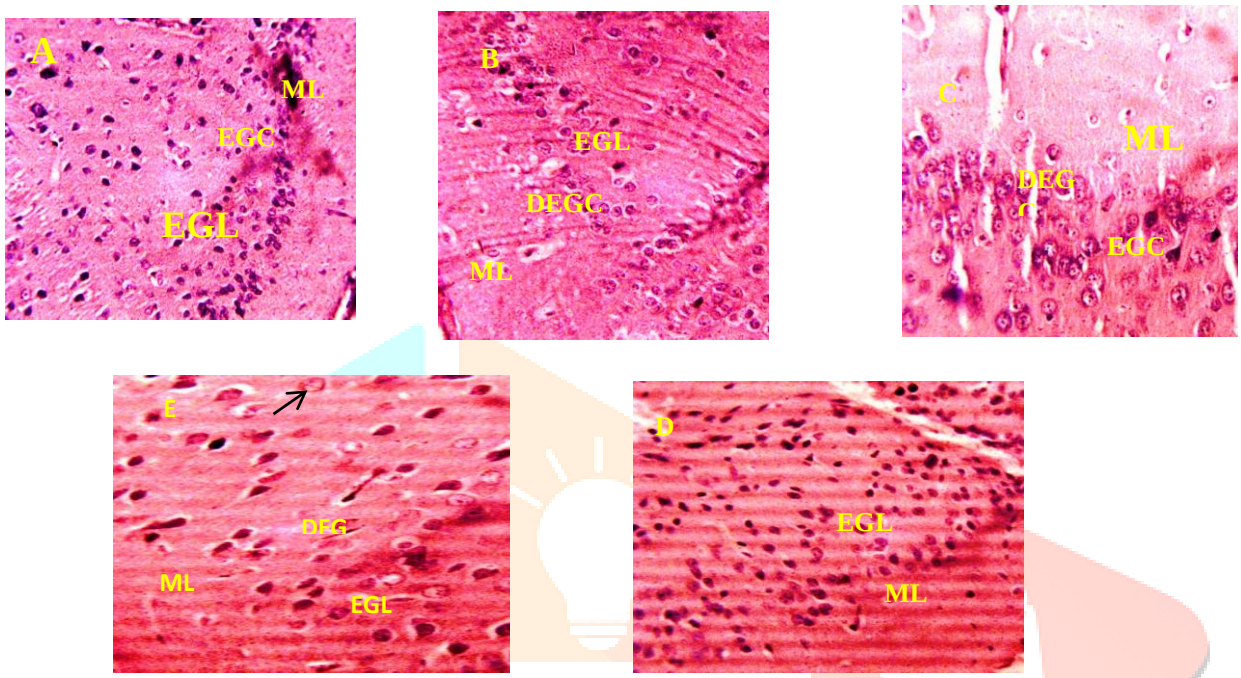


Plate I: A- Treated with Distilled Water, B- Infected and Not Treated, C-treated with 2500mg/Kg Of Extract, D-treated With 1250mg/Kg Of Extract, E- Treated With Chloroquine 10mg/Kg. EGC- External Granular Cells, EGL-external Granular Layer, ML-molecular Layer, DEGC-degenerated External Granular Cells. (H and E X 250)

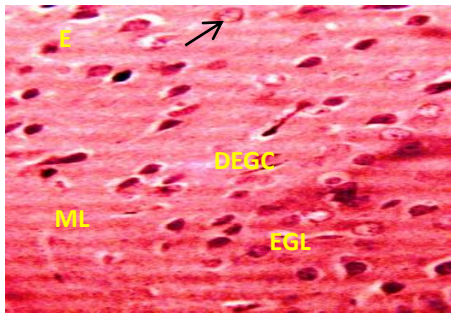
Cell count and volume estimation

Using the principle of affected cells will pick up much dye when stained characterized with necrotized nucleus the number of cell in layers I and II were counted and shows increment in group 2 compare to group 3, 4 and 5 respectively as shown table 3 below. There is no significant difference at $p< 0.05$ and indicates anti-plasmodial activity. It further establish the correlation of parasiteamia level and percentage suppression. Between layer I and II across the groups shows no significant difference which means even spread of the parasite and prove d the efficiency of stereology tool used.

Table 3: Showing cell count of layer I and II expressed in Mean ± SEM

	Grp 1	Grp 2	Grp 3	Grp 4	Grp 5
L I	0.00 ± 0.01	12.6 ± 0.16	10.0 ± 0.61	10.0 ± 0.41	10.8 ± 0.21
L II	0.00 ± 0.01	11.0 ± 0.30	9.6 ± 0.70	10.2± 0.30	11.0± 0.20

$p< 0.05$



The Volume estimation shows no significant difference amongst the groups and across the layers observed. There is increase in volume of layers I and II in group 2 compared to the groups of 3, 4 and 5. Below is table 4 showing the estimated volume?

Table 4: Showing volume of layer I and II expressed in Mean ± SEM

	Grp 1	Grp 2	Grp 3	Grp 4	Grp 5
L I	0.00 ± 0.00	0.045 ± 0.10	0.036 ± 0.22	0.036 ± 0.12	0.38 ± 0.81
L II	0.00 ± 0.00	0.039 ± 0.21	0.034 ± 0.03	0.035 ± 0.21	0.039 ± 0.21
$p < 0.05$					

DISCUSSION

In order to deal with the expanding problem of drug resistance which continues to challenge malaria control efforts, new antimalarial drugs are needed. The indigenous people are exploiting a range of herbals for effective treatment of various diseases involving malaria. Modern drugs have been deducted from folklore and traditional medicine. There are about 1200 plant species from 160 families used to treat malaria (Willeox *et al*., 2004). According to WHO, about 60 % of world's people use herbal medicine for treating their sickness (WHO, 2000). The extracts were considered active when parasitaemia was reduced by > 30% (Carvalho *et al*., 1990). The current study showed that the ethanol extract of *Vernonia amygdalina* had a dose dependent he extracts of *V. amygdalina* had a Curative effect on *P. berghei* in mice, especially at the dose of 250 mg/kg. This suggests that *V. amygdalina* leaf extract can suppress parasite growth if given orally for curative purposes. The oral treatment with *V. amygdalina* on Day 7 revealed that the ethanol extracts had reduced the level of parasitaemia to 45.6% and 50.6%, respectively. In addition, in curative test of the current study, the effect of the ethanol extract of *V. amygdalina* on the established malaria infection had shown a significant parasitaemia suppression with maximum of 50.6 % at the dose of 125mg/kg, respectively. These findings lend credence to the study of Adebisi, (2007) which observed that the aqueous leaf extract of *V. amygdalina* had significant curative effect of 73.9% at the dose of 200mg/kg. The difference might be due to route of administration.

Physical dissectors were chosen for this study because they bear the advantage of being thin enough to apply typical staining methods as well as the ability to use a whole-slide scanner to create virtual slides. These digital files can then be imported into a stereology software system, and analysis can be performed directly on the virtual file, without the aid of a live microscope. Certain software platforms (such as the AutoDissector; Visiopharm, Hørsholm, Denmark) have the ability to automatically capture matching microscopic fields of view from both of the sections and display them side by side for counting. One section is typically termed the "reference" section, and the other is termed the "look-up" section. Cells are counted when a unique counting feature (such as the nucleus or nucleolus) is present on one section and not the other. These technological advances have made use of the physical dissector both practical and efficient.

5.0 CONCLUSIONS

The spread of resistant malaria parasites to the available antimalarial drugs call for a new chemotherapeutic agent to control the disease. Medicinal plants are constantly screened for bioactivity in our quest for the discovery of new and effective therapeutic agents. The results of this study could help encourage more identification and validation of natural products. The use of stereology in this study has minimize the assumption associated with the effect of ELVA extract on the cerebral cortices and drug host interaction.

In conclusion, the results obtained from current study indicated that;

- Based on the results of the studies it was concluded that ethanol extract of *V. amygdalina* shows parasitaemia suppression and some promising curative activities in dose dependent manner.
- From the histological observations it could be inferred that the ethanol extracts of *V. amygdalina* ameliorate the effects of *P. berghei.*
- Cellular count and volume estimation shows that the pattern of distribution of the extract is uniform by not showing any significant difference across the group or within the group
- Thus the ethanol extracts showed antimalarial effects in young mice, which justify the traditional usage of this plant as malaria remedy.

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