

## Research Article

# Calabash Chalk's Geophagy Affects Gestating Rats' Behavior and the Histomorphology of the Cerebral Cortex

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**Introduction.** Calabash chalk contains heavy metals, and this led to this study on the effect of this chalk on the behavior and the histomorphology of the cerebral cortex of gestating rats. **Material & Methods.** 24 female rats were equally divided into 4 groups and were mated at preoestrus with the males. The day after mating was designated as day 1 of gestation. On gestation days 7–20, groups 1, 2, 3, and 4 animals were treated with 1 mL of distilled water, and 1 mL (200 mg/kg), 2 mL (400 mg/kg), and 3 mL (600 mg/kg) of calabash chalk suspension, respectively. On pregnancy day 21, behavioral tests using the open field and the light/dark mazes were carried out and the animals subsequently euthanized and their brains were routinely processed. **Results.** There was no difference in ambulatory activities, but group 4 animals had more ( $P < 0.05$ ) transition frequency and were more averse to the dark in the light and dark field, while sections of the cerebral cortex showed a higher ( $P < 0.05$ ) cellular population, hypertrophied pyramidal cells, and vacuolations in the treatment groups. **Conclusion.** Calabash chalk may have anxiolytic effect especially at high dose in the light and dark field but not in the open field and can stimulate maternal cerebral cortical cellular changes.

## 1. Introduction

Geophagy is the practice of eating soil, clay, or chalk, a form of pica [1, 2]. This practice is in place in almost every part of the world, although less frequent in developed societies [3, 4]. Geophagy occurs with animals, as well as humans, in both sexes, and in all races [5]. It is most often seen in rural or preindustrial societies among children and pregnant women [4, 6, 7].

Clay consumption is correlated with pregnancy, and some women eat clay to eliminate nausea, possibly because the clay coats the gastrointestinal tract [8, 9]. This process may also result in the absorption of dangerous toxins and eggs of parasites that may have been passed in animal faeces [10, 11]. Occupation, marital status, and gestational age are factors associated with geophagy. Anaemia and red blood cell characteristics suggestive of iron deficiency, as well as

*Ascaris lumbricoides* infection, are some of the other factors associated with geophagy [10]. One other complication of the act of geophagy is “geophagic syndrome,” which is marked by growth retardation, delayed sexual maturity, and liver and spleen enlargements [12].

The act of geophagy is a common practice in Nigeria and some other subsaharan African countries [10]. One of such is the eating of a clay and chalk mixture called calabash chalk. Calabash chalk is also known as calabash clay, Calabar stones, poto, la craie or argile in French, nzu by the Igbos, and ndom by the Efiks/Ibibios of Nigeria, as well as mabele by the Lingala of Congo. It is commercially available and may be sold in blocks, as large pellets and in powder forms [13].

Calabash chalk is generally made up of aluminium silicate hydroxide, which is a known member of the kaolin clay group, with the formula:  $Al_2 Si_2 O_5 OH_4$  [14]. Several other substances which could be poisonous to the body have also

TABLE 1: Treatments schedule for the control and the experimental groups.

Groups ( <i>n</i> = 6)	Treatment	Duration
Group 1 (control)	Distilled water	14 days
Group 2	200 mg/kg body weight of the suspension of <i>Calabash chalk</i>	14 days
Group 3	400 mg/kg body weight of the suspension of <i>Calabash chalk</i>	14 days
Group 4	600 mg/kg body weight of the suspension of <i>Calabash chalk</i>	14 days

been reported [14–17]. These includes metals, metalloids, and persistent organic pollutants [14]. The metals include iron, aluminium, potassium, titanium, barium, chromium, zinc, manganese, nickel, rubidium, copper, and tin with the metalloids being lead and arsenic [14–17]. The organic pollutants as reported include alpha lindane, endrin, endosulphan II, and *p*, *p*<sup>I</sup>-dichloro diphenyl dichloroethane (DDD) [14].

Few research works have been reported with calabash chalk. Reports on the calabash chalk carried out on animal models revealed hepatic sinusoidal enlargements, fragmented liver parenchyma, and depletion of red blood cells [18–20]. Recent reports showed that calabash chalk geophagy resulted in oedema with haemorrhages in the mucosa of the stomach, acanthosis, hyperkeratosis, and koilocytic changes in the mucosa of the oesophagus, as well as alteration of growth rate and demineralization of the femur bone [21, 22].

Kaolin, a constituent of calabash chalk has been reported to cause reactive astrocytosis, microgliosis, inflammation, and brain damage [23, 24]. Another constituent, lead can cause problems in pregnancy, as well as, learning and behaviour problems in young children [25, 26], as it is known to induce a broad range of physiological, biochemical, and behavioral dysfunctions in laboratory animals and humans [27], including central and peripheral nervous systems [28]. Acute arsenic exposure can damage many tissues and organ systems including the nervous, respiratory, and cardiovascular systems, gastrointestinal tract, and skin [29].

These reports on calabash chalk and/or its constituents on different body organs show a disturbing trend that can affect the brain of gestating animals. Thus, this study on the effect of the chalk on the behavior and the histomorphology of the cerebral cortex of gestating rats was borne.

## 2. Materials and Methods

Thirty-six-month-old sexually mature rats (24 female and 4 male rats) were used for the experiment. They were purchased from the Animal House of the Faculty of Basic Medical Sciences, University of Uyo, Uyo, Nigeria, where they were also kept. Ethical approval for the study was obtained from the Ethical Board of the institution. The animals were allowed to acclimatize for two weeks, and the female rats were equally divided into 4 groups (6 animals per group) and housed three animals per cage. Group 1 was the control, while groups 2, 3, and 4 were the treatment groups. The male rats served only

during mating. The animals were fed on Vital Feed's pelletized growers feed (Grand Cereals, a subsidiary of UAC of Nigeria PLC, Jos). The feed and water were allowed *ad libitum*.

The calabash chalk (non-salted) was purchased as a large block from a local market in Calabar, Nigeria. It was chopped into small pieces and grounded into fine powder with the aid of a manually operated grinder. 40 g of the calabash chalk was weighed and dissolved in 1000 mL of distilled water in a glass jar. Since the calabash chalk is partially miscible with water, it was administered as suspension, stirred prior to the administration.

The oestrous cycles of the female rats were determined by vaginal lavage as described by Marcondes et al. [30], and this was carried out every morning between 8 and 9 am for 4 days. Briefly, vaginal secretion was collected with a glass pipette filled with 0.2 mL of normal saline (NaCl 0.9%) by inserting the tip into the rat's vagina but not deeply, and the fluid obtained was placed on glass slides. The slides were observed under a light microscope, without the use of the condenser lens, using the  $\times 10$  objective lenses. Three types of cells were recognized: round and nucleated ones were epithelial cells; irregular ones without nucleus were the cornified cells; and the little round ones were the leukocytes. The proportion among them was used for the determination of the oestrous cycle phases.

The female rats were mated at preoestrous with the males. The day after mating was designated day 1 of gestation [31–33]. On gestation days 7–20, group 1 animals were treated with 1 mL of distilled water and groups 2, 3, and 4 animals were treated with 1 mL (200 mg/kg), 2 mL (400 mg/kg), and 3 mL (600 mg/kg) of calabash chalk suspension, respectively (Table 1). Each milliliter of calabash chalk suspension contained 40 mg of calabash chalk. On gestation day 21, behavioral tests using the open field and the light and dark mazes were carried out consecutively one hour apart.

The apparatus used for "open field" and "light and dark field" tests was constructed of white plywood. The open field maze had the following dimensions: 72  $\times$  72 cm with 36 cm walls. One of the walls was clear Plexiglas, so the animals could be visible and the floor lined with clear Plexiglas. Blue lines were drawn on the floor with a marker and this was visible through the clear Plexiglas floor. These lines divided the floor into sixteen 18  $\times$  18 cm squares. A central square of 18  $\times$  18 cm was drawn in the middle of the open field. The light and dark field maze was a rectangular box with open roof of 45  $\times$  27 cm. It was divided into a small chamber (18  $\times$  27 cm) and large chamber (27  $\times$  27 cm) by a flat board, with an opening (7.5  $\times$  7.5 cm) at the floor level linking the two chambers. The small chamber was painted black, with the large chamber painted white. The floor was covered by Plexiglas and the large chamber was divided into nine squares (9  $\times$  9 cm) by blue lines, while the floor of the small chamber was divided into six squares (9  $\times$  9 cm) by white lines. The mazes were located in a 1.8  $\times$  4.6 cm test room lit by a 60-watt red lamp for background lighting.

Rats were carried to the test room in home cages an hour before the test and were handled by the base of their tails at all times. Each rat was placed in the proximal right-hand corner of the maze (large chamber in light and dark maze) and

TABLE 2: Weekly body weights of the dam.

Group (n = 6)	Week 1 (g) P = 0.990 F = 0.03900	Week 2 (g) P = 0.720 F = 0.4483	Week 3 (g) P = 0.892 F = 0.2060	Week 4 (g) P = 0.724 F = 0.4424
Group 1 (control)	191.25 ± 6.88	196.17 ± 7.37	200.17 ± 8.33	215.17 ± 9.09
Group 2 (200 mg/kg of chalk)	192.25 ± 8.35 <sup>NS</sup>	202.25 ± 7.69 <sup>NS</sup>	206.58 ± 8.37 <sup>NS</sup>	226.42 ± 9.77 <sup>NS</sup>
Group 3 (400 mg/kg of chalk)	188.92 ± 6.93 <sup>NS</sup>	193.00 ± 7.36 <sup>NS</sup>	205.50 ± 5.76 <sup>NS</sup>	221.00 ± 6.66 <sup>NS</sup>
Group 4 (600 mg/kg of chalk)	190.67 ± 5.92 <sup>NS</sup>	191.75 ± 5.35 <sup>NS</sup>	200.42 ± 6.71 <sup>NS</sup>	214.75 ± 7.27 <sup>NS</sup>

Data were presented as mean ± standard error of mean.

<sup>NS</sup>Not significantly different from the control group at  $P < 0.05$ .

TABLE 3: Results of the behavioral test using the open field maze.

Groups (n = 6)	Line crossing P = 0.41 F = 1.00	Groom P = 0.05 F = 2.97	Rearing P = 0.47 F = 0.86	Stretch attend P = 0.07 F = 2.61	Central square entry P = 0.48 F = 0.84	Central square duration P = 0.47 F = 0.87	Def. P = 0.57 F = 4.58	Urin. P = 0.41 F = 1.00
Group 1 (control)	20.63 ± 5.98	2.63 ± 0.65	12.63 ± 3.52	0.75 ± 0.31	0.38 ± 0.37	0.96 ± 0.96	1.25 ± 0.84	0.00 ± 0.00
Group 2 (200 mg/kg of chalk)	19.38 ± 3.87 <sup>NS</sup>	2.38 ± 0.63 <sup>NS</sup>	8.38 ± 1.40 <sup>NS</sup>	0.25 ± 0.16 <sup>NS</sup>	0.00 ± 0.00 <sup>NS</sup>	0.00 ± 0.00 <sup>NS</sup>	1.13 ± 0.44 <sup>NS</sup>	0.00 ± 0.00 <sup>NS</sup>
Group 3 (400 mg/kg of chalk)	12.50 ± 4.11 <sup>NS</sup>	6.00 ± 1.54 <sup>NS</sup>	17.50 ± 6.56 <sup>NS</sup>	0.25 ± 0.16 <sup>NS</sup>	0.00 ± 0.00 <sup>NS</sup>	0.00 ± 0.00 <sup>NS</sup>	2.50 ± 1.02 <sup>NS</sup>	0.00 ± 0.00 <sup>NS</sup>
Group 4 (600 mg/kg of chalk)	25.25 ± 6.56 <sup>NS</sup>	3.88 ± 0.72 <sup>NS</sup>	12.13 ± 2.79 <sup>NS</sup>	0.00 ± 0.00 <sup>NS</sup>	0.25 ± 0.16 <sup>NS</sup>	0.25 ± 0.16 <sup>NS</sup>	1.30 ± 0.63 <sup>NS</sup>	0.25 ± 0.25 <sup>NS</sup>

Data were presented as mean ± standard error of mean.

<sup>NS</sup>Not significantly different from the control group at  $P < 0.05$ .

allowed to explore the apparatus for five minutes. After the five-minute test, the rat was returned in its home cage and the open field was cleaned with 70% ethyl alcohol and permitted to dry before the introduction of the next rat. Behavior was scored manually, and each trial was recorded for subsequent analysis using a video camera positioned above the apparatus. The counting was also done manually. The parameters scored included central square frequency and duration (open field only), transition frequency, duration spent in the light and dark chambers (light and dark field only), line crossing, rearing, grooming defecation, and urination.

Immediately after the behavioral tests, each rat was anaesthetized by exposure to chloroform vapour in a chamber and subsequently euthanized. The brains of each rat was excised and routinely processed for histological studies using the Haematoxylin (H) and Eosin (E) staining method. Briefly, after seven days fixation, the brain samples were dehydrated with ascending grades of alcohol, cleared in xylene, impregnated in molten paraffin, and embedded in fresh molten paraffin. A longitudinal orientation of the brain was cut and serial sections were obtained at 10  $\mu\text{m}$  using the rotary microtome. The sections were brought to water on slides and stained with H and E. Stereology was applied to the sections of cerebral cortices of the animals (ten sections

per brain sample) using ImageJ software. Photomicrographs were obtained by a digital camera connected to both the microscope and a computer.

**2.1. Statistical Analysis.** Using Primer software, one-way analysis of variance (ANOVA) was applied for the statistical analysis of all data, followed by post hoc Student-Newman-Keuls test. All results were regarded as significant at  $P < 0.05$ .

### 3. Results

**3.1. Morphology.** The results showed body weight gain by the dams in all the groups, but no difference existed in the body weight gains of the treatment groups compared to the control group (Table 2).

**3.2. Behavioral Test.** There was no difference in all the parameters measured in the open field test (Table 3). In the light and dark field test, the treatment groups were significantly ( $P < 0.05$ ;  $F = 9.65$ ) lower in rearing activity compared to the control group. Group 4 treated with 600 mg/kg of calabash chalk suspension was significantly ( $P < 0.05$ ) higher in stretch attend ( $F = 3.11$ ), duration in the dark ( $F = 7.25$ ), and

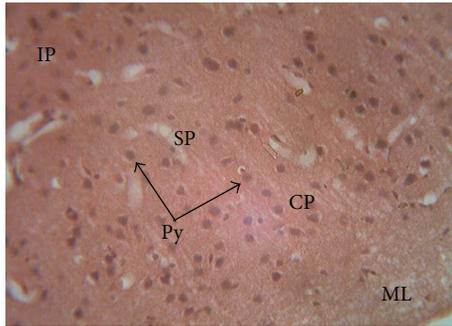


FIGURE 1: Photomicrograph of the cerebral cortex of the control group dams showed four cortical layers: ML: marginal layer, CP: cortical plate, SP: subplate, and IP: intermediate plate. Within these layers were the pyramidal cells (Py). Mag.  $\times 400$ . H & E.

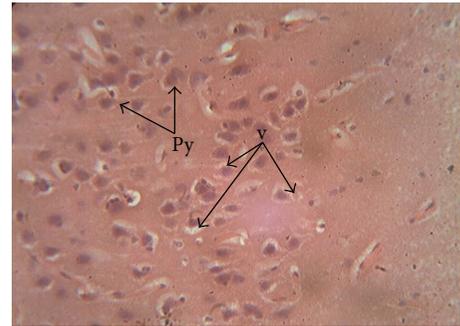


FIGURE 3: Photomicrograph of the cerebral cortex of group 3 dams treated with 400 mg/kg of calabash chalk suspension showed hypertrophy of pyramidal cells (Py) and vacuolations (v) around them. Mag.  $\times 400$ . H & E.

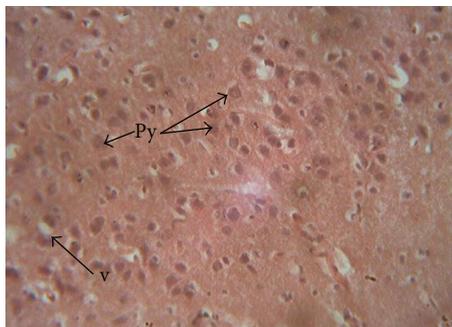


FIGURE 2: Photomicrograph of the cerebral cortex of group 2 dams treated with 200 mg/mL of calabash chalk suspension showed hyperplasia of cells and hypertrophy of pyramidal cells (Py) with some vacuolations (v) around them. Mag.  $\times 400$ . H & E.

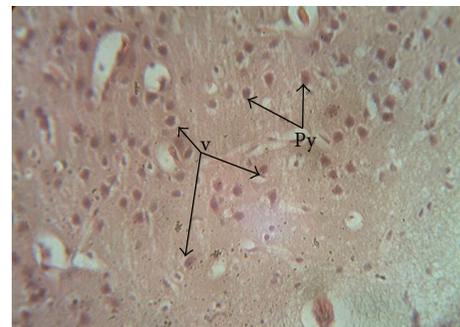


FIGURE 4: Photomicrograph of the cerebral cortex of group 4 dams treated with 600 mg/kg of calabash chalk suspension showed hypertrophy of pyramidal cells (Py) and vacuolations (v) around them. Mag.  $\times 400$ . H & E.

transition frequency ( $F = 5.91$ ), and they spent significantly ( $P < 0.05$ ;  $F = 7.25$ ) lower time in the dark compared with the control group. Groups 2 and 3 treated with 200 mg/kg and 400 mg/kg of calabash chalk suspension, respectively, were significantly ( $P < 0.05$ ;  $F = 5.21$  and  $F = 7.25$ , resp.) lower in line crossing and duration spent in the dark but significantly ( $P < 0.05$ ;  $F = 7.25$ ) higher in the time spent in the light arena compared with group 4 treated with 600 mg/kg of calabash chalk suspension (Table 4).

**3.3. Histomorphology/Stereology.** Group 1. The section of the cerebral cortex of the control group dams showed four cortical layers: marginal layer, cortical plate, subplate, and intermediate plate. Within these layers were the pyramidal cells, astrocytes, and oligodendrocytes (Figure 1).

Group 2. The section of the cerebral cortex of group 2 dams treated with 200 mg/kg of calabash chalk suspension showed hyperplasia of cells and hypertrophy of pyramidal cells (Py) with some vacuolations (v) around them compared with the control group (Figure 2).

Group 3. The section of the cerebral cortex of group 3 dams treated with 400 mg/kg of calabash chalk suspension showed hypertrophy of pyramidal cells (Py) and vacuolations (v) around them compared with the control group (Figure 3).

Group 4. The section of the cerebral cortex of group 4 dams treated with 600 mg/kg of calabash chalk suspension showed hypertrophy of pyramidal cells (Py) and vacuolations (v) around them compared with the control group (Figure 4).

The cellular population of the treatment groups was significantly ( $P < 0.05$ ;  $F = 2.020E + 07$ ) higher than that of control group. The average sizes and surface area covered by the cells were however not different from the control group (Table 5).

## 4. Discussion

This study investigated the effect of calabash chalk on the behavior and the histomorphology of the cerebral cortex of gestating rats. No difference existed in body weight gain by the dams and ambulatory activities, but treatment group 4 animals had more ( $P < 0.05$ ) transition frequency and were more averse to the dark in the light and dark field compared with the control. The section of the cerebral cortex showed a higher ( $P < 0.05$ ) cellular population, hypertrophied pyramidal cells, and vacuolations in the treatment groups. These results indicate that calabash chalk may have anxiolytic effect especially at high dose in the light and dark field but

TABLE 4: Results of the behavioral test using the light and dark field maze.

Groups (n = 6)	Line crossing P = 0.01 F = 5.21	Transition P = 0.00 F = 5.91	Groom P = 0.24 F = 1.50	Rearing P = 0.00 F = 9.65	Stretch attend P = 0.04 F = 3.11	Duration in light P = 0.00 F = 7.25	Duration in dark P = 0.00 F = 7.25	Def. P = 0.01 F = 5.25	Urin. P = 0.10 F = 2.33
Group 1 (control)	23.00 ± 2.30	2.88 ± 0.58	3.75 ± 1.63	14.50 ± 1.94	0.00 ± 0.00	0.21 ± 0.05	4.38 ± 0.05	0.50 ± 0.33	1.00 ± 0.65
Group 2 (200 mg/kg of chalk)	15.00 ± 0.42 <sup>d</sup>	1.38 ± 0.26 <sup>d</sup>	1.00 ± 0.00 <sup>NS</sup>	5.00 ± 0.00 <sup>*</sup>	0.50 ± 0.19 <sup>NS</sup>	0.03 ± 0.00 <sup>d</sup>	4.56 ± 0.00 <sup>d</sup>	2.00 ± 0.27 <sup>*</sup>	0.00 ± 0.00 <sup>NS</sup>
Group 3 (400 mg/kg of chalk)	14.75 ± 3.81 <sup>d</sup>	3.50 ± 0.85 <sup>NS</sup>	2.50 ± 0.57 <sup>NS</sup>	5.50 ± 1.70 <sup>*</sup>	0.50 ± 0.19 <sup>NS</sup>	0.16 ± 0.05 <sup>d</sup>	4.43 ± 0.05 <sup>d</sup>	0.50 ± 0.19 <sup>b</sup>	0.00 ± 0.00 <sup>NS</sup>
Group 4 (600 mg/kg of chalk)	27.63 ± 3.26 <sup>NS</sup>	5.50 ± 0.93 <sup>*</sup>	2.75 ± 0.67 <sup>NS</sup>	9.50 ± 1.18 <sup>*</sup>	1.00 ± 0.38 <sup>*</sup>	0.54 ± 0.14 <sup>*</sup>	4.05 ± 0.14 <sup>*</sup>	1.50 ± 0.46 <sup>NS</sup>	0.00 ± 0.00 <sup>NS</sup>

Data were presented as mean ± standard error of mean.

<sup>\*</sup>Significantly different from the control group at  $P < 0.05$ .

<sup>b</sup>Significantly different from group 2 at  $P < 0.05$ .

<sup>d</sup>Significantly different from group 4 at  $P < 0.05$ .

<sup>NS</sup>Not significantly different from the control group at  $P < 0.05$ .

TABLE 5: Results of the stereology of the cerebral cortices of the dams.

Group (n = 6)	Cellular population $P < 0.0001$ $F = 2.020E + 07$	Total surface area of cells ( $\mu\text{m}^2$ )	Average cellular size ( $\mu\text{m}$ )
Group 1 (Control)	3689 $\pm$ 0.37	17070	5 $\pm$ 0.00
Group 2 (200 mg/kg of chalk)	7257 $\pm$ 0.37*	17650	2 $\pm$ 0.00 <sup>NS</sup>
Group 3 (400 mg/kg of chalk)	4367 $\pm$ 0.37 <sup>*,c,d</sup>	15820	4 $\pm$ 0.00 <sup>NS</sup>
Group 4 (600 mg/kg of chalk)	6324 $\pm$ 0.37 <sup>*,b</sup>	18790	3 $\pm$ 0.00 <sup>NS</sup>

Data were presented as mean  $\pm$  standard error of mean.

\*Significantly different from the control group at  $P < 0.05$ .

<sup>b</sup>Significantly different from group 2 at  $P < 0.05$ .

<sup>c</sup>Significantly different from group 3 at  $P < 0.05$ .

<sup>d</sup>Significantly different from group 4 at  $P < 0.05$ .

<sup>NS</sup>Not significantly different from the control group at  $P < 0.05$ .

not in the open field and can stimulate cellular changes in the maternal cerebral cortex.

Though there were weekly body weight gains by the dams, there was no difference between the treatment and the control groups. As all the experimental animals were exposed to the same handling method during the treatment period, this indicates that the animals may have had the same baseline age, growth, and pregnancy rates. Thus, the chalk may not have had effect on the body weight/growth of the dams. Prenatal treatments may not always affect the dam's body weights and pregnancy outcome. This is in line with a previous report [34]. Ferguson et al. [34] showed no effect of folate treatment differences in dam's body weights or pregnancy outcomes.

The open field test showed no difference in the treatment groups, an indication that the chalk may not have affected the parameters measured. The open field test is used to determine locomotion, exploratory, and anxiety related activities [35]. It provides objective measures of emotionality in rats [36]. Higher measured activities usually indicate anxiogenic effects, with a lower activity indicating anxiolytic effects. In this study, these were not the case.

The light and dark maze test showed no difference ( $P < 0.05$ ) in ambulatory activities. The light and dark test is based on the innate aversion of rodents to brightly illuminated areas and on the spontaneous exploratory behavior of rodents in response to mild stressors, that is, novel environment and light [37]. Light and dark test is useful to predict the anxiolytic-like or anxiogenic-like activities [38]. As no difference was observed in the ambulatory activities, this indicates that the chalk may not have affected the parameters. These supports the report of the chalk in the open field test. However, group 4 animals treated with 600 mg/kg of the calabash chalk had more ( $P < 0.05$ ) transition and were more averse to the dark compared with the control group. This indicates that the chalk at a higher dose has a potential to be anxiolytic in the light and dark field.

Pharmacological data involving different anxiety tests are often inconsistent across studies. This can be due to factors such as variations in genetic background, test protocol, and laboratory environment [39]. However, intertest variations within the same pharmacological study should indicate construct differences between tests. In Lewis rats, chlordiazepoxide produced anxiolytic-like effects in the elevated plus maze but not in the open field, whereas in spontaneously hypertensive rats (SHRs), chlordiazepoxide in addition to a tachykinin NK1-receptor antagonist produced anxiolysis in the open field but not in the elevated plus maze. Such results corroborate the idea that these tests measure different aspects of anxiety [40]. This may be the case in this study, where no difference was observed in the open field unlike the light and dark field.

Sections of the cerebral cortex showed significant ( $P < 0.05$ ) cellular hyperplasia, as well as neuronal hypertrophy and vacuolations within the brain sections of the treatment groups. This is an indication that the substance gain access to the brain and that the brain was traumatized.

The components of the calabash chalk such as lead, arsenic, aluminum, and kaolin are known to cross the blood-brain barrier and to cause different effects in different parts of the brain [41–44]. Thus, the effects observed in this study may be associated with these components.

As neurons are not known to proliferate when traumatized, the higher cellular population in the treatment groups may be due to gliosis, as gliosis usually result when there is brain trauma [45, 46]. Gliosis may lead to the formation of astrocytic scar [47] that may interfere with the normal functions of the neurons likely to result in death.

## 5. Conclusion

The results indicate that calabash chalk may have anxiolytic effect especially at high dose in the light and dark field but not in the open field and can stimulate cellular changes in the maternal cerebral cortex which may lead to neuronal death.

## Conflict of Interests

The authors declare that they have no conflict of interests pertaining to the research and the paper.

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