The role of *Calotropis procera* in phenytoin-induced toxicity in the postnatal developing cerebellum of Wistar rats - histological and gross morphometric studies

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SUMMARY

The role of methanolic leaf extracts of *Calotropis procera* in phenytoin-induced toxicity in the postnatal developing cerebellum of Wistar rat was studied.

Forty sexually mature female rats, weighing about 160 g of the Wistar strain were randomly divided into five groups of eight animals per group. They were mated and pregnancy confirmed by the presence of a vaginal plug. The animals were fed with a standard diet of rat pellets and water provided *ad libitum*. The control animals received water, while the test groups received 50 mg/kg of phenytoin, 300 mg/kg, methanolic extracts of C*alotropis procera* and 200 mg/kg vitamin C orally, both separately and in combination during and after pregnancy. At the end of the experiment, the offspring for days 1, 7, 14, 21, 28 and 50 post-partum, five per group, were weighed and killed. The brains and cerebella were dissected out and weighed and the cerebella processed for histological studies.

In the phenytoin-treated animals the results showed a non significant reduction in the body weight of the animals, $P>0.05$, and a significant reduction in the brain and cerebellar weights, $P<0.05$, was observed. The administration of extracts of *Calotropis procera* and vitamin C reversed these changes when compared with the phenytoin-treated group, but not significantly when compared with the control. Histologically, the outer molecular, Purkinje and inner granular layers of the cerebellar cortex were intact, and in all the groups the external granular layer was not seen on day 21 post-partum.

In conclusion, supplementation with methanolic extracts of *Calotropis procera* reduced the rate at which phenytoin induced toxicity in the postnatal developing cerebellum of Wistar rats.

Key words: Phenytoin – Toxicity – Cerebellar development – *Calotropis procera* – Vitamin C

INTRODUCTION

While investigating the intrauterine and early postnatal development of the human brain, Dobbing and Sands (1973) coined the term "brain growth spurt": the period of major growth and development of the brain.

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In humans it occurs during the second half of pregnancy and in the first year of life. At that time there is a rapid and continuous growth of the human brain, manifested by cell multiplication and growth, myelinization and synapse formation. Being in the phase of its active development, actively growing parts of the brain such as the cerebral hemispheres and cerebellum are also most vulnerable to the deleterious effects of the environment (Brazelton, 1986). Hence, neuroteratogens such as antiepileptic drugs (AEDs) may affect brain development in the second half of pregnancy and to some extent also postnatally. Intrauterine exposure to AEDs such as valproic acid, phenytoin, carbamazine and phenobarbital may result in increased rates of major and minor congenital anomalies, especially if exposure occurred during the first trimester of pregnancy (Ornoy, 2003). Phenytoin, a potent and widely used antiepileptic drug has long been suspected of causing cerebellar dysfunction (Merrit and Putnam, 1939), or even cerebellar atrophy, as shown by computed tomography (Baier et al, 1984) or histological examination (Haberland, 1962). Phenytoin is thought to cause chronic intrauterine hypoxia / ischaemia and embryofoetal toxicity via reacting oxygen intermediates. Reacting oxygen species (ROS) can oxidize molecular targets such as deoxyribonucleic acid (DNA), proteins and lipids in a process called oxidative stress, resulting in cellular dysfunction and *in utero* death or teratogenicity (Wells et al, 1996; Zablocka and Janusz, 2008). Oxidative stress has been implicated in degenerative processes such as ageing (Ames et al., 1993), cardiovascular diseases, cancer, Alzheimer's disease, stroke, Parkinson's disease and other neurodegenerative diseases, (Ames, 1983; Temple, 2000). However, the antioxidant system helps to prevent free radical-induced cellular damage.

Vitamin C functions as an antioxidant in the body by readily donating electron to regenerate other antioxidants, such as vitamin E and GSH (Halpner et al, 1998) and can inhibit ROS generation and lipid peroxidation by chelating free transition metals such as copper and iron (Zablocka and Janusz, 2008).

Calotropis procera, also known as the giant milk weed, is a flowering plant commonly found in the tropics worl-wide. The plant is found in almost all parts of Nigeria but is more abundant in the Northern part of the country (Sofowora, 1984). It gets its common name, giant milk weed, from the thick white sap that oozes from a cut stem or from the stem when a leaf is plucked off. Hence, the family to which the plant belongs is referred to as the milkweed family (Ghazanfar, 1989). *Calotropis procera* extract has been shown to prevent loss of body weight in diabetic rats and increased hepatic levels of the endogenous antioxidants in alloxan-induced diabetes in rats, and it has been reported that its antioxidant and antidiabetic properties are comparable to the standard antidiabetic drug glibenclamide (Roy et al, 2005).

Apart from its important function in controlling and coordinating muscle activity, the cerebellum also assists the rest of the brain in regulating autonomic activities such as respiration, cardiovascular function, control of papillary size, learning and classical conditioning, as well as cognitive processing and sensory discrimination (West, 1995; Chizhikov and Millen, 2003). Thus, the objective of this research was to test the hypothesis that *calotropis procera* could protect or reduce the rate at which phenytoin induces toxicity in histological and gross morphometric analyses in the postnatally developing cerebellum of the Wistar rat.

Materials and Methods

Breeding of Animals

Forty sexually mature female rats weighing about 160 g of the Wistar strain were obtained from the central animal house of the Faculty of Basic Medical Sciences, University of Ibadan, Ibadan, Oyo State (Nigeria). The animals were randomly divided into five groups of eight animals per group. They were mated and pregnancy was confirmed by the presence of a vaginal plug. The animals were fed with a standard diet of rat pellets and water provided *ad libitum*. All procedures used in animal handling conformed to acceptable guidelines on the ethical use of animals in research.

Drug Preparation and Administration

The experimental animals received phenytoin, vitamin C (standard antioxidant) and *Calotropis procera* leaf extracts, separately and in combination, while the control animals received water.

Phenytoin, dissolved in tap water was administered orally during pre and postnatal

life at a dose of 50 mg/kg. The sodium salt of Phenytoin (capsule) manufactured by Mancare Pharmaceuticals PVT Ltd India purchased from the pharmacy of the University College Hospital (UCH), Ibadan, was used for the experiment.

200 mg/kg body weight vitamin C was administered orally one hour prior to phenytoin administration to the experimental animals during pre and post natal life.

Extraction of Calotropis Procera leaves

The leaves of *Calotropis procera* were harvested at the main campus of the University of Ibadan and authenticated by Dr. O.A. Ugbogu of the Forestry Research Institute of Nigeria (FRIN), Ibadan, receiving a Forestry Herbarium Identification number (FHI) 108221. They were air-dried at room temperature for two and a half months, blended, and made into powdered form, obtaining about 1.2 kg. Cold methanolic extraction for about 72 hours was performed to facilitate a better extraction of flavonoids. The methanolic solution was concentrated with a rotatory evaporator at a temperature below 50°C for 7 hours. The concentrated extract (187.0 g) was stored in the refrigerator until use. The concentrated extract was reconstituted with water before administration.

Phytochemical studies of the leaves of *Calotropis procera* were carried out at the Department of Pharmacognosy, Faculty of Pharmacy, University of Ibadan, Ibadan. The following compounds were screened for: alkaloids, flavonoids, cardenolides, saponins and tannins.

A 300 mg/kg of body weight methanolic extract of *Calotropis procera* was administered orally one hour prior to phenytoin administration to the experimental animals during pre- and postnatal life. The dose of *Calotropis procera* extracts used in the research was based on safety evaluation studies carried out by Mossa et al (1991) showing that the use of the extract at a single high dose (up to 3 g/kg body weight) does not produce any visible toxic symptoms or mortality.

Grouping of Animals

Group I: Control group, receiving normal saline.

Group II: Receiving 50mg/kg of phenytoin.

Group III: Receiving 300mg/kg *Calotropis procera*.

Group IV: Receiving *Calotropis procera* + phenytoin.

Group V: Receiving phenytoin + 200 mg/kg Vitamin C.

At the end of the experimenst, the animals of days 1, 7, 14, 21, 28 and 50 postpartum, five per group, were weighed and killed. The brains and cerebella were dissected out, weighed and the cerebella were fixed in 10% formol saline. The cerebellar tissues were processed employing a routine paraffin embedding technique and stained with Haematoxylin and Eosin (H and E) for histological observation.

Gross Morphometric Studies

The following gross parameters were measured:

i. Mean body weight of the animals, using a Swiss microwa balance (type 7720)
ii. Mean relative weight of the brain, using a Swiss microwa analytical balance (type 5540), and\
iii. Mean relative weight of the cerebellum, using a Swiss microwa analytical balance (type 5540).

Histological Observations

These were accomplished by studying the processed cerebellar tissues with the aid of a light microscope.

Statistical Analysis

The data obtained were further analyzed with an unpaired Student's t-test, using Microsoft Excel computer software,. The Mean, Standard Deviation, SD, and the level of significance at 95% Confidence Interval were calculated.

Results

Phytochemistry

Phytochemical studies carried out on the leaves of *Calotropis procera* showed that it mainly contained flavonoids, alkaloids and some cardenolides.

General Observations

There was no mortality in either the control or test groups along the duration of the experiment and they all tolerated the procedure without any complications. Also, no focal neurological deficits were observed, since the

animals moved about freely in their cages throughout the experiment.

Gross morphometric studies

– Mean body weight of the developing animals:

There was a non-significant reduction in the average body weight of the developing animals in the phenytoin-treated groups on days 1, 7, 14, 21, 28 and 50 postpartum when compared with the control animals (P>0.05). The administration of *Calotropis procera* extracts and vitamin C to the phenytoin-treated animals improved the average body (P>0.05) (Table 1).

Table 1. Mean body weight of the animal in the control and treated groups on days 1, 7, 14, 21, 28 and 50 postpartum.

Days/Gp	1	7	14	21	28	50
Control	7.5±0.2	13.6±0.6	27.4±1.5	45.0±3.8	49.4±2.1	82.3±2.7
Pheny	7.3±0.2	12.8±0.9	26.1±1.5	43.0±4.0	47.5±1.7	80.2±2.6
Cp	7.4±0.4	13.1±0.6	28.4±2.1	45.0±3.1	49.4±2.8	84.0±2.5
Pheny+*Cp*	7.4±0.3	13.2±0.9	27.4±1.7	45.5±3.1	50.1±2.5	82.1±3.0
Pheny+vC	7.1±0.4	13.5±1.0	27.2±1.3	45.3±3.6	49.2±2.5	81.3±5.1

Values are given as means ± SD, (n=5). Mean body weight of animals is expressed in grams. Gp= group; Pheny= phenytoin; vC= vitamin C; Cp= *Calotropis procera* extract. P>0.05 in the treated group versus the control group.

– Mean relative weight of the developing brain:

Phenytoin-induced toxicity was associated with reduced brain weight on days 1, 7, 14, 21, 28 and 50 postpartum but significantly different on days 14 (1.96±0.26% vs 3.58±0.12%) and 21(2.25±0.35% vs 2.94±0.22%) postpartum when compared with the control group (P< 0.05).

The administration of *Calotropis procera* extract to phenytoin-treated animals improved the mean relative weight of the developing brain on days 1, 7, 14, 21 and 28 postpartum when compared with phenytoin-treated group but significantly on days 1 and 14 postpartum (P<0.05) (2.89±0.30 vs 2.45±0.21% and 2.94±0.17 vs 1.96±0.26% respectively). The administration of vitamin C to phenytoin significantly improved the weight of the developing brain on days 1, 7 and 14 postpartum when compared with the phenytoin group (P<0.05) (2.98±0.08% vs 2.45±0.21%, 3.65±0.55% vs 2.89±0.37% and 3.92±0.23% vs 1.96±0.26% respectively) (Table 2).

Table 2. Mean relative weight of the brain in the control and treated groups on days 1, 7, 14, 21, 28 and 50 postpartum.

Days/Gp	1	7	14	21	28	50
Control	2.73±0.28	2.83±0.29	3.58±0.12	2.94±0.22	2.91±0.16	2.05±0.07
Pheny	2.45±0.21	2.89±0.37	$1.96±0.26^{c}$	$2.25±0.35^{b}$	2.66±0.21	2.01±0.15
Cp	2.33±0.35	2.95±0.34	$2.73±0.27^{ce}$	$2.30±0.25^{b}$	2.88±0.16	$1.92±0.04^{b}$
Pheny+*Cp*	$2.89±0.30^{d}$	3.17±0.28	$2.94±0.17^{ce}$	$2.61±0.23^{a}$	2.81±0.14	$1.92±0.07^{a}$
Pheny+vC	$2.98±0.08^{f}$	$3.65±0.55^{ad}$	$3.92±0.23^{df}$	$2.50±0.37^{a}$	2.70±0.31	1.98±0.12

Values are given as means±SD, (n=5). The mean relative brain weight of the animals is expressed in %. Gp=group; Pheny=phenytoin; vC=vitamin C; *Cp*= *Calotropis procera* extract. ^{a}P<0.05, ^{b}P<0.01, ^{c}P<0.001 versus control group; ^{d}P<0.05, ^{e}P<0.01, ^{f}P<0.01 versus phenytoin group.

– Mean relative weight of the developing cerebellum:

There was a significant reduction in the mean relative weight of the developing cerebellum in the phenytoin-treated group on days 1, 7, 14, 28 and 50 postpartum when compared with the control group (P< 0.05) (0.47±0.04% vs 0.63±0.09%, 0.86±0.08% vs 1.01±0.07%, 0.60±0.03% vs 0.79±0.04%, 0.53±0.03% vs 0.68±0.05% and 0.41±0.03% vs 0.62±0.05%, respectively).

Calotropis procera administered to phenytoin treated animals improved the mean relative developing cerebellar weight on days 1, 14, 21, 28 and 50 postpartum when compared with the phenytoin-treated animals but significantly so on days 1, 14, 28 and 50 postpartum (P<0.05) (0.75±0.06% vs 0.47±0.04%, 0.71±0.10% vs 0.60±0.03%, 0.64±0.04% vs 0.53±0.03% and 0.54±0.11% vs 0.41±0.03% respectively). The administration of vitamins C to the phenytoin-treated animals improved the mean relative cerebellar weight on days 1, 14, 21 and 28 postpartum when compared with the phenytoin-treated animals, at P> 0.05 (Table 3).

Table 3. Mean relative weight of the cerebellum in the control and treated groups on days 1, 7, 14, 21, 28 and 50 postpartum.

Days/Gp	1	7	14	21	28	50
Control	0.63±0.09	1.01±0.07	0.79±0.04	0.56±0.05	0.68±0.06	0.62±0.05
Pheny	$0.47±0.04^{b}$	$0.86±0.08^{a}$	$0.60±0.03^{c}$	0.50±0.07	$0.53±0.03^{c}$	$0.41±0.03^{c}$
Cp	0.51±0.09	0.94±0.08	0.74±0.07e	0.53±0.56	$0.59±0.05^{ad}$	$0.45±0.02^{c}$
Pheny+*Cp*	$0.75±0.06^{df}$	$0.81±0.07^{a}$	$0.71±0.10^{d}$	0.55±0.04	$0.64±0.04^{e}$	$0.54±0.11^{d}$
Pheny+vC	0.51±0.10	$0.65±0.12^{ce}$	0.76±0.19	0.53±0.06	$0.55±0.04^{b}$	$0.37±0.03^{c}$

Values are given as means±SD, (n=5). The mean weight of cerebellum of animals is expressed in %. Gp=group; Pheny=pheny-

toin; vC=vitamin C; *Cp= Calotropis procera* extract. [a]P<0.05, [b]P<0.01, [c]P<0.001 versus control group; [d]P<0.05, [e]P<0.01, [f]P<0.001 versus phenytoin group.

Histological observations

Employing routine paraffin wax embedding histological preparations with the Haematoxylin and Eosin (H&E) staining technique revealed normal cerebellar cytoarchitecture in both the control and the test groups. The external granular layer in all the groups was not seen on day 21 post partum (Figures 1-3).

Discussion

Altman (1987) stated the central nervous system is the target of harmful environmental agents result in behavioural abnormalities in humans with no quantitatively evident neuropathy. He called this microneuronal hypoplasia, which is a retardation of brain development characterized by a quantitative reduction in the normal population of late-generated, short-axoned neurons in specific brain regions. The cerebellum is known to be affected by situations of nutritional deficiency (Clark et al., 1973), cyanide (Pavlakovic et al.,

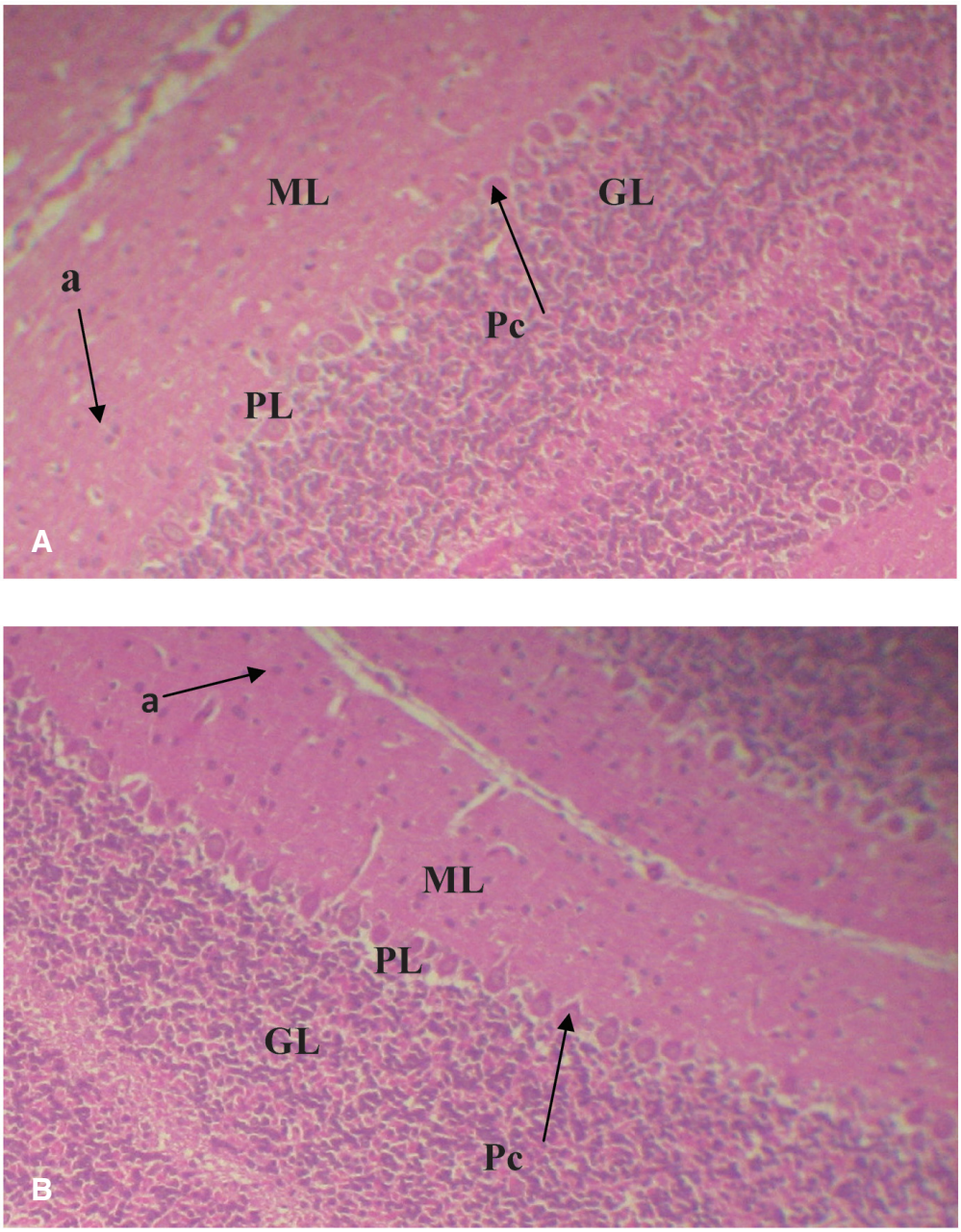

Fig. 1. 21-day-old postpartum rat cerebellar cortex of **a)** Control group and **(b)** Phenytoin-treated group showing normal cerebellar cytoarchitecture and complete disappearance of the external granular layer (EGL). Molecular layer, ML, Purkinje layer, PL, Granular layer, GL, White matter, W, Purkinje cells, Pc, astrocyte, a. H&E staining. x100.

1994; Malomo et al., 2004), irradiation, (Sugihara et al., 2000), alcohol (West et al., 1990), and anticonvulsant drugs, such as valproic acid, carbamazine, phenytoin and phenobarbital (Szot et al., 1986). Phenytoin has been found to induce neurotoxicity by generating free radicals, resulting in oxidative stress which then leads to cellular damage and dysfunction (Liu et al., 1997).

The present postnatal study was carried out to investigate the role of methanolic extracts of *Calotropis procera* in the *in vivo* model of phenytoin-induced toxicity in rats by assessing the gross morphometric and histological changes in the developing cerebellum of Wistar rats. In this study, the no- significant reduction in the average body weight in the phenytoin treated animals may have been a result of caloric restriction arising from the loss of appetite induced by phenytoin. Craig (2005) reported that phenytoin intoxication manifested predominantly as nausea, central nervous system dysfunction (particularly confusion, nystagmus and ataxia), with a depressed conscious state, coma and seizure occurring in more severe cases. The adminis-

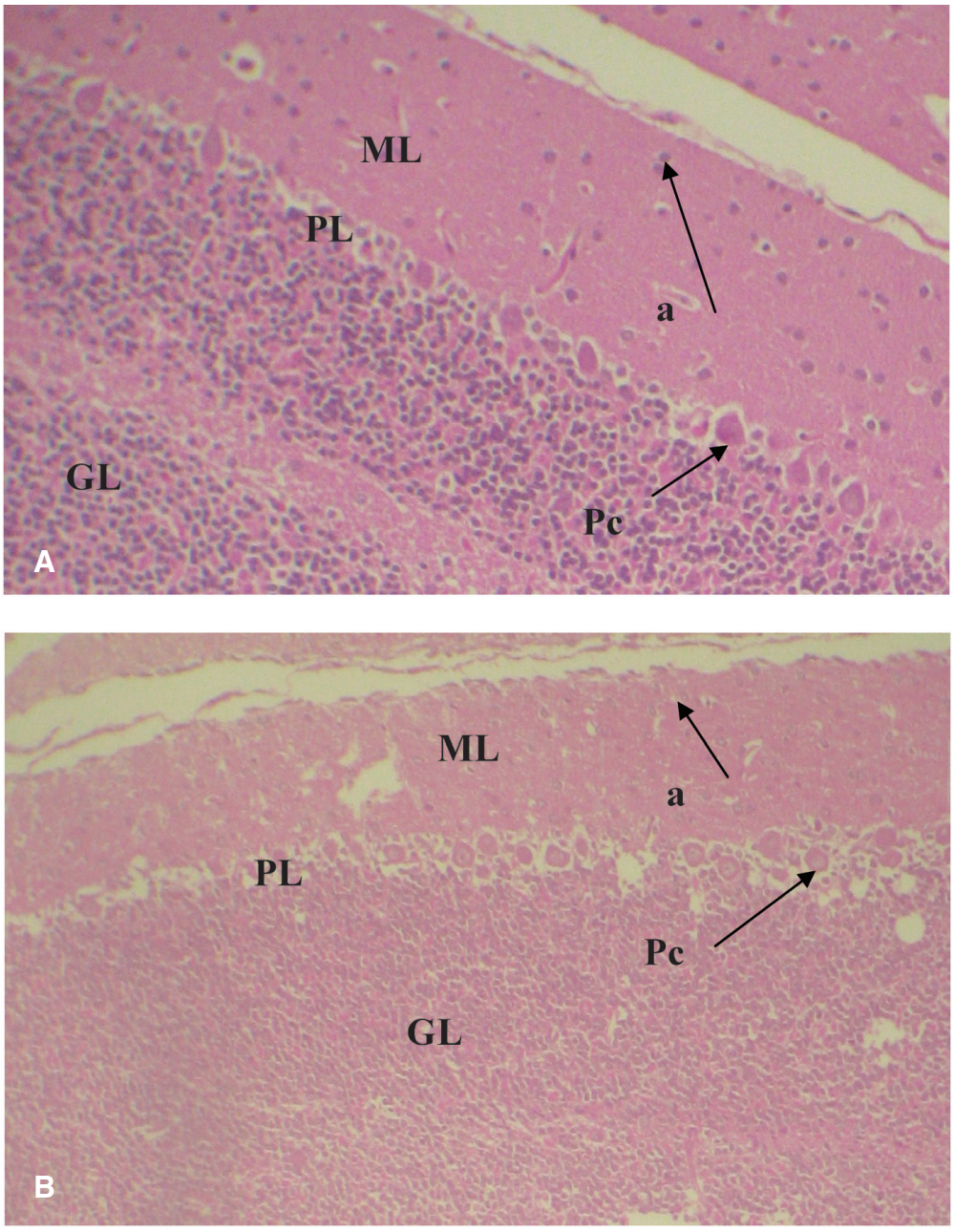

Fig. 2. 21-day-old postpartum rat cerebellar cortex of **a)** Phenytoin + vitamin C and **(b)** Phenytoin+*calotropis procera*-treated group showing normal cerebellar cytoarchitecture and complete disappearance of the external granular layer (EGL). Molecular layer, ML, Purkinje layer, PL Granular layer, GL and White matter, W, Purkinje cells, Pc. and astrocyte, a. H&E staining. x100.

tration of phenytoin to rats for 7 days has been shown to cause a reduction in body weight and the cell density of the pyramidal cell neurons in the CA 1 (cornus ammonis) region of the hippocampus (Osuagwu et al., 2007).

The significant reduction in the mean relative weights of the brain and cerebellum in the postnatal phase of the developing brain and cerebellum in the phenytoin-treated animals, confirmed the works of other researchers that phenytoin has a high teratogenic potential (Ujhazy et al., 2004) and causes neurobehavioural alterations (Dubovicky et al., 2004). These findings support the concept of phenytoin-induced chronic intrauterine hypoxia/ischaemia and embryo-foetal toxicity (Wells et al., 1996), and also the notion that the central nervous system during the neonatal period is more vulnerable to phenytoin neurotoxicity because of higher brain phenytoin concentrations (Kaneko et al., 1988). Ohmori et al. (1997) reported that oral administration of low-dose phenytoin (10, 17.5, 25, or 35 mg/kg) to newborn mice once a day during postnatal days 2-4 caused a reduction in total brain weight and size and in cerebellar weight in the 25 and 35mg/kg phenytoin-treated groups. The reduction in the average weight of animals, and the mean relative weights of the developing brain and cerebellum may be due to the accumulation or gestational carry-over effect of phenytoin metabolites, which may generate free radical intermediates.

The medicinal effects of *Calotropis procera* in the treatment of different diseases and ailments have been documented (Sahu, 1984). The flavonoid content of *Calotropis procera* extracts enables it to scavenge free radicals and as such may play an important role in preventing free radical-induced cellular damage. *Calotropis procera* extracts significantly improved the mean relative brain and cerebellar weights when compared with the phenytoin-treated animals. Roy et al. (2005) reported the antioxidant and protective properties of *Calotropis procera* extracts against alloxan-induced diabetes in rats, preventing a loss of body weight in diabetic rats. Vitamin C administration did not significantly reverse the reduction in the body, brain and cerebellar weights but did decrease the rate at which such reduction occurred. However, the antioxidant activity of vitamin C is well documented. Lee et al. (2004) reported that birth weight and body length were highest when levels of vitamin C was high in maternal serum, indicating the importance of the antioxidant nutrient balance for pregnant women exposed to various oxidants through food, drinking water or inhaled air.

The external granular layer (EGL) is the most metabolically active part of the developing cerebellum since the differentiation of the cells of this layer gives rise to the outer stellate, basket, and granule and Golgi type II cells of the cerebellar cortex. This differentiation process requires energy. In this study, the EGL was absent on postnatal day 21, confirming the findings of Jacobson (1991) and Hatten and Heintz (1995) that in the mouse the EGL disappears by about postnatal day 20. The cerebellar cortex in adult rats showed the normal three-layered cytoarchitecture of the outer molecular layer containing relatively few cells, an extremely cellular inner granule layer,and a single row of intervening Purkinje cell layer which suggests that phenytoin has no effect on the patterning of the cerebellar cortex.

In conclusion, the present study shows that phenytoin administered to animals during pre- and postnatal life causes gross morphometric changes in the brain and cerebellum of the neonate and post-weaning rats but that the administration of extracts of *Calotropis procera* and vitamins C does not completely protect the developing rat cerebellum from the neurotoxic effects of phenytoin but reduces the rate at which such toxicity occurs in the postnatal developing cerebellum of the Wistar rat.

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