Niger. J. Physiol. Sci. 36 (December 2021): 211– 220
www.njps.physiologicalsociety.com
https://doi.org/10.54548/njps.v36i2.10

Niger. J. Physiol. Sci.

Full-Length Research Article

Aqueous Extracts of *Daucus carota* (Linn) Protected the Developing Cerebellum of Wistar Rats Against Perinatal Arsenic-Induced Oxidative Stress

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Summary: The neuroprotective effects of the aqueous extract of *Daucus carota* (Dc) tuber against arsenic-induced oxidative damage on the developing cerebellum of Wistar rats were studied. Twenty-five pregnant rats (110-200g) were divided into five groups (n=5) – control received distilled water; Arsenic (As); Dc (200mg/kg); Dc (200mg/kg) +As; Vitamin C (Vc) (100mg/kg) +As. The pregnant rats in all the groups were treated orally from the first day of pregnancy to postnatal day 21. The Dc extract and Vc were administered one hour before the administration of As. Body weight of the pups on days 1, 7, 14, 21 and 28 were recorded, while neurobehavioural (forelimb grip strength and negative geotaxis) tests were done on day 21 pups. The rats were sacrificed and cerebellar tissues were collected for oxidative stress, histological (H and E), and immunohistochemical studies. Decreased forelimb grip strength, increased lipid peroxidation and decreased glutathione, glutathione peroxidase, catalase and superoxide dismutase was observed in the As group compared with the control and other treated groups. Histologically, the cerebellar cortex of the As pups showed persistent external granular layer (EGL) on postnatal day 21, reduced thickness of the molecular layer (ML) on postnatal day 28, pyknotic and depleted Purkinje cells compared with the control and other treated rats. Immunohistochemical evaluations of the cerebellar cortex showed astroliosis in the As-treated group on day 21 pups compared with the control and other treated groups. Aqueous extracts of *Daucus carota* and Vitamin C reversed the toxicity caused by arsenic. From the results of the study, arsenic-induced oxidative stress with morphological alterations in the perinatal developing rat cerebellum. Extracts of *Daucus carota* exhibited antioxidant activity as such may be a potential neuroprotective agent.

Keywords: Arsenic, *Daucus carota*, oxidative stress, developing cerebellum, Purkinje cells

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Manuscript received- October 2020; Accepted- May 2021

INTRODUCTION

The nervous system, in which the cerebellum is an important part of, is one of the earliest systems to develop embryologically but completes its developmental process postnatally (Hill *et al*., 2018), and as a result, it is highly prone to any form of injury in prenatal and postnatal life. The cerebellum lies in the posterior cranial fossa of the cranial cavity and functions in the control of smooth and skilful movements, balance, and higher cognitive and emotional functions (Hall *et al*., 1995; Imosemi and Osinubi, 2011; Hashimoto and Hibi, 2012; Buckner, 2013; Balaei *et al.,* 2017). The cerebellum is highly susceptible to oxidative stress during prenatal and perinatal periods (Lopez, *et al.,* 2009). Neurotoxins disrupt the function of the nervous system by destroying brain cells or nerves which carry signals around the body. Although metals are required for normal homeostasis, when taken in excess amounts, they accumulate in the brain, inducing neurotoxicity which eventually results in neurodegeneration (Chen *et al*., 2016). Arsenic is a naturally occurring metalloid found in groundwater (Izah and Srivastav, 2015; WHO, 2017) and from anthropomorphic sources (Ettinger *et al.,* 2017). Arsenic, a neurotoxin (Sodini, 2015), poses a major public health issue which has been linked to impairments in cognitive development (WHO, 2017). Arsenic toxicity continues to be a problem because it is difficult to have a global assessment of the burden of arsenic toxicity due to its long latency period and in most cases where water is polluted, there are usually more than one pollutants (Tyler and Allan, 2014; Chitsulo, 2018; UNICEF-WHO, 2018). In Nigeria, studies have shown that the level of arsenic in drinking water (Egbinola and Amanambu, 2014; Odebunmi, *et al.,* 2014; Izah and Srivastav, 2015; Etim, 2017) is higher than the WHO recommended level (10µg/L) (WHO, 2017) with the acceptable limit of 0.01 mg/L recommended by Standard Organization of Nigeria (SON) (Izah and Srivastav, 2015). Arsenic can induce oxidative stress by alternating between oxidation states of metals or by interaction with antioxidants, thereby increasing inflammation, accumulation of free radicals in cells and causing cellular dysfunction (Halliwell and Whiteman, 2004). Arsenic is capable of crossing the blood-placenta and blood-brain barriers, adversely effecting pregnancies leading to low-birth-weight, spontaneous abortions, infant death, foetal loss (Milton *et al.,* 2005; Rahman *et al.,* 2007; Rahman *et al.,* 2008; Kile *et al.,* 2016) and causing a disruption in neurological development (Benedetti, 1996; Ding, *et al.,* 2013; Tyler and Allan, 2014; Punshon, *et al.,* 2015; Sodini, 2015; WHO, 2017; UNICEF-WHO, 2018; Htway, *et al.,* 2019). The cerebellum has been shown to be highly susceptible to environmental insults and

oxidative damage (Kern and Jones, 2006). In a developmental study, maternal exposure to arsenic was reported to have altered the developmental process of the cerebellum by slowing down the proliferation of cells especially in the granular layer (Ding, *et al.,* 2013).

Neurological disorders have significantly been on the rise in developing and developed countries (Malomo, *et al.,* 2004; Ajibade, *et al.,* 2015) and in a bid to reduce this rising burden and as an alternative to synthetic drugs, plants are being employed as they are rich sources of phytochemicals (Prajna and Hedge, 2018). A regular intake of vegetables and fruits is believed to be effective in improving antioxidant activities in the brain (Potter, *et al.,* 2011; Yuan, *et al.,* 2017). *Daucus carota* Linn (Carrot) is a root vegetable from *Apiaceae* family (Mani, *et al.,* 2010; Silva Dias, 2014; El-masry and El-rhman, 2017; Praja and Hedge, 2018) that is grown globally and is available all year round. It is rich in carotenoids, flavonoids, polyacetylenes, vitamins and minerals (Silva Dias, 2014) such as ascorbic acid, and tocopherol (Prajna and Hedge, 2018). Consumption of *Daucus carota*, either as food or as juice, has been reported to prevent cancerous growths, impaired vision, control diabetes, blood pressure and boosting the immune system (Silva Dias, 2014; Praja and Hedge, 2018).

The characteristic orange colour of *Daucus carota*s is as a result of their rich content of beta-carotene (an antioxidant), and *Daucus carota*s are also rich sources of vitamins A, B, C and K as well as other minerals (Ca, F, Se, and Mg) (Bjarnadotti, 2015; Bystricka, *et al.,* 2015; Praja and Hedge, 2018). *Daucus carota* contains phytochemicals such as phenolic acid, flavone and flavonols (Alarcón-Flores, *et al.,* 2015). Fruit vegetable juice from *Daucus carota* has been shown to up-regulate mRNA levels in the brain (Yuan *et al.,* 2017) and protect tissues against toxins (Nayeem, *et al.,* 2010; Lee, *et al.,* 2011; El-masry and El-rhman, 2017). Praja and Hedge (2018) reported the cytotoxic, antioxidant, anti-diabetic, antimicrobial, gastro-protective, nephron-protective, cardio-protective, and anti-inflammatory effects of *Daucus carota*.

The primary targets of arsenic toxicity are the gastrointestinal tract, the heart, the brain and kidneys (Benedetti, 1996), Chronic exposure to arsenic may be deleterious to the developing brain (Tyler and Allan, 2014; Sodini, 2015) with the cerebellum highly vulnerable to such intoxication and poisoning (Manto, *et al.,* 2012). Humans and animals cannot survive without water, however, consumption of high amounts of arsenic or small amounts in water over a long period of time exposes man to a wide range of diseases and affects the developing cerebellum (Ding, *et al.,* 2013). This study therefore, evaluated the protective effects of aqueous extracts of *Darcus carota* on arsenic-induced oxidative stress in the postnatal developing cerebellum of rats.

MATERIALS AND METHODS

Drug/Plant materials: Fresh *Daucus carota* (carrots) were bought from Bodija market, Bodija, Ibadan and identified at the Forestry Research Institute of Nigeria (FRIN), Forestry Hill, Jericho, Ibadan with FHI: 112306. Standard vitamin C supplement, batch number 20VCII, manufactured by Nuel Pharm Ltd, Owode-Egba, Ogun State, Nigeria, was purchased from a reputable Pharmarcy in Ibadan. Sodium Arsenite, 10.1g of 98% produced by Qualigens (Shanon Co. Clare, Ireland) was bought from Ad-Folak scientific limited, Ibadan, Nigeria.

Procedure for aqueous extraction of *Daucus Carota*: The samples of *Daucus carota* (carrot) was washed with clean tap water and chopped into pieces. The chopped pieces (2.170kg) was blended using a laboratory electric blender Warring commercial blender, USA, sieved using muslin clothe and filtered. The filtrate was concentrated using a rotary evaporator at 40°C. The concentrate was air-dried in a glass desiccator in order to have the extract in a paste-like form. The final weight was determined as 80.50g.

Animals: Twenty-five (25) female albino Wistar rats, weighing 110-200g, were purchased from the Central Animal House of the College of Medicine, University of Ibadan. The animals were housed in the animal house of the Department of Veterinary Physiology and Biochemistry, University of Ibadan and acclimatised (12 hours light/dark cycle) in well-ventilated cages for two weeks with access to clean tap water and rat chow *ad libitum*. The rats were mated and pregnancy confirmed by the presence of vaginal plug and smear, and this was then regarded as the first day of gestation. All animals received humane care according to criteria outlined in the Guide for the Care and Use of Laboratory Animals published by the US Department of Health and Human Services, Washington (PHS, 1996).

Experimental design: The pregnant rats were divided into five groups (n=5) rats as follows; I- Rats received distilled water and served as the control group; II- Rats received 10% of LD50 (41mg/kg) of arsenic as sodium arsenite daily (Bashir *et al.,* 2006); III: Rats received 200mg/kg/body weight of aqueous extract of *Daucus carota* daily (Sodimbaku *et al.,* 2016); IV- Rats received aqueous extract of *Daucus carota* 200mg/kg per body weight and 10% of LD_{50} (41mg/kg) of arsenic daily; V-Rats received 100 mg/kg of Vitamin C (Okey and Ayo, 2015) and 10% of LD_{50} (41mg/kg) of arsenic daily.

Extracts of vitamin C was administered one hour before the administration of arsenic. All treatments were orally done using oral cannula from the first day of pregnancy to postnatal day 21.

Gross morphological studies: After birth, the body weights of days 1, 7, 14, 21 and 28 pups was recorded and the pups, sacrificed.

Neurobehavioural Tests

On day 21 for each group, forelimb grip strength and negative geotaxis tests were done.

Forelimb grip strength test: This test involves the forepaws of the rats being placed on a horizontally suspended metal wire (measuring 2 mm in diameter and 1m in length), placed one meter above a landing area filled with soft bedding. The length of time each rat was able to stay suspended before falling off the wire was recorded with a stopwatch. A maximum time of 2 minutes was given to each rat after which it was removed. This test reflects muscular strength in the animals (Tamashiro *et al*., 2000).

Negative geotaxis: The unlearned response to gravitational cues is referred to as negative geotaxis (Mortz and Alberts, 2005). This test was done to measure balance/equilibrium. Pups of day 21 were placed head down on a 45° (forty-five degrees) inclined plane and then observed for the time taken to orient in a head-up direction. Time taken was recorded using a stopwatch.

Sacrifice of animals and collection of samples: Pups of days 1, 7, 14, 21 and 28 from the various groups were sacrificed by quick cervical dislocation, and the brain dissected out and weighed. Some of the cerebella of day 21 pups were preserved in phosphate-buffered saline (PBS) at 4^{O}C and at pH 7.4 for oxidative stress and antioxidant assay, while others of days 1, 7, 14, 21 and 28 pups were fixed in 10% formol-saline for histomorphological and immunohistochemical (day 21pups) evaluations.

Oxidative stress and Antioxidant assay: The cerebella of the control and treated day 21 pups were homogenized in eight volumes of 50 mM of Tris-HCl buffer (pH 7.4) containing 1.15% potassium chloride. The homogenate was, centrifuged at 10,000 g for 15 minutes at 4 ºC. The supernatant collected from the centrifugation was used for the estimation of Malondialdehyde (MDA), Reduced Glutathione (GSH), Superoxide dismutase (SOD), Catalase (CAT) and Glutathione peroxidase (GPx). The level of MDA determined lipid peroxidation according to the method described by Varshney and Kale (1990), Reduced glutathione was determined at 412 nm in a colorimeter using the method described by Beutler et al., (1963). Catalase activity (CAT) was measured spectrophotometrically by the method of Claiborne (1985). Superoxide dismutase (SOD) activity was determined by the method of Misra and Fridovich (1972) and Glutathione peroxidase (GPx) activity was measured according to the procedure of Rotruck et al., (1973) with some modifications.

Histological preparations: Cerebellar tissues from the pups of all groups were fixed in 10% formo-saline, processed employing routine paraffin embedding and stained with Haematoxylin and Eosin for histological evaluation. The slides were examined and evaluated under a 500-pixel Leica digital binocular microscope.

The following parameters were evaluated in the cerebellar cortex; thickness of the external granular layer (EGL) and molecular layer (ML), and Purkinje cell density and astrocyte population using the computer software, image-j.

Immunohistochemistry: Cerebellar tissues were immunostained with Glial fibrillary acidic protein (GFAP) for astrocyte population (neuroglia) using the Avidin biotin immunoperoxidase method. Briefly, cut formalin-fixed paraffin sections were treated with 3% hydrogen peroxide (H_2O_2) for 15min, to block endogenous peroxidase. Then, washed in phosphate-buffered saline (PBS) and treated with GFAP primary antibody (GFAP, mouse monoclonal antibody 1:100 dilution, Leica Biosystems Inc. Ilinois, USA) at room temperature for 60min. The sections were washed in 3 changes of PBS for 5min each, incubated with horseradish peroxidase (HRP) secondary biotinylated anti-mouse antibodies and washed in 3 changes of PBS for 5mins. The sections were then incubated with diaminobenzidine (DAB) for 3 to 5min and counterstained with Haematoxylin solution for 2 mins and blued briefly. Sections were dehydrated in alcohol, cleared in xylene and mounted in DPX. Images were captured from the cerebellar cortex with a 500-pixel Leica binocular microscope. Astrocyte population was counted using the software, image-j.

Statistical analysis: Data collected was further analysed as mean±SEM employing the one-way analysis of variance (ANOVA) followed by Tukey Posthoc for multiple comparisons using the GraphPad prism 6.0 at $p<0.05$.

RESULTS

Effect of treatment on Body weight: There was no significant difference in the mean body weight of all treated groups compared with the control group at $p>0.05$ (Table 1).

Effect of treatment on Brain Weight: There was no significant difference in the mean brain weight of all treated groups compared with the control group at $p>0.05$ (Table 2).

Table 1: Effect of treatment on body weight (g) of pups of the control and treated groups

Group	Day 1	Day 7	Day 14	Day 21	Day 28
Control	7.50±0.32	8.78±0.27	14.54±0.31	25.44±0.69	30.08±0.42
As	6.78±0.29	8.26±0.16	14.66±0.60	25.26±1.44	28.36±0.74
Dc	6.88±0.35	8.52±0.29	14.98±0.57	23.04±0.52	28.64±0.97
DcAs	7.00±0.27	9.02±0.31	14.52±0.80	24.38±0.70	28.90±0.67
VcAs	6.76±0.24	8.18±0.19	14.58±0.64	23.30±0.51	30.30±0.36

Values (n=5) are expressed as Mean±SEM As- Arsenic, Dc- Daucus carota, DcAs- Daucus carota+Arsenic, VcAs- vitamin C+Arsenic. The result showed that arsenic administered at 41mg/kg did not significantly affect the body weight of the treated pups studied, p>0.05.

Table 2: Effect of treatment on body weight (g) of pups of the control and treated groups

Group	Day 1	Day 7	Day 14	Day 21	Day 28
Control	0.25±0.02	0.68±0.01	1.13±0.05	1.26±0.04	1.46±0.03
As	0.23±0.01	0.65±0.03	1.08±0.06	1.24±0.04	1.44±0.03
Dc	0.23±0.01	0.65±0.02	1.11±0.05	1.23±0.05	1.36±0.02
DcAs	0.25±0.02	0.66±0.01	1.08±0.07	1.24±0.04	1.39±0.02
VcAs	0.25±0.02	0.63±0.01	1.13±0.05	1.21±0.04	1.43±0.02

Values (n=5) are expressed as Mean±SEM As- Arsenic, Dc- Daucus carota, DcAs- Daucus carota+Arsenic, VcAs- vitamin C+Arsenic. There was progressive increase in the brain weight of the pups, however, exposure to 41mg/kg arsenic did not significantly affect the brain weight of control and treated groups, p>0.05.

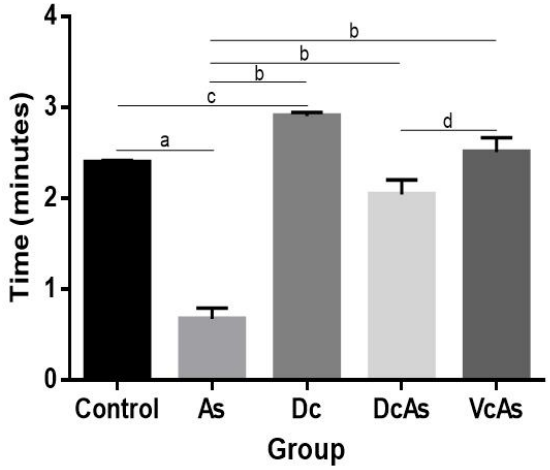


Figure 1:
Forelimb grip strength test. Values (n=8) are expressed as mean±SEM, As- arsenic, Dc- *Daucus carota*, DcAs- *D. carota* + arsenic, VcAs- vitamin C + arsenic. Arsenic, administered at 41mg/kg decreased the forelimb grip strength, while Dc extract and Vc increased the forelimb grip strength.[a]p<0.05 vs control; [b]p<0.05 vs As; [c]p<0.05 vs Dc; [d]p<0.05 vs VcAs.

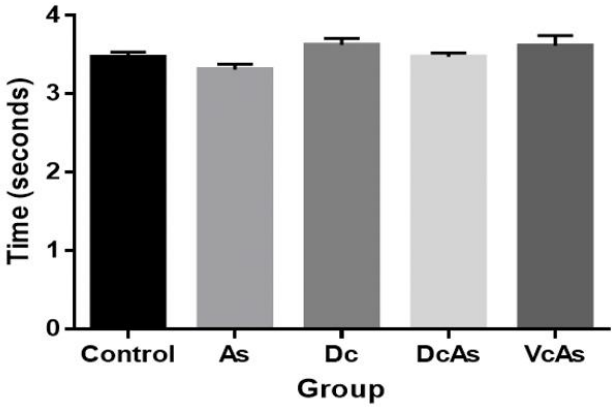


Figure 2: Negative geotaxis in seconds of pups of day 21. Values (n=8) are expressed as mean±SEM, As- arsenic, Dc- *Daucus carota*, DcAs- *D. carota*+arsenic, VcAs- vitamin C+arsenic. Arsenic, administered at 41mg/kg arsenic did not significantly affect negative geotaxis in control and treated groups at the level of this study, p>0.05.

Table 3:
Oxidative stress markers and antioxidants in the cerebellum of the control and treated pups on day 21

Group	LPO µmol/mg tissue	GSH (µg/mg tissue)	CAT (Unit/mg tissue)	SOD Unit/mg tissue	GPx µg/mg tissue
Control	0.18±0.01	0.81±0.03	0.38±0.01	0.70±0.05	44.72±0.60
As	0.29±0.01[a]	0.41±0.04[a]	0.21±0.02[a]	0.46±0.05[a]	31.54±3.37[a]
Dc	0.15±0.02[b]	0.74±0.04[b]	0.32±0.02[b]	0.68±0.01[b]	43.04±2.50[b]
DcAs	0.16±0.01[b]	0.66±0.02[b]	0.31±0.03[b]	0.61±0.01[b]	38.96±2.74
VcAs	0.18±0.01[b]	0.66±0.06[b]	0.26±0.01[a]	0.58±0.01	40.40±1.02

Values (n=5) are expressed as Mean±SEM As- Arsenic, Dc- Daucus carota, DcAs- Daucus carota+Arsenic, VcAs- vitamin C+Arsenic. Arsenic exposure at 41mg/kg significantly caused increased lipid peroxidation, decreased GSH concentration as well as SOD, CAT and GPx activities. Daucus carota and Vc reversed the oxidative marker and antioxidants evaluated, [a]p<0.05 vs control; [b]p<0.05 vs As.

Effect of treatment on forelimb grip strength test: A shorter time was spent in the forelimb grip strength by the arsenic-treated pups on day 21 compared with the control and other treated rats at p<0.05 (Figure 1).

Effect of treatment on Negative geotaxis: The time (in seconds) taken for the pups of day 21to turn against gravity on an inclined (45°) flat surface was not significantly different at p>0.05 in both the control and treated groups (Figure 2).

Effect of treatment on Oxidative stress markers and antioxidant assays: A significant increase in lipid peroxidation and decrease in glutathione, catalase, superoxide dismutase and glutathione peroxidase was seen in the developing cerebellum of arsenic-treated pups on day 21 compared with the control and other treated groups at p<0.05. Catalase activity was significantly decreased in Vc+As-treated group compared with the control pups on day 21 at p<0.05 (Table 3).

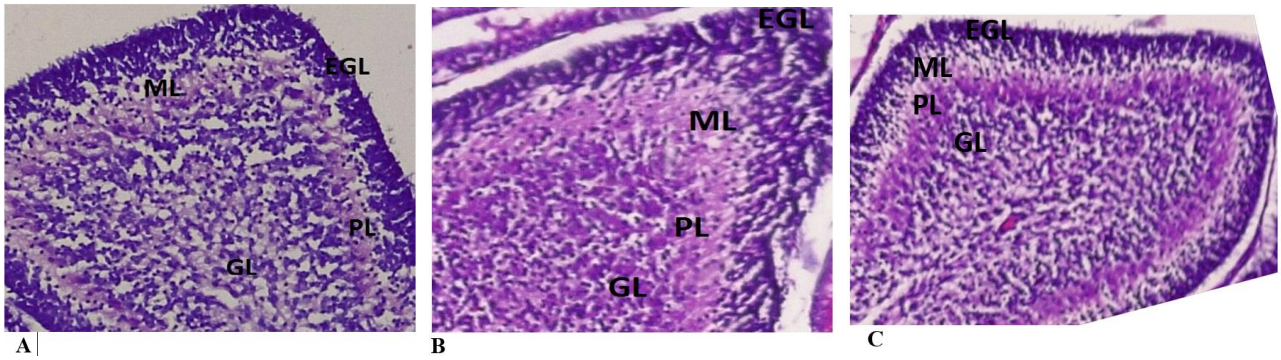
Microscopic observations of the cerebellar cortex Histological analysis: There was no significant difference in the thickness of the external granular layer (EGL) of the cerebellar cortex in the control and treated rat pups on postnatal day 7 at p>0.05. However, a significantly increased EGL thickness was seen in arsenic-treated pups on day 14 compared with the control and *Daucus carota* groups at p<0.05 (Table 4, Plates 1 and 2).

Table 4:
Effect of treatment on the thickness of the external granular layer of the cerebellar cortex on postnatal day 7 and 14.

Group	Day 7 EGL (µm)	Day 14 EGL (µm)
Control	86.00 ±16.31	24.00±2.45
As	80.00 ±4.47	42.00±7.35[a]
Dc	80.00 ±15.17	24.00±2.43[b]
DcAs	69.00 ±5.83	34.00±2.49
VcAs	68.00 ±18.44	30.00±2.41

Values (n=5) are expressed as mean ± SEM. EGL- External Granular Layer, As- arsenic, Dc- Daucus carota, DcAs- D. carota + arsenic, VcAs- vitamin C + arsenic. [a]p<0.05 vs control; [b]p<0.05 vs As.

Effect of treatment on cerebellar cortex of day 21 pups: Employing routine paraffin-embedding, H and E staining technique, the cerebellar cortex of day 21 pups of the control and treated rat pups showed the normal cytoarchitecture of Molecular layer (ML), Purkinje layer (PL) and Granule layer (GL). However, there was persistent 2-3 cell layer thick external granular layer (EGL) in the cerebellar cortex of the As-treated, and one cell layer thick in the DcAs and VcAs pups compared with the control on day 21 (Plate 3).



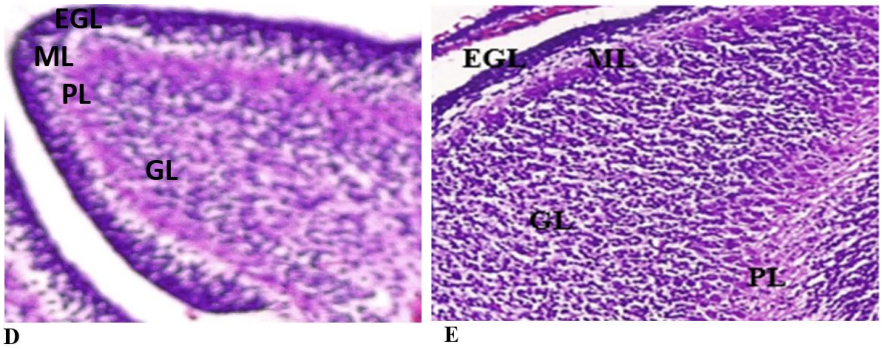


Plate 1:
Photomicrographs of the cerebellar cortex of day 7 pups H&E ×100. (A): Control, (B): As, (C): Dc group, (D): DcAs group, (E): VcAs group. EGL- external granular layer; ML- molecular layer; PL- Purkinje cell layer; GL- granular layer. As- arsenic, Dc- *Daucus carota*, DcAs- *D. carota* + arsenic, VcAs- vitamin C + arsenic.

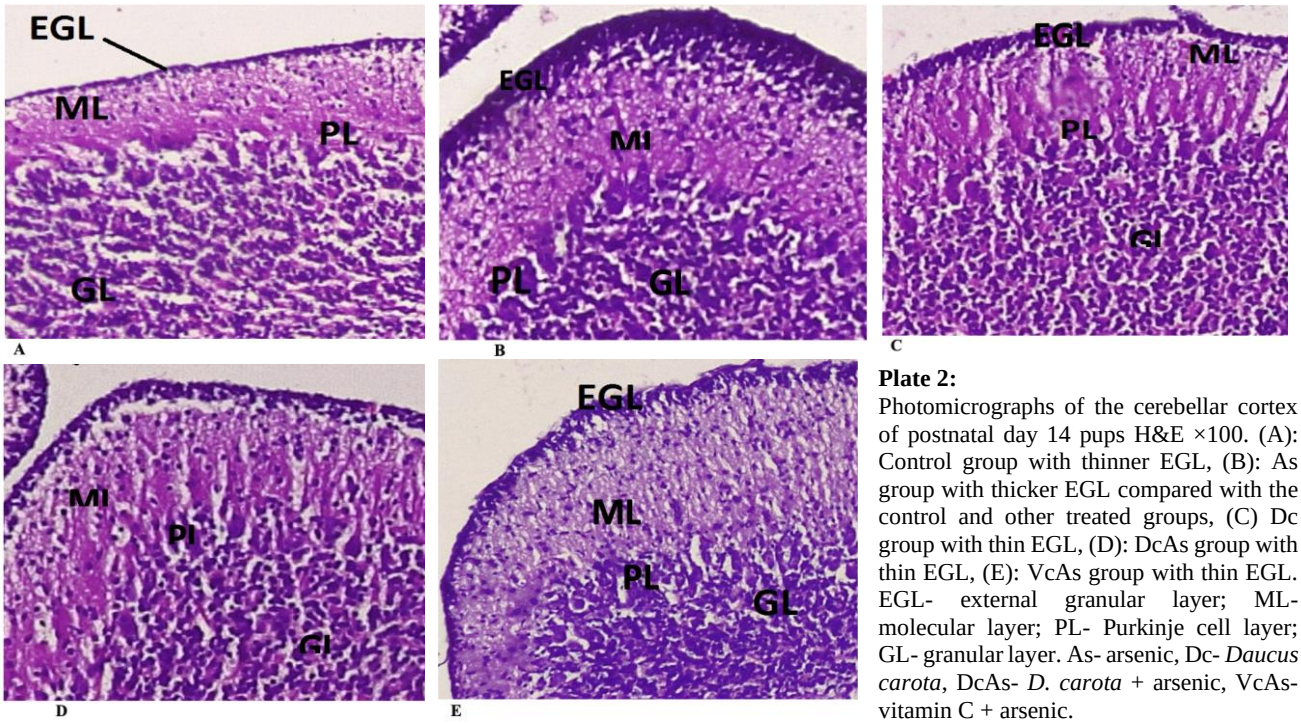


Plate 2:
Photomicrographs of the cerebellar cortex of postnatal day 14 pups H&E ×100. (A): Control group with thinner EGL, (B): As group with thicker EGL compared with the control and other treated groups, (C) Dc group with thin EGL, (D): DcAs group with thin EGL, (E): VcAs group with thin EGL. EGL- external granular layer; ML- molecular layer; PL- Purkinje cell layer; GL- granular layer. As- arsenic, Dc- *Daucus carota*, DcAs- *D. carota* + arsenic, VcAs- vitamin C + arsenic.

Effect of treatment on the thickness of the molecular layer and Purkinje cell density of the cerebellar cortex on postnatal day 28: A significant decrease in the thickness of the molecular layer (ML) and density of the Purkinje cells of the cerebellar cortex was observed in the As, DcAs and VcAs groups on postnatal day 28 compared with the control- and Dc-treated rat pups at $p<0.05$. However, a significant increase in the thickness of the molecular layer (ML) and density of the Purkinje cells of the cerebellar cortex was seen in the Dc and DcAs groups compared with the As-treated pups on day 28 at $p<0.05$ (Table 5, Plate 4).

Table 5:
Effect of treatment on the thickness of the Molecular layer and Purkinje cell density of the cerebellar cortex in the control and treated rats on postnatal day 28.

Group	ML thickness (μm)	Pc density/1.3mm
Control	280.80±4.24	41.00±1.00
As	215.20±3.18[a]	18.80±1.53[a]
Dc	256.20±5.37[b]	32.80±3.71[b]
DcAs	246.30±8.43[ab]	29.20±2.71[ab]
VcAs	229.80±7.83[a]	27.00±2.35[a]

Values (n=5) are expressed as mean±SEM, ML- Molecular Layer, Pc- Purkinje cell, As- arsenic, Dc- Daucus carota, DcAs- D. carota + arsenic, VcAs- vitamin C + arsenic. [a]p<0.05 vs control; [b]p<0.05 vs As.

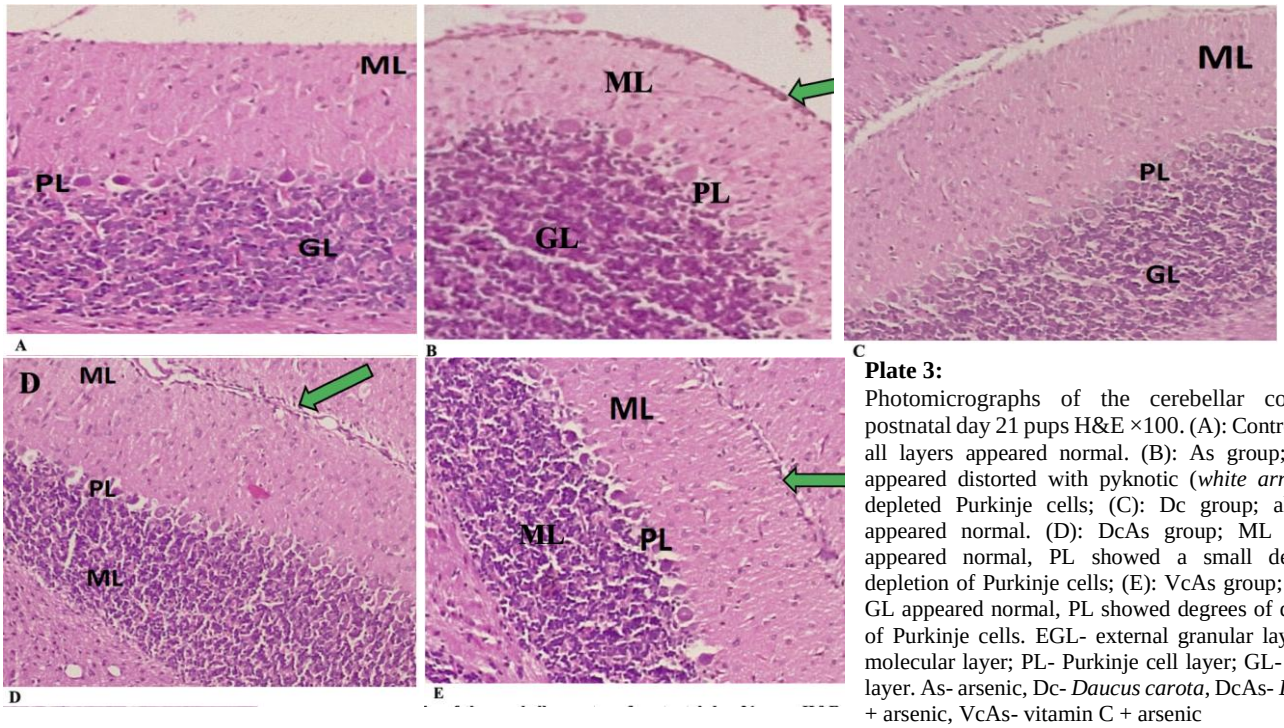


Plate 3:
Photomicrographs of the cerebellar cortex of postnatal day 21 pups H&E ×100. (A): Control group; all layers appeared normal. (B): As group; the PL appeared distorted with pyknotic (*white arrow)* and depleted Purkinje cells; (C): Dc group; all layers appeared normal. (D): DcAs group; ML and GL appeared normal, PL showed a small degree of depletion of Purkinje cells; (E): VcAs group; ML and GL appeared normal, PL showed degrees of depletion of Purkinje cells. EGL- external granular layer; ML- molecular layer; PL- Purkinje cell layer; GL- granular layer. As- arsenic, Dc- *Daucus carota*, DcAs- *D. carota* + arsenic, VcAs- vitamin C + arsenic

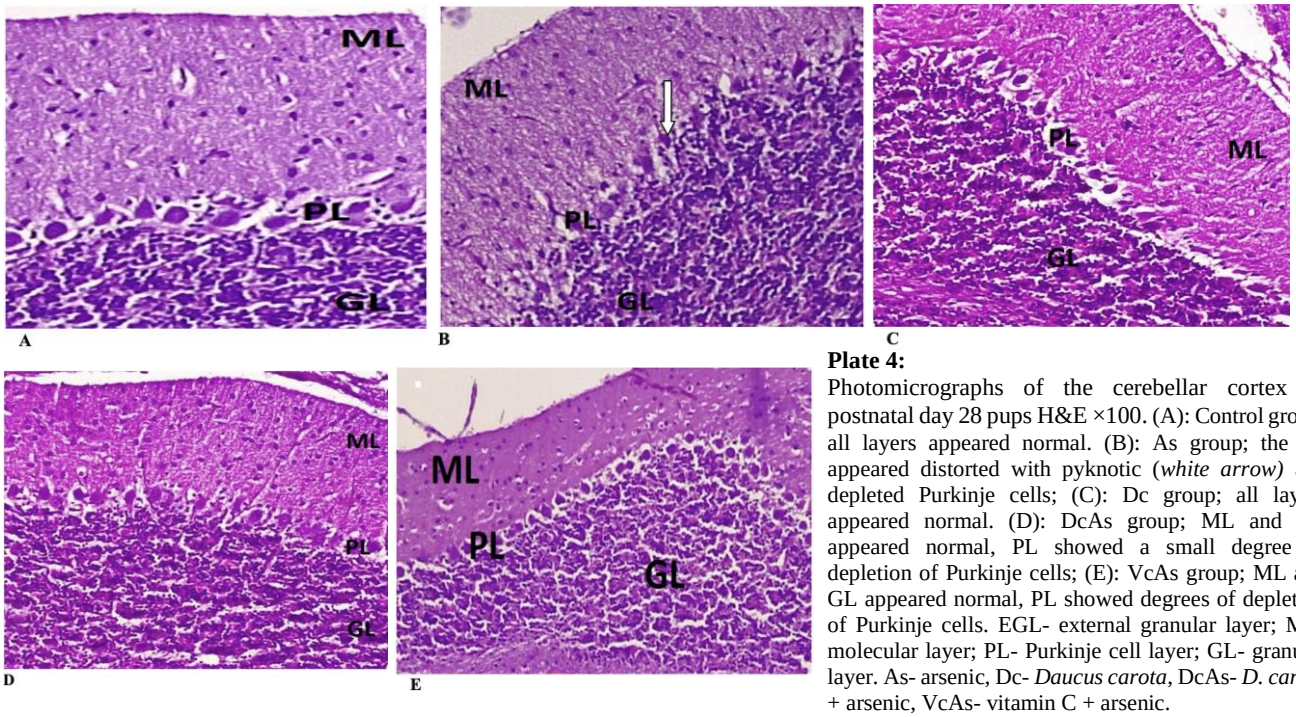


Plate 4:
Photomicrographs of the cerebellar cortex of postnatal day 28 pups H&E ×100. (A): Control group; all layers appeared normal. (B): As group; the PL appeared distorted with pyknotic (*white arrow)* and depleted Purkinje cells; (C): Dc group; all layers appeared normal. (D): DcAs group; ML and GL appeared normal, PL showed a small degree of depletion of Purkinje cells; (E): VcAs group; ML and GL appeared normal, PL showed degrees of depletion of Purkinje cells. EGL- external granular layer; ML- molecular layer; PL- Purkinje cell layer; GL- granular layer. As- arsenic, Dc- *Daucus carota*, DcAs- *D. carota* + arsenic, VcAs- vitamin C + arsenic.

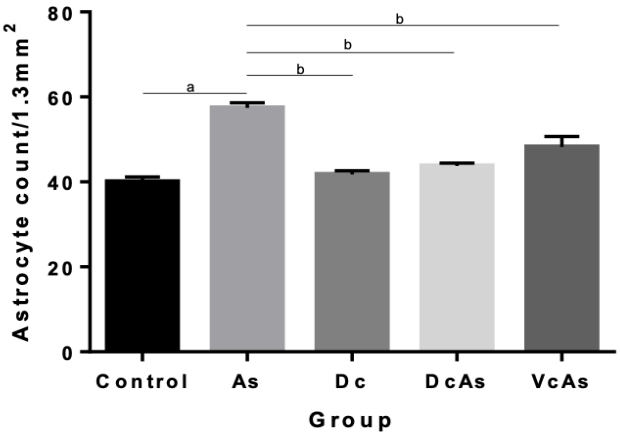


Figure 3:
Astrocyte population in the cerebellar cortex of day 21 pups. Values (n=5) are expressed as mean±SEM, As- arsenic, Dc- *Daucus carota*, DcAs- *D. carota*+arsenic, VcAs- vitamin C+arsenic. [a]p<0.05 vs control; bp<0.05 vs As

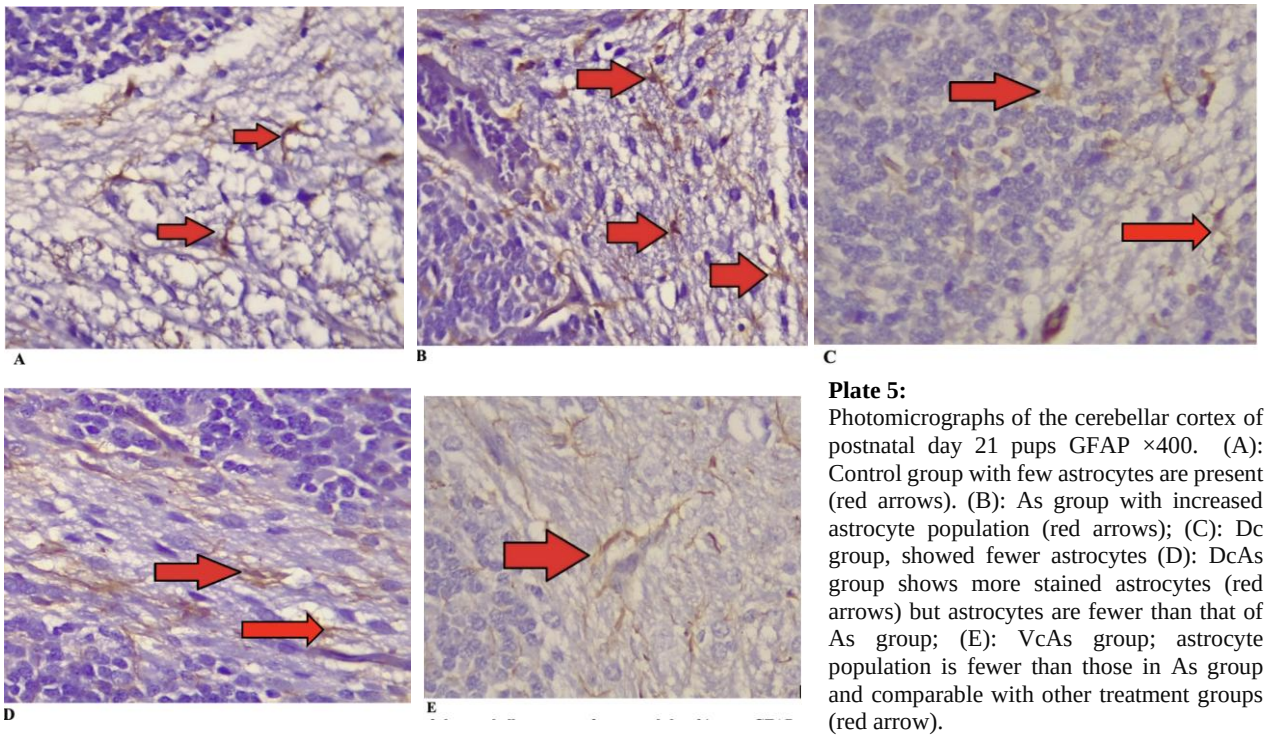


Plate 5:
Photomicrographs of the cerebellar cortex of postnatal day 21 pups GFAP ×400. (A): Control group with few astrocytes are present (red arrows). (B): As group with increased astrocyte population (red arrows); (C): Dc group, showed fewer astrocytes (D): DcAs group shows more stained astrocytes (red arrows) but astrocytes are fewer than that of As group; (E): VcAs group; astrocyte population is fewer than those in As group and comparable with other treatment groups (red arrow).

Effect of treatment on Astrocyte Count on Postnatal Day 21: There was significant increase in astrocyte population in the cerebellar cortex of the As-treated pups compared with the control and other treated pups on day 28 at $p<0.05$ (Figure 3 and Plate 5).

DISCUSSION

In this study, the protective effects of *Darcus carota* (Dc) on arsenic-induced neurotoxicity on the postnatal developing cerebellum was observed. The results from our study showed that the body and brain weight of the control and treated rat pups was not significantly affected. These findings are in agreement with the reports of Grosicki and Kowalski (2006); Potter *et al*. (2011). Experimental studies have linked the toxic effect of arsenic to a decrease in body and organ weight (Avani and Rao, 2009; Kozul-Horvath *et al.*, 2012; Negishi *et al.*, 2013; Tolins *et al.*, 2014; Barai *et al.*, 2017). Herrera *et al.* (2013) observed that there was no decrease in body or organ weight in perinatal arsenic-treated pups. Other researchers have also supported that body and organ weight are unaffected in pups whose dams were prenatally and or perinatally exposed to arsenic (Gandhi *et al.*, 2011; Ince *et al.*, 2012; Aung *et al.*, 2016). Reduction in body weight has been reported to be more in adult rats than in pups (Bikashvili *et al.*, 2017) and differences observed may be as a result of a diet which can reduce the effect of arsenic toxicity (UNICEF-WHO, 2018).

Neurobehavioural evaluations showed decreased forelimb grip strength, an indication of decreased muscular strength and tone in the arsenic-treated group resulting in muscular weakness. Muscle coordination and orientation, a function of negative geotaxis involving the re-orientation against gravity was not significantly affected. Arsenic toxicity has been shown to affect the development of the cerebellum (Ding *et al.*, 2013), affecting smooth and skilful movements, balance, emotion and cognition (Buckner 2013; Balaei *et al.*, 2017). Research has shown that oxidative stress induces generation of ROS which causes increase in protein loss, reduction of force generation and increased muscle atrophy (Gumucio and Mendias, 2013). Increased muscular strength and tone observed in the group administered Dc and vitamin C may be attributed to their antioxidant and protective properties.

Oxidative stress occurs as a result of a distressing imbalance in the production of reactive oxygen species and antioxidant activity (Betteridge 2000; Halliwell and Whiteman, 2004). It has been proposed that oxidative stress may be the mechanism by which arsenic toxicity exerts its effects (Tolins *et al.*, 2014) and the cerebellum is susceptible to oxidative damage especially during prenatal and perinatal periods (Lopez *et al.*, 2009). The elevated level of MDA and reduction of GSH concentration as well as SOD, CAT and GPx activities in the developing cerebellum of arsenic-treated day 21 pups indicated oxidative stress. Cerebellar tissue is known to be highly sensitive to damage by free radicals because of its high concentration of polyunsaturated fatty acids, low concentration of cytosolic antioxidants and high use of oxygen (Ebokaiwe et al., 2013). The results indicated that arsenic induced oxidative stress in the brain of the arsenic exposed pups. These findings are similar to those of other researchers (Rao and Avani, 2004; Bashir *et al.*, 2006; Ince *et al.*, 2012; Herrera *et al.*, 2013; Mundey *et al.*, 2013; Negishi *et al.*, 2013; Bonetto *et al.*, 2017; Khodayar *et al.*, 2019). Antioxidants have the ability of reducing free radicals and preventing oxidation. Day 21 pups administered extracts of Dc and vitamin C had reduced MDA levels and elevated GSH concentration as well as SOD, CAT and GPx activities suggesting antioxidant activity of Dc, which helped in mitigating the oxidative damage induced by arsenic toxicity in the developing rat cerebellum. The results suggested that Dc demonstrated ameliorative effect against lipid peroxidation probably due

to the high antioxidant activity associated with its high phenolic content (Dada et al., 2017).

Neurons and astrocytes expend a high amount of oxygen in carrying out their function (Gandhi and Abramov, 2012) and may be the major target of arsenic neurotoxicity (Piao *et al.,* 2005; Ma *et al.,* 2010). These may be the reasons behind the histomorphometric observations found in this study. The developing cerebellar external granular layer (EGL) on postnatal day 14 arsenic-treated pups was found to be significantly thicker than that of the control group, although the difference was not significant on postnatal day 7. Ding *et al.* (2013) reported a decreased thickness of the cerebellar EGL on postnatal days 10 and 13 rats treated with arsenic. The thicker EGL observed in the arsenic-treated pups on day 14 may be due to delayed differentiation of the EGL because the EGL is a highly metabolic layer which disappears in humans at approximately two years of age and in mouse, at about postnatal days 20 (Hatten and Heintz, 1995; Altman and Bayer, 1985; Imosemi and Osinubi, 2011; Butts *et al.,* 2014). The external granular layer (EGL) consists of highly metabolic cells whose differentiations result in the synthesis of the outer stellate, basket, Golgi type II and granule cells of the cerebellar cortex. In this study, there was complete disappearance of EGL in the control and other treated groups on day 21 pups but 2-3 layers thick of EGL persisted in arsenic-treated group. The mechanism for the persistent EGL in the arsenic-treated group is not clearly understood but delayed maturation of cells of the EGL have been reported by some workers (Malomo et al., 2004; Imosemi and Osinubi, 2011) which may be due to the oxidative stress induced by arsenic. The molecular layer (ML) of the cerebellar cortex is usually smaller than the other cortical areas in early development but shows a rapid increase between postnatal days 9-25. It becomes the most superficial layer when the external granular layer disappears (Heinsen 1977). There are numerous synapses of the Purkinje cells (Pc) with basket and stellate cells in the ML layer (Balaei, *et al.,* 2017). In the present study, it was observed that arsenic significantly decreased the thickness of the ML in pups of day 28. Ding *et al.,* (2013) reported that arsenic cause condensation, vacuolation and nuclear fragmentation in the ML, causing reduction in the thickness of the ML. The Purkinje cells of the cerebellum serve as the sole output neuron of the cerebellar cortex, therefore they are highly important (McKay and Turner, 2005; Balaei, *et al.,* 2017) and are easily affected by genetic and environmental insults (Minai, 2014). It was observed in this study, that the number of Purkinje cells was significantly reduced in the arsenic-treated pups on day 28. The mechanism involved in the loss of Purkinje cells is not completely clear but arsenic has been reported to generate ROS which inhibits the activity of antioxidant enzymes in some tissues, inducing oxidative stress and causing cell deaths. Co-administration of extracts of Dc and vitamin C with arsenic increased the ML thickness and density of the Pc count, probably by its free radical scavenging activity and preventing further oxidative damage of the ML and Pc of the developing cerebellum.

Astrocytes play an important role in protecting the blood brain barrier and they also provide biochemical support to other neurons (Tolins, *et al.,* 2014). In this study, arsenic toxicity caused an increase in astrocyte population probably by the generation of free radicals in the developing cerebellum on postnatal day 28 resulting in astrogliosis. Cantanzaro *et al.* (2010) and Tolins *et al.* (2014) reported that arsenic exposure to rats increased apoptosis in primary culture of astrocytes. Research has shown that oxidative stress can up-regulate the expression of glial fibrillary acidic protein (GFAP) and ROS in astrocytes thereby promoting excessive reactive astrogliosis and glial scar formation (Sofroniew, 2009). *Daucus carota* containing phenolic compounds and flavonoids, and vitamin C reduced astrogliosis probably by scavenging the free radicals generated by arsenic toxicity.

From our results, perinatal arsenic exposure induced oxidative stress, affected forelimb grip and caused morphological changes (delayed maturation and differentiation of the EGL, reduced thickness of the ML, loss of Pc and astolgliosis) in the developing cerebellum of rats, and that extracts of *Daucus carota* and Vitamin C reduced the rate at which arsenic induced oxidative stress as such may be a potential neuroprotective agent.

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