**Effects of aqueous extract of kolanut (*Cola nitida*) on Sprague Dawley dams and exposure on the hippocampus of the progeny**

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**A research proposal presented to the School of Anatomical Sciences, University of the Witwatersrand, as the requirement towards the fulfilment of the Degree of Doctor of Philosophy (PhD) in Anatomical Sciences.**

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## DEDICATION

This research is dedicated to my children, Adebola, Adetola, Adeoluwa and to all pregnant women.

## ACKNOWLEDGEMENTS

To You oh God, be all the glory and honor. Thank you for life! The journey that started in 2019 August plus 10 months of COVID has come to a successful end. It could only have been you, Lord! I ascribe greatness to you, the keeper of my soul and my strength. Yes, Lord another phase has ended, may you continue to guide me forever.

My profound gratitude goes to my supervisors; Professor Amadi Ihunwo and Professor Felix Mbajiorgu. Thank you, Sirs, for your constructive criticism, advice, support, and guidance in bringing this work to completion. God bless you Sirs.

My appreciation goes to School of Anatomical Sciences, University of the Witwatersrand, Johannesburg, South Africa for providing me an enabling environment and great opportunity to carry out this study. To all the staff, thank you for the encouragement and support. Mrs. Alli (Mrs. A) and Brady, Mr. Jacob, and Tony, thank you for going the extra miles for me. I appreciate other members of the teaching and non-teaching staff for your support and encouragement.

To my funders, Consortium for Advanced Research Teaching in Africa (CARTA) and Staff Needs Assessments, University of Ibadan. I could not have done this without your support and financial backing. Thank you for creating a good platform and being the shoulders upon which I stand.

To my mentors, Professors V.O. Adetimirin and Adefolarin Malomo, thank you for the encouragement, support and numerous times of reading my thesis.

My sincere appreciation goes to my siblings, Mr. I. A., and Princess O. A. Salawu, Princess Dolapo Oluniyi, Eng. and Mrs. O. D. Adekoya, my niblings and the rest of the royal family. Thank you for the support, encouragement, and prayers, especially during the difficult times. I appreciate you all. Maami, Queen Victoria Aduke Alade (Wura), thank you for the prayers and support. You are indeed a real mother, abiyamo tooto.

Finally, to my wonderful children Adebola, Adetola and Adeoluwa (Baba), thank you for your understanding, prayers, and reporting me to the right quarters once you noticed I was slacking. Thank you for your encouraging words and finally the work is done! Your mama is **Ph**innishe**D**. I pray that you shall be greater than us and fulfil destinies. I love you my angels and forever proud of you.

## ABSTRACT

**Background:** Kolanut, a tropical nut eaten by people across sub-Sahara Africa, contains caffeine, theobromine, catechins, and tannins. Pregnant women often eat it to suppress morning sickness. This study investigated the effects of kolanut on the structure and functions of the developing hippocampus.

**Methods:** Kolanut extract at 400 mg/kg of body weight infused in gel cubes was fed to 6 female and gel cubes without kolanut to control 6 female *Sprague Dawley* rats from the first day of mating till parturition. Several behavioral tests were administered on the pups namely surface righting (SR), cliff avoidance (CA) across different age group, post-natal day (PND) 4, 5, 6 & 7, head rising, head pivoting, locomotion, open field, novel object recognition (NOR) and location (NOL), and radial-arm maze (RAM) PND 21 and 56. Their hippocampi were subjected to histology [Nissl, Golgi-cox], immunohistochemistry (Ki67 and DCX), biochemistry [malondialdehyde (MDA), superoxide dismutase (SOD), reduced glutathione (GSH), glutathione peroxidase (GPX), transcription factors [brain-derived neurotrophic factor (BDNF), and acetylcholine (ACh)]. Expression of immediate (*c-fos* & *c-jun)* and memory genes (*dlg3*, *dlg4*, *creb1* & *creb2)* were also determined.

**Results:** Difficulty to get pregnant was observed in 33.3% of the dams; 16.7% had a still birth while 4.0% of the pups from dams fed kolanut-treated diet had paralysis of the limb. Kolanut significantly reduced the body weight ($p < 0.001$ – $p < 0.0001$) and increased brain weight, especially at PNDs 56 & 70 ($p < 0.001$ – $p < 0.0001$) of the dams and pups compared to the control. Food consumption was significantly ($p < 0.05$) lower for dams on kolanut-treated diet, but their water intake was significantly higher ($p < 0.001$). Kolanut significantly affected the behavioral indices of the animals; it significantly increased the latency of CA and SR tests across age groups ($p <0.0001$ and $p < 0.0001$ respectively). The frequency of head rising, frequency of pivoting and its latency, and locomotion were significantly ($p < 0.01$ – $p < 0.0001$) lower in animals fed kolanut-treated diet. Animals fed kolanut-treated diet showed anxiety during the open field tests on PNDs 21 and 56, and exhibited increased line crossing, corner time, distance covered and velocity ($p < 0.05$ – $p < 0.0001$). Frequencies of freezing episodes, grooming, rearing, fecal bolus and urination were also significantly ($p < 0.01$ – $p < 0.0001$) higher in animals that received kolanut-treated diet. The discrimination ratio of NOR and NOL were significantly

lower in animals fed kolanut-treated diet; they took longer duration to complete the tasks in RAM. Results from Golgi staining showed that kolanut caused pyknosis ($p < 0.0001$), inflamed soma, reduced aborization and synapses ($p < 0.0001$), reduction in spine quantity ($p < 0.0001$), change in morphology, fragmentation and constriction of the dendrites of the hippocampal neurons. When stained with DCX, the hippocampi of animals fed kolanut showed decrease in mean density of neuronal cells ($p < 0.0001$), increase number of pyknosis ($p < 0.0001$) and chromatolytic cells ($p < 0.0001$), neuronal atrophy, clumping, multi-layering and gliosis of the DG cells. In addition, the Ki67 showed a significant loss of proliferating cells ($p < 0.0001$) in the sub-granular zone of the DG. Kolanut significantly ($p= 0.0067$) increased the MDA and glutathione peroxidase levels. BDNF and ACh were significantly ($p = 0.0001$) increased by kolanut. A significant and positive correlation was found between SRT and MDA in PND 7 ($r = 0.99$, $p = 0.0059$), grooming and MDA in PND 56 ($r = 0.77$, $p = 0.0252$), grooming and BDNF in PND 21 ($r = 0.76$, $p = 0.0297$). A significant negative correlation was obtained between rearing and ACh in PND 56 ($r = -0.83$, $p = 0.0115$), Ki67 and MDA ($p = 0.0213$), and DCX cells and BDNF ($p = 0.017$). Prenatal kolanut consumption caused downregulations of *cfos* mRNA and *cjun* mRNA and *creb1*levels, an increase in *creb2* level and a significant reduction in the levels of *dlg3* mRNA and *dlg4* mRNA, thus affecting the dendrites and spine morphology.

**Conclusion:** Prenatal kolanut consumption adversely affected food intake, behavior, neuronal morphology, decreased neurogenesis and neuroplasticity and resulted in downregulation of genes important for normal development of the neurons and synapses. It exerted anti-neuroprotective effects by inducing oxidative stress, altering cholinergic system activity, stimulating over-expression of BDNF protein and concomitantly causing changes in morphology of the hippocampal neurons. Pregnant women and those of reproductive status need to be made aware of the adverse effects of kolanut consumption during pregnancy.

**Keywords:** Kolanut, Hippocampus, MDA, Ach, DCX, Ki67, *cfos*, *cjun*, *dlg*, *creb*

## CERTIFICATION

We certify that this work was carried out by Dr Foluso Ayobami Atiba in the School of Anatomical Sciences, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa.

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## ABBRRVATIONS

| | | |
|---|---|---|
| ACh | - | Acetylcholine |
| ACTB | - | Beta Actin |
| BDNF | - | Brain Derived Neurotrophic Factor |
| BSA | - | Bovine Serum Albumin |
| CA | - | Cliff Aversion |
| CARTA | - | Consortium for Advanced Research Training in Africa |
| CNS | - | Central Nervous System |
| CREB | - | Camp-Response Element Binding Protein |
| DCX | - | Doublecortin protein |
| DLG | - | Disc Large Homologue |
| DTNB | - | 5,5-dithio-bis-(2-nitrobenzoic acid) |
| EC | - | Entorhinal Cortex |
| G | - | Grams |
| GABA | - | Gama-aminobutyric Acid |
| GAPDH | - | Glyceraldehyde-3-Phospate Dehydrogenase |
| GPX | - | Glutathione Peroxidase |
| GSH | - | Reduced Glutathione |
| IGE | - | Independent Gene Expression |
| KG | - | Kilogram |
| LPO | - | Lipid peroxidation |
| MDA | - | Malondialdehyde |
| MIL | - | Milliliters |

| | | |
|---|---|---|
| NBT | - | Nitro Blue Tetrazolium |
| NHP | - | Non-Human Primates |
| NOL | - | Novel Object Location |
| NOR | - | Novel Object Recognition |
| PB | - | Phosphate Buffer |
| PBF | - | Phosphate Buffered Formalin |
| PFC | - | Prefrontal Cortex |
| PND | - | Post Natal Day |
| RAM | - | Radial Arm Maze |
| SFN | - | Society for Neuroscientists |
| SOD | - | Superoxide Dismutase |
| SVZ | - | Subventricular Zone |
| SGZ | - | Subgraular Zone |
| SRT | - | Surface Righting Test |
| TETFUND | - | Tertiary Trust Fund |

# CHAPTER ONE

## 1.1 INTRODUCTION

Despite the lack of research-based evidence on the safety of some substances being consumed during pregnancy, the documented utilization of complementary and alternative medicines (local substances/herbs) during pregnancy to subdue features of morning sickness has continued to rise in different populations and groups (Gibson 2001; Pangle 2006; Onyiapat 2014). The attraction to these alternatives emanates from easy availability at little or no cost compared to the scarcity and exorbitant cost of conventional medication (Ologe et al., 2008). Nigerian pregnant women are no exception in this regard, as they also consume substances during pregnancy that are not part of conventional medicine (Onyiapat, 2014). One of such substances consumed by Nigerian pregnant women is kolanut (*Cola nitida)* (Atiba et al., 2023). It is commonly observed that the consumption of kolanut amongst Nigerian pregnant women is heightened during the early stages of pregnancy, when the embryo is undergoing rapid differentiation of its structures (Yildirim et al., 2016; Atiba et al., 2023), thereby, very susceptible to teratogenic agents. The agents may obstruct/interfere with the migration of various differentiating cell lines at that critical period of development (Navari, 2009; Källén, 2016; Atiba et al., 2020, 2021). Since kolanut is commonly consumed amongst pregnant women, the need to investigate and document the teratogenic effects, if any, and its postnatal effect are invaluable to the total well-being of the individual, both in early life and in adulthood relative to the cognitive function amongst others.

Studies have shown that kolanut is cultivated in sub-Sahara Africa because of its stimulatory function and prevention of thirst and hunger (Ash and Ash, 1995; Burdock, 2005; Burdock et al., 2009). The kola plant is a member of the cacao family. The classification is in transition, in 2003, following the angiosperm phylogeny, it shifted from Sterculiacea to Malvaceae. *Cola acuminata* belongs to Malvaceae family native to tropical Africa, while *Cola nitida* belongs to Sterculiaceae family (Onaolapo and Onaolapo, 2019). *Cola (Vent.) Schott & Endl*. is the genus, and the species is *Cola. nitida* (Vent.) Schott & Endl. The taxonomy of kolanut is presented thus:

**Table 1.1: Taxonomy of Kolanut**

| **Kingdom** | **Plantae** | **Plants** |
|---|---|---|
| Sub-kingdom | Tracheobionta | Vascular plants |
| Super-division | Spermatocyte | Seed plants |
| Division | Magnoliophyta | Flowering plants |
| Class | Magnoliopsida | Dicotyledons |
| Sub-class | Dileniidae | |
| Order | Malvales | |
| Family | Sterculiaceae | Cacao family |
| Genus | Cola Schott & Endl | Cola |
| Species | | *Cola nitida* (Vent.) |

*Source: (Simpson, 2006)*

Its usage has increased over the years to include sports enhancement, weight reduction (Olsen, 2005) and dispelling of fatigue, especially among long-distance drivers and ancient soldiers (Starin 2013a; Tijani and Adetutu 2013). Traditionally, every part of the tree can be prepared into a tonic and used to relieve cough, diarrhea and vomiting (Ayensu, 1978). It is also claimed that the husk can be made into local concoctions to lessen labor pain. The trunk sheath is used in treating bumps, sores and lacerations and the rootstock provides excellent brushing tufts for cleansing the teeth (Tachie-Obeng and Brown, 2004; Starin, 2013). Opeke also reported that its alkaloids are being used by pharmaceutical companies in the production of drugs (Opeke, 1992).

Kolanut also holds an important role in the sociocultural stand in many countries especially, Nigeria. This socio snack that is chewed by both genders, the young and the old, is usually found at important functions and ceremonies. Kolanut stands as a key element in weddings, naming ceremonies and in divination from gods (Tachie-Obeng and Brown, 2004). Oftentimes, the absence of kolanut signifies an incomplete ceremony. It is sometimes used as part of the death rites (Tachie-Obeng and Brown 2004; Starin 2013).

Kolanut is also processed into making drinks like Coca-Cola and beverages (Ash and Ash, 1995; Burdock, 2005), with one nut giving about two big cups of American coffee (Kiple and Ornelas, 2000). It is also present in food condiments and helps in aiding digestion (Ash and Ash, 1995; Burdock, 2005). Also, kolanut can be consumed as a substitute for alcohol by those of Islamic faith in West Africa (Blumenthal, 2000).

However, kolanut is not without its adverse effects in animal models. These include hepatotoxicity, renal toxicity and increased heart rate (Ikegwuonu et al., 1981), gastric ulcer (Ojo et al., 2010), reduction in sperm count and body weight (Obidike et al., 2010; Umoren et al., 2011; Ogunwole et al., 2015), adverse changes in the behavioural and endocrine system (Ettarh et al., 2000; Celec and Behuliak, 2010) with erratic brain sodium pump activity (Obochi et al., 2007c) when taken in excess. Kolanut has also been found to induce oxidative stress via the generation of free radicals in rats (Atiba et al. 2016). Caffeine, one of its constituents, has been found to cross the placenta (Fabro and Siever, 1969) and crosses the blood-brain barrier in animal models(Galli et al. 1975; Tanaka and Nakazawa 1990). However, there are no documented reports on the effects of maternal consumption of kolanut (intrauterine consumption of aqueous crude extract of kolanut) on the developing brain in relation to neurogenesis and memory.

### 1.2 Brain sensitivity

In the first trimester, the fetus has a rapid growth and involves the critical formation of the central nervous system (CNS). This period is extremely prone to toxicity (Horn, 2008) whose effects might be seen immediately after birth, during milestones or as a young adult which could later have effects on the general output of an individual.

Though the toxicity of kolanut has been reported, no report exists on its beneficial or adverse effects on memory-dependent behavior as well as neurogenesis in the developing brain in relation to the normal development of neuronal elements responsible for memory. Therefore, I proposed to study the effects of kolanut on the developing and adult hippocampi of the *Sprague Dawley* brains. This study detailed information on morphological, cellular, molecular, and morphometric changes associated with the consumption of this social nut of the developing *Sprague Dawley* brain.

#### 1.2.1. Neurogenesis

Early brain development is mostly influenced by genetic factors which stimulate a process called neurogenesis (Gage, 2002; Rao and Shetty, 2004) and environmental factors which affect mostly later development (Pletiko et al., 2014; Igado et al., 2017). The mammalian CNS stem cell is multipotent, self-renewing and produces all brain neurons (Götz and Huttner, 2005). Neurogenesis is known to occur at two locations, the subventricular zone (SVZ) lining the lateral ventricles and the subgranular zone (SGZ) in the hippocampal dentate gyrus (Götz and Huttner 2005; Chopp et al. 2007; Olude et al. 2014). The ability of the brain to renew itself has given hope to humankind

and debunked the old paradigm that once a brain undergoes an injury or trauma, it can never be repaired (Bordey, 2006). Neurogenesis, apart from its self-renewal function also helps in brain plasticity and, in the presence of pathology, it helps in the rate of replacement of old neurons with new neurons (Kitabatake et al., 2007).

Adult neurogenesis and regulation in the SGZ is genetically influenced by contributing conditions like aging, gender, steroids, stress and pathologies (Kitabatake et al., 2007) and these influencing factors can be divided into positive and negative regulators depending on their outcomes on neurogenesis (Zhao et al. 2015; Schmitz et al. 2017). An example of these factors can be substances like drugs and medications ingested during pregnancy, affecting adult hippocampal neurogenesis (Boldrini et al., 2018) thereby resulting in weakened or delayed synapses (Kitabatake et al., 2007) which can later lead to some functional damage in the developing brain.

Successful neurogenesis is modulated by both genetic factors and neurotransmitters which are stimulated by growth factors. The proto-oncogenes, especially *c-jun* and *c-fos* have significant roles in mitogenesis (Stiles and Jernigan, 2010) and neuronal plasticity (Ramirez-Amaya, 2007; Minatohara et al., 2016). The *c-fos* and *c-jun* genes are also termed immediate early genes because their transcription does not need prior protein synthesis (Lau and Nathans, 1987). These genes help in coupling extracellular stimuli with intracellular adaptations (Hill and Treisman, 1995). However, *c-fos* and *c-jun* transcription are regulated by transmitters like GABA, dopamine, glutamate and serotonin (Lau and Nathans, 1987). Another gene required for neurogenesis is the DCX gene which is initiated by doublecortin protein that helps in the process of migration of neurons to their proper sites (Reiner et al., 2006; Ayanlaja et al., 2017).

### 1.2.2. Hippocampus: Memory

The hippocampus is situated within the medial temporal lobe of the forebrain, made up of five regions, subiculum, cornu ammonis (CA) 1, 2, 3 and dentate gyrus. The c- shaped hippocampal formation is responsible for recollection of an item based on their ability to recall such an item due to past exposure or learning experience (Eichenbaum et al., 2007). In its cognitive function, the hippocampus interprets memory to be bits of experiences of short discrepancy across space and time (Fuhs and Touretzky 2007; Ekstrom and Ranganath 2018), hence, any assault or lesion in any of its regions may affect the cognitive function of the subject (Miller and Leslie, 1994; Horn, 2008; Balaban and Yates, 2017).

The ability to learn, retain and recall a piece of information or event by the hippocampus over a period is termed memory. This newly formed information can exist for a while during the labile phase but after a while with no disruption by any circumstances, then the memory becomes consolidated (Ramirez-Amaya, 2007).

Memory formation is regulated by genes and transcription factors like cAMP-response elements binding protein (*CREB*), disc large homologue (*DLG*), *c-fos, c-jun*, and the independent gene expression (IGE) transcription factors; which include brain-derived neurotrophic factor (BDNF) and cAMP-response elements binding protein factor (Ramirez-Amaya, 2007; Kandel, 2012; Minatohara et al., 2016; Mallei et al., 2018).

The *CREB* genes are involved in short- and long-term memory. *CREB 1* promotes the formation of long-term memory and its potentiation, while *CREB 2*, a memory suppressor gene, restricts the maturation of new synaptic interconnections thereby blocking the formation of new memory. Another gene involved in memory formation is the *c-fos* gene. Changes in its expression can be used in observing the plasticity of long-term memory formation and maintenance of neuronal activity (Ramirez-Amaya, 2007; Minatohara et al., 2016).

The *DLG* gene belongs to the guanylate kinase protein family and are cytosolic scaffolding proteins that maintain neuronal integrity relating to synaptic plasticity and memory formation especially in spatial learning (Bordiuk et al. 2014; Uddin and Singh 2017; Soler et al. 2018). The IGE transcription factors are also mediators of the long-term response of the neuron to external stimuli and affect the learning phase. For example, BDNF and acetylcholine (ACh) are highly expressed in the hippocampus, cognition, neuroplasticity, synapse function and dendrite remodeling (Mallei et al., 2018).

Therefore, a mutation in these genes that regulate the hippocampal functions or its structures, could lead to neurodevelopmental problems and loss of cognitive and spatial functions.

### 1.2.3. Dendritic Arborization

Dendritic arborization is an important factor in neuronal connectivity and participates in the critical role of learning, memory and behavioral function (Jones et al., 2018). Dendrites are the primary sites of information input into the neurons with each neuron having its own peculiarity in branching patterns (Urbanska et al., 2008). The integration of information (from individual synapses) within the neuron (Urbanska et al., 2008) is dependent on the type of dendritic branching pattern which is associated with a degree of neuronal connectivity (Schmuck et al.,

2020). Different conditions can affect the branching or the pattern of dendrites such as transcription factors (Jan and Jan, 2010), learning, enriched environment and hormonal fluctuations (Uylings and Van Pelt, 2002), neurodevelopmental disorders, traumatic brain injury and brain tumors (Gilabert-Juan et al., 2017). Also, kolanut (*Cola nitida*) was found to cause oxidative stress via generation of free radicals (Ajarem and Ahmad, 1994; Atiba et al., 2016) in developing pups. Therefore, kolanut effects on hippocampal neurogenesis relative to its impact on its cognitive function are not known.

### 1.3 Rationale

Kolanut is a stimulant (McIlroy, 1963; Kiple and Ornelas, 2000) that is vastly consumed in most African countries especially Nigeria as an everyday social snack (Tindall, 1998). It has a slightly bitter taste, prevents fatigue and dispels sleepiness (Graham, 1978). It is also frequently consumed in Nigeria by pregnant women (Ologe et al., 2008; Ifesanya and Oke, 2013; Atiba et al., 2023) as anti-emetic. However, there is scarcity of available data on the effects of kolanut on the structure of the developing hippocampus.

Considering the critical role of neural function in the well-being of an individual, it has become a necessity to study the likely implications of prenatal exposure of crude aqueous extract of kolanut on the morphological, morphometric, cellular, and genetic changes and the possible effects on the cognitive function later in life. To do this, experimental animals were employed. This study focused on the kolanut-induced histomorphology and neurochemical changes in the hippocampi and memory of the experimental animal pups on postnatal days (PND) 0, 7, 21, 56 and 70. This study may advance knowledge on the likely implications of intrauterine kolanut exposure on neurogenesis and cognitive function in adulthood.

### 1.4 Aim

To determine the effects of aqueous extract of kolanut (*Cola nitida*) on Sprague Dawley dams and its exposure on the hippocampus of the progeny.

### 1.5 Objectives

1. To determine the effects of aqueous extract of kolanut on the dams (body and brain weights).
2. To assess the effect of intrauterine exposure to aqueous extract of kolanut on hippocampal-dependent memory using cliff aversion test, surface righting test, spontaneous movement

on PND 4, 5, 6, and 7; open field, novel object test and radial arm maze on PND 21 and 56 *Sprague Dawley* pups.

3. To determine the effect of intrauterine exposure to aqueous extract of kolanut on density of the prefrontal cortex and hippocampus of PND 0, 7, 21, 56 and 70 *Sprague Dawley* rats using Cresyl violet staining technique and serology.
4. To determine the effect of intrauterine exposure to aqueous extract of kolanut on dendritic arborization in the cerebral cortex and hippocampus of PND 0, 7, 21, 56 and 70 *Sprague Dawley* rats using Golgi-Cox staining technique and Sholl analysis.
5. To deduce the effect of intrauterine exposure to aqueous extract of kolanut on neurogenic activity in the hippocampus of PND 0, 7, 21, 56 and 70 *Sprague Dawley* rat pups using Ki-67 and DCX immunohistochemistry.
6. 
    i. To determine the effect of intrauterine exposure to aqueous extract of kolanut on the expression of immediate early genes (*c-fos, c-jun*), memory genes (*dlg3 and dlg4, creb-1 and creb-2*), IGE transcription factors (*BDNF and ACh)* PND 0, 7, 21, 56 and 70 *Sprague Dawley* rat pups using quantitative polymerase chain reaction (qPCR) technique.
    ii. To determine toxicity indices (malondialdehyde, glutathione peroxidase, reduced glutathione and superoxide dismutase) in the hippocampi of PND 0, 7, 21, 56 and 70 *Sprague Dawley* rat pups using biochemical analyses technique.

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 Physiology of nausea and vomiting

Nausea and vomiting are protective mechanisms that prevent the ingestion or digestion of potentially harmful substances (Taylor et al., 2017). Their etiopathogenesis, signs, symptoms, and assessment are often considered independently in literature, although there were many clinical trials but many studies fail to distinguish between them (Andrews, 1992; Singh et al., 2016). Nausea is the unpleasant sensation of wanting to vomit, while vomiting is the forceful ejection of stomach and intestinal contents through the mouth (Wood et al., 1999; Tan et al., 2018). Nausea is a complex sensation involving cognitive, emotional, and interceptive domains in the brain, and is more common, disabling, and difficult to control than vomiting (Singh et al., 2016). It serves as a warning signal without any adaptive purpose, and is triggered by various stimuli causing stress responses in the endocrine system, gastrointestinal tract, autonomic nervous system (ANS), and prodromal stress responses (Balaban and Yates, 2017).

Vomiting can have severe consequences, including acid-base imbalances, volume and electrolyte depletion, starvation, and aspiration pneumonia (Sanger and Andrews, 2006). Nausea is a term that has changed over the years, it is defined as an awareness of impending vomiting (Stern et al., 2011; Horn et al., 2014; Lackner, 2014). Vomiting occurs in two stages: retching and expulsion (Pleuvry, 2015). Retching involves the simultaneous contraction and relaxation of the abdominal muscles and diaphragm, while expulsion involves sustained contraction of the abdominal muscles, intercostal muscles, and muscles of the pharynx and larynx, resulting in the retrograde contraction of the intestinal muscles with relaxation of the gastric fundus (Pleuvry, 2015). Vomiting is often preceded by retching, where the contents of the gastrointestinal tract are driven into the esophagus without being expelled (Tan et al., 2018).

Nausea and vomiting can be caused by a variety of stimuli and are mediated by a bidirectional relationship between the brain and the gut (Zhong et al., 2021). Several mechanisms have been reported to trigger nausea and vomiting, involving different anatomical areas in the central and

peripheral nervous systems. Scholars have found out that several mechanisms can cause nausea and vomiting. These include:

- The entry of toxins/drugs/bacteria/viruses/fungi into the gastrointestinal lumen. This indirectly stimulates the brainstem emetic nuclei located in the dorsal vagal complex through the release of local emetic neurotransmitters in the upper gastrointestinal tract and subsequent activation of corresponding receptors present on vagus nerves or splanchnic nerves (Wood et al., 1999; Sanger and Andrews, 2006).
- Disruption of the vestibular nuclei and cerebellum. Nausea and vomiting result from ongoing interactions between the gastrointestinal tract, including its enteric neural system, the central nervous system, and the autonomic nervous system (Zhong et al., 2021).
- Diseases in the gastrointestinal tract that stimulate vagal afferent neurons or other internal tissues (e.g., cardiac) that stimulate visceral afferent neurons (Breit et al., 2018).
- The entry of toxic or drug-like substances or infectious organisms into the body systemically. These may act directly on the dorsal vagal complex emetic nuclei in the brainstem (Cangemi and Kuo, 2019; Zhong et al., 2021).
- Cognitive and emotional stimuli in the central and peripheral nervous system, including the frontal lobe and limbic system (Breit et al., 2018)

According to American College of Obstetricians and Gynecologists, morning sickness, characterized by symptoms such as tiredness, dizziness, nausea, and vomiting, is a common phenomenon associated with pregnancy (Gadsby et al., 1993; Lee and Saha, 2011; Herrell, 2014). These symptoms are not limited to mornings and can occur at any time of the day or period of pregnancy, depending on individual hormonal responses. As reported by Herrell, approximately 70% of pregnancies experience nausea and vomiting, often beginning around six weeks and lasting for weeks or months (i.e.- first trimester) (Herrell, 2014). Symptoms usually improve during the second trimester (13-27 weeks). Lee and Saha (2011) were of the opinion that morning sickness can occur throughout pregnancy in about 10% of women; about 82.8% of pregnant women will experience morning sickness at some point during their pregnancy (Ebrahimi et al., 2010; Einarson et al., 2013); and up to 80% of all pregnant women experience some form of nausea and vomiting during their pregnancy (Gadsby et al., 1993). Nausea, which affects about 75% of pregnant women, is associated with the actual ejection of gastric content, termed vomitus

(Pleuvry, 2015), whereas, up to 90% of pregnancies experience some level of nausea, with or without vomiting (Matthews et al., 2015).

In a prospective study of nearly 800 patients who were followed from conception, 57% reported nausea and 27% reported both nausea and vomiting by 8 weeks of gestation (Hinkle et al., 2016). There is strong evidence that patients who experience nausea and vomiting during early pregnancy have a lower risk of miscarriage than patients who do not experience these symptoms (Weigel and Weigel, 1989). For instance, in one meta-analysis done by Weigel & Weigel (1989), the odds of miscarriage were 0.36 (95% CI 0.2-0.42) in patients with nausea and vomiting in the first 20 weeks of pregnancy. Although, the analysis found no correlation between outcome and severity of the disorder; most patients in the studies had mild symptoms rather than hyperemesis; most data were collected retrospectively; and patients with pregnancy losses prior to pregnancy recognition were excluded. Symptoms of morning sickness vary from mild to severe.

Hyperemesis gravidarum is a severe and chronic form of nausea (Lee and Saha, 2011; Koot et al., 2018). Symptoms can include excessive vomiting (more than 3 times a day), severe dehydration leading to dark-coloured urine, dizziness when standing, little-to-no urine production, and loss of appetite leading to weight loss (London et al., 2017). A study reported that patients with hyperemesis gravidarum who had a cesarean delivery appeared to be at increased risk for postoperative nausea and vomiting, which tended to be more severe compared to those without a history of hyperemesis (Jacobs et al., 2020).

The exact cause of morning sickness is still medically unknown, but medical experts were of the opinion that it could be due to factors such as low blood sugar or an increase in pregnancy hormones like human chorionic gonadotropin (HCG) or estrogen. Factors like stress, fatigue, certain foods, or motion sensitivity can exacerbate morning sickness (Herrell, 2014). While nausea and vomiting during pregnancy can disrupt a woman’s daily routine, studies have shown that these symptoms do not negatively affect the cognitive development of the offspring (Nulman et al., 2009). Due to the discomfort and energy-draining effect of these symptoms, some pregnant women resort to taking various substances to alleviate them. These can include prescribed medications or alternative remedies based on the experiences of other pregnant women or advice passed down from relatives (Mohammed et al., 2013; Yildirim et al., 2016).

### 2.1.2 Substances consumed in pregnancy.

Substance use during pregnancy is a significant public health issue that can lead to various negative perinatal outcomes (Forray, 2016). The consumption of substances such as drugs and alternative medications during pregnancy has been increasing (Van Gelder et al., 2013). The type of drug used, the degree of use, and the timing of exposure can all impact the pregnancy and pose challenges to fetal development.

Medications are often used to manage pregnancy-related disorders such as nausea and vomiting. Studies indicate that between 44% and 99% of women use medications during pregnancy (Bakker et al., 2006; Van Gelder et al., 2013). Physicians, especially, the Obstetricians prescribe various methods and medications to alleviate nausea and vomiting. These include drinking plenty of fluids, especially water, drinking tea with fresh grated ginger, taking prenatal vitamins with a snack (e.g., pyridoxine, doxylamine), ensuring adequate ventilation and fresh air, and smelling pleasant scents such as mint or orange (ACOG, 2018). Antiemetics, a type of medication used to prevent nausea during pregnancy, include antihistamines (most extensively used) such as dimenhydrinate, meclizine, and diphenhydramine. Others include benzamides (metoclopramide), butyrophenones (droperidol), and phenothiazines (promethazine, prochlorperazone, and ondansetron) (Taylor et al., 2017).

Some pregnant women resort to self-medication due to pregnancy-related symptoms such as backache, headache, nausea, vomiting, and fatigue (Gibson, 2001; Pangle, 2006). Inappropriate use of medications and substances, which has been reported in many developing countries (Bamgboye et al., 2006), can cause structural and functional defects in the developing fetus (Mohammed et al., 2013) as the fetus is susceptible to the adverse effects of these substances (Gibson, 2001). Some pregnant women also resort to the use of illicit substances to alleviate nausea and vomiting. Younger women, especially those between the ages of 18 and 29 has been reported to be more likely to develop substance use disorders during their reproductive years. Substance use during pregnancy continues to increase, with pregnant women increasingly reporting its use to relieve pregnancy-related nausea and vomiting in the USA, Europe, and Australia (Young-Wolff et al., 2018). Illicit substances used during pregnancy include nicotine, alcohol, cannabis, marijuana, cigarettes, cocaine, and other illicit substances (Herrell, 2014; Forray, 2016). A 2012 national survey reported that 5.9% of pregnant women use illicit drugs.

Studies have shown that the use of illicit substances during pregnancy can lead to miscarriage, stillbirth, low birth weight, congenital anomalies, preterm delivery, small-for-gestational age, and child mortality (O'Leary et al., 2012; Forray, 2016). Long-term effects include cognitive and behavioral difficulties, poor speech and language outcomes, executive functioning deficits in children, and psychosocial consequences in adulthood (Bakoyiannis et al., 2015; Rangmar et al., 2015).

The use of alternative substances, such as herbal medicines, is common amid pregnant women in combating nausea and vomiting. Some pregnant women opt for herbal medicines and concoctions over medically prescribed drugs, primarily because of their faith in herbs, being natural, are safer and have less harmful effects than common western drugs (Heitmann et al., 2015; Petry et al., 2018; Peprah et al., 2019). This practice is traditionally and culturally recognized in regions like Sub-Saharan Africa (El Hajj and Holst, 2020).

Common medicinal herbs used by pregnant women in Sub-Saharan Africa include ginger (*Zinger officinale*), garlic (*Allium sativum*), kolanut (*Cola nitida*), and bitter cola (*Garcinia kola*) (Kennedy et al., 2013; Ahmed et al., 2018b). A paper reviewed by S.M. Ahmed et al. (2018) involving over twenty thousand of African pregnant and lactating mothers found that the mean occurrence of herbs used during pregnancy differed by location, ranging from 32% in Central Africa to 45% in East Africa. There was a significant association between the use of herbs and families of lower-level demographic and socioeconomic status and among rural dwellers for treating symptoms associated with pregnancy, like nausea and vomiting (Chrubasik et al., 2005; Shewamene et al., 2017; Ahmed et al., 2018a, 2018b; Peprah et al., 2019).

## 2.2 Kolanut (*Cola nitida)*

Kolanut (*Cola nitida*) is a seed pod of kola tree, primarily seen in the sub-Saharan regions of Africa (Tachie-Obeng and Brown, 2004). The kola plantation is common in Nigeria, Sierra Leone, Ivory Coast, Gabon, and Congo (Asogwa et al., 2011). Over time, kola trees have been introduced to other regions of the world, such as tropical Americas, Brazil, Sri Lanka, Malaya, West Indies, and East Africa, and thrive in humid climates (Tachie-Obeng and Brown, 2004; Unya, 2021).

Kolanut plays a significant role in different customs, beliefs and social settings. Sometimes, it can replace food and water in certain circumstances (Adisa et al., 2010). Nigeria, a once major producer of kolanut, accounted for 88.0 % of the world's production before the oil boom (Quarco T, 1973). However, 90.0 % of this was locally consumed, while the remaining 10.0 % was exported (Quarco T, 1973; Tachie-Obeng and Brown, 2004). In Nigeria, the Igbos call kolanut "*Oji*", the Hausas "*Gworo*", and the Yorubas "*Obi*" (Adejoke Adebusola Adelusi et al., 2020). Other names given to kolanut in other part of the world include Guru Nut, Arbre à Kola, Bissy Nut, Cola acuminata, Kolatier, Noix de Gourou, Noix de Kola, Noix du Kolatier, Noix de Soudan, Soudan Coffee, *Sterculia acuminata, Sterculia nitida, Sterculia acuminate* (Adisa et al., 2010; Adejoke Adebusola Adelusi et al., 2020)

### 2.2.1 Kolanut Description

There are two most common varieties of kolanut: red and white kola, which are produced by the same plant species as fresh dicotyledon seeds and are sometimes found within the same pod (Tachie-Obeng and Brown, 2004). The difference in coloration can be due to species type, genetics and duration after the harvest (Tachie-Obeng and Brown, 2004). They have acidic flavor when fresh and has nutmeg and aromatic aroma with less bitterness when dry (Burdock et al., 2009). Caffeine, tannins, theobromine and phenolics, including d-catechin, 1-epicatechin, and kolanin, are the main chemical constituents of kolanut with phlobaphens, proteins, and starch (Kanoma et al., 2014). Kolanut has different species that are cultivated in tropical Africa (Tachie-Obeng and Brown, 2004), but the most common types indigenous to West Africa are *Cola nitida* and *Cola acuminate* (Oestreich-Janzen, 2016; Unya, 2021).

Figure 2.1A: **The Red *Cola nitida* seeds**

Figure 2.1b: **The White *Cola nitida***

Source: (Atiba et al., 2021)

### 2.2.2 Propagation

*Cola nitida*, one of the common species of kola tree is cultivated and highly supported by dry soil and temperature of about twenty-three to twenty-eight degrees Celsius (Tachie-Obeng and Brown, 2004; Tende et al., 2011). The peak period of production is from October to December. The kola tree can withstand altitudes of up to 1,000 feet (300 meters) above sea level if rich and deep soil is available, but it is typically thought of as a lowland forest species. It requires a tropical climate with an annual average temperature of at least 75$^{o}$F (24$^{o}$C) and can tolerate a brief dry season, so it can be grown in dry areas if it is near ground water. Shade is required for the tree to grow properly, and better results can be obtained by adding good fertilizers (Adelusi et al., 2020; Adesida et al., 2021). The tree starts producing seeds approximately from twelve to fifteen years and this continues throughout the tree life span which can be up to one hundred (100) years (Lovejoy, 1980).

### 2.2.3 Composition of *Cola nitida*

The composition of *Cola nitida* has been examined by different scholars and similar results have been presented (Adeniyi et al., 2017, 2022). Kolanuts are known to consist of xanthine alkaloids which include caffeine, theobromine, and theophylline (Somorin, 1973), and phenolic such as phlobaphens, tannic acid, d-catechin, and epicatechin, catechines, procyanidins, proanthocyanidins, and sterols (Elusiyan et al., 2018). Other components include sugar, water, fatty acids and alcohols (Adesanwo et al., 2017) (Table 2.1). The diagram in figure. 2.2 shows main chemical constituent of kolanut.

**Table 2.1**. Composition of the nuts of *Cola nitida* and *Cola acuminata* reported in four articles

| | 1 | | 2 | | 3 | | 4 | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *C. acum.* | *C. nitida* | *C. acum.* | *C. nitida* | *C. acum.* | *C. nitida* | *C. acum.* | | *C. nitida* | |
| Parameter | | | | | | | Red | White | Red | White |
| | % | | | | | | | | | |
| Moisture | 5.8 | 2.3 | 10.2 | 12.5 | 13.3 | 12.9 | 10.5 | 8.9 | 9.5 | 11.0 |
| Crude Protein | 9.4 | 10.5 | 10.6 | 10.1 | 9.7 | 8.8 | 10.1 | 10.1 | 10.0 | 9.6 |
| Fat | 15.8 | 13.3 | 1.2 | 0.2 | 10.8 | 9.2 | 2.7 | 2.4 | 1.2 | 2.2 |
| Crude Fibre | 12.5 | 17.5 | 7.4 | 4.3 | 5.5 | 6.5 | 7.6 | 7.3 | 7.5 | 7.0 |
| Ash | 4.2 | 3.1 | 3.5 | 3.0 | 2.6 | 2.3 | 5.0 | 4.1 | 4.2 | 4.9 |
| Carbohydrate | 52,2 | 53.2 | | | 58.3 | 60.7 | 64.1 | 67.2 | 67.7 | 67.1 |
| | mg/g | | | | | | | | | |
| Calcium | | | 1.7 | 4.3 | | | 4.7 | 6.3 | 5.3 | 9.5 |
| Magnesium | | | 10.0 | 11.5 | | | 0.4 | 0.5 | 0.4 | 0.5 |
| Potasium | | | | | | | 0.9 | 0.9 | 0.9 | 0.8 |
| Sodium | | | | | | | 0.2 | 0.2 | 0.2 | 0.2 |
| Zinc | | | 1.4 | 0.7 | | | | | | |
| Iron | | | 2.8 | 4.4 | | | 1.4 | 1.4 | 1.21 | 1.2 |
| Copper | | | 0.7 | 0.6 | | | | | | |
| Total Sat. Fatty Acid (%) | | | | | 31.5 | 30.9 | | | | |
| Total Unsat. Fatty Acid (%) | | | | | 68.6 | 69.1 | | | | |
| Total Monunsat. Fatty Acid (%) | | | | | 23.4 | 25.7 | | | | |
| Total Polyunsat. Fatty Acid (%) | | | | | 45.2 | 43.3 | | | | |
| Total Phenolic (µgEqAG/100g) | | | 445.0 | 679.9 | | | | | | |
| Total Flavonoid (µgEqQ/100g) | | | 537.4 | 374.6 | | | | | | |
| Total Flavonoid (µgEqQ/100g) | | | 537.4 | 374.6 | | | | | | |
| Vitamin C (mg/100 ml) | | | 0.5 | 6.3 | | | | | | |
| Alkaloids (%) | | | 1.8 | 2.1 | | | | | | |
| Saponin (%) | | | 8.3 | 4.7 | | | | | | |
| Tanin (mg TAE/100 mg) | | | 7.1 | 5.7 | | | | | | |

In column headings, 1= Abolude, 2004; 2 Nouvlessounon *et al*., 2015; 3 = Adeyeye *et al*., 2017 ; 4 = Adeniyi *et al*., 2022. C. acum. = *Cola acuminata ;* units of fatty acids, Vitamin C and antinutritional factors are indicated against them.

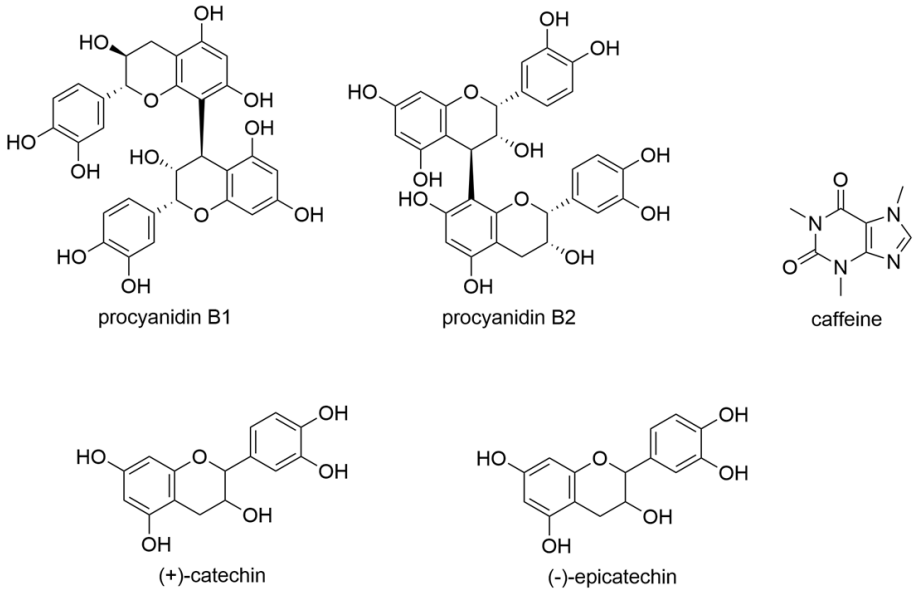



Figure 2.2: Some chemical structures of kolanut
Mbembo et al., 2022

### 2.2.5 Uses of Kolanut in West Africa

Kolanut is highly valued across various demographics in West Africa (Starin, 2013), serving multiple purposes globally. In Nigeria, it is one of the most popular masticatory plants and holds significant social, traditional, and economic value in West Africa (Asogwa et al., 2011; Ndagi et al., 2012). It is a common phenomenon, as well as a potent traditional belief icon.

#### 2.2.5.1 Traditional and Sociocultural uses

Kolanut is commonly used for social and ceremonial purposes in West Africa. They are integral to traditional hospitality, cultural, and social ceremonies in Nigeria and many West African countries, to the extent that their absence often signifies an incomplete ceremony (Tachie-Obeng and Brown, 2004; Starin, 2013; Oestreich-Janzen, 2016). The extract of kolanut is also used in it native form to treat physical and mental exhaustion (Blumenthal et al., 2000; Adeosun et al., 2017). Kolanut is important and a must-have during weddings, naming ceremonies, and divination activities. It is given as a sacred offering and to show respect. It is an essential component of community or religious rites. Muslims believe that Prophet Muhammad loved kolanut and often gift them out, leading his followers to distribute kola during important social gatherings as

handouts (Lovejoy, 1980). Kolanut has been reported as a means of alleviating poverty among rural dwellers (Adebayo and Oladele, 2012). In some parts of West Africa, kolanut was once dedicated as legal tender and duty fees (Lovejoy, 1980), and served as second most important indigenous cash crop (Ndagi et al., 2012; Starin, 2013)

#### 2.2.5.2 Economic/ Industrial uses

The edible fruit of the kola tree is used as a flavoring ingredient in beverages, giving rise to the term 'cola' (Burdock et al., 2009). It is also utilized in the making of soda, pharmaceuticals, wines, and confectioneries (Umoren et al., 2009). Kolanut is considered a special diet to boost energy and wellbeing, as well as to flavor sodas. The nut is commonly chewed raw but can also be used as a powder after drying and grinding. Boiling can be used to extract caffeine and other constituents (Adejoke Adebusola Adelusi et al., 2020). Kolanut is known to contain caffeine, which is likewise seen in coffee, mate, tea, and little in chocolate. Kolanuts also contain more caffeine than coffee, more reason they are commonly chewed as a stimulant in different parts of Africa (Unya, 2021). The nut is exported around the world for extraction and used for the production of methylxanthine-based pharmaceuticals (Burdock et al., 2009). The seed of kolanut can be consumed; it is quite bitter, but when the whole fruit is chewed, it has a sweet fruity flavor. It also has a pleasant aroma that reminds you of rose petals (Adelusi et al., 2020; Adesida et al., 2021)

#### 2.2.5.3 Medicinal uses

Kolanut (*Cola nitida*) has been used as a caffeine stimulant for a long time (Oestreich-Janzen, 2016). In the United States, before the banning of caffeine products in soft drinks after 1904, kolanut extract was used in the production of these beverages. Even today, the recipe used by Coca-Cola is a chemical synthetic of kolanut. Extracts of Eugenol, G. kola, Vitamin A, Vitamins A+D, Vitamin D, *Cola acuminata* (white), *Cola nitida* (pink), and *Cola nitida* (red) have been found to be effective antioxidants in red cell survival and viability (Adebayo and Oladele, 2012). According to Ibikunle & Ogbadoyi (2011), kolanut extracts are used to treat and control trichomoniasis due to its trichomonacidal potential. In Europe, roasted seeds are used to treat various disorders and as a stimulant (Balagizi et al., 2017). Both the European Commission and the US Food and Drug Administration have consented to kolanut being one of the food additives.

Kolanut is used to treat tiredness, exhaustion, headache and migraine and some psychological conditions like melancholia, also, diarrhea and decrease in muscle tone (atony) (Adejoke Adebusola Adelusi et al., 2020). Traditionally, especially in Nigeria, people believe that kolanut is used in treating fatigue, starvation, germs and bacteria, eczema, skin sores and ulcers, oral diseases, nausea and vomiting, difficult in child births, irregular menstrual cycles, decrease in sexual urge, eye diseases and so on (Adebayo and Oladele, 2012; Starin, 2013b).

A nutrition research study conducted on 100 women who delivered newborn babies in Nigeria showed that 76% of them consumed kolanut within 13 – 91 grams/week. Out of these, 47% took kolanut during pregnancy to control morning sickness, while 53% did it habitually. Likewise, it showed that kolanut consumption has a significant correlation with head circumference, chest circumference but no significant correlation in birth weight of babies (Abidoye and Chijioke, 1990). In folk medicine, *Cola nitida* is aphrodisiac, an appetite suppressant, and to treat migraine headaches, morning sickness and indigestion (Esimone et al., 2007; Balagizi et al., 2017).

### 2.2.6 Side effects of Kolanut

It is widely assumed that caffeine is responsible for most of the actions of kolanut (Umoren et al., 2009). Caffeine affects people differently, and most people can safely tolerate 400mg of caffeine per day (Adejoke Adebusola Adelusi et al., 2020). The side effect of kolanut and caffeine have been compared. Ogutuga (1975) proposed that the caffeine content of kolanut could be as high as 7.0 %, and that caffeine is often regarded as the agent responsible for the physiological or clinical effects of kolanut in humans and other mammals (Chukwu et al., 2006). An experimental study conducted by Umoren et. al. (2009) confirmed this. In their study, they determined and compared the effects of chronic kolanut consumption and its active principle, caffeine, on locomotor behavior and body weight using mouse. The study discovered that diets high in kolanut and caffeine reduced food intake and body weight over a period and caffeine diet consumption also reduced water intake and locomotor activity significantly but diets high in kolanut had no effect on water intake or locomotor activity. As a result, they concluded that the effect of kolanut on locomotor behavior and water intake may be due to factors other than caffeine.

According to Umoren et al. (2011), kolanut as an active stimulant with a strong effect can cause anxiety and tremors, which are difficult to manage, it can then have a negative impact on the Central Nervous System (CNS) and the Peripheral Nervous System (PNS). Kolanut contains a lot

of caffeine, so it should be consumed with moderation. Kolanut as a stimulant when taken in excess can affect the liver and kidneys and cause an increase in heart rate of animal models (Ikegwuonu et al., 1981). Kolanut has also been found to induce oxidative stress via generation of free radicals (Atiba et al., 2016). Studies have also shown that overconsumption of kolanut affects the male reproductive system by reducing sperm counts and body weights of the tested animals (Obidike et al., 2010; Umoren et al., 2011; Ogunwole et al., 2015), behavioral and endocrine system (Celec and Behuliak, 2010) and brain sodium pump activity (Obochi et al., 2007c). Kolanut has Caffeine, as one of its constituents. It crosses the placenta, enters into the fetal circulation, penetrates the blastocyst, crosses blood brain barrier, and accumulates in the fetal brain (Tanaka & Nakazawa 1990).

The effect of kolanut consumption on brain is beginning to surface in literature in recent years. Imam-Fulani et al. (2018) found out that acetone extract of *Cola nitida* exerted a significant increase and restorative effect on the brain Na+/K+-ATPase activity and spatial memory of STZ-induced diabetic female Wistar rats when compared with the group with diabetes. Atiba et al. (2021) discovered that pregnant rats treated with *Cola nitida* exhibited some symptoms such as reduction in brain weights, body, and loss of Bergmann glia, increased molecular layer thickness, and persistent external granular layer of the cerebellum at the initial stage. Alterations were also discovered in the Purkinje cells such as reduction in density, loss of dendrites and multiple layering, while the white matter showing spongiosis and GFAP and BCL- 2 over-expression.

Glial fibrillary acidic protein (GFAP) is the main marker for brain astrocytes. The presence of injury or toxicity in the CNS increases the production of GFAP thereby causing an increase in GFAP-positive cells. This leads to hypertrophied cell bodies and processes of the astrocytes and this phenomenon is called astrogliosis (Sun and Jakobs, 2012). Therefore, expression of GFAP is directly proportionate to the rate of injury or trauma to the brain tissues (Zhang et al., 2016c). Also, overexpression of B Cell Lymphoma 2 (BCL-2) is associated with injury or toxicity in the brain in order to sustain the lifespan of neurons without further cell division (Tsuyama et al., 2017).

## 2.3 Development of the Brain

### 2.3.1 Overview of the Brain

Brain, with approximately 100 billion neurons and over 100,000 kilometers of interconnections, has an estimated storage capacity of $1.25 \times 10^{12}$ bytes (Hofman, 2012). It is enveloped by a layer of tissue known as the meninges. It comprises several specialized areas, including the cortex, the brain stem, the basal ganglia, and the cerebellum, all of which interact to facilitate brain function. The cortex is responsible for thinking and voluntary movements, the brain stem aids in breathing and sleeping and connects the spinal cord to the rest of the brain, the basal ganglia coordinate messages between different parts of the brain, and the cerebellum assists in coordination and balance. The hippocampus, located in the temporal part of the cerebral cortex, plays a crucial role in learning and memory (Hofman, 2014).

The brain has different lobes: the frontal lobes, the parietal lobes, the temporal lobes, the occipital lobes, and the limbic lobes (figure 2.3). The frontal lobes are involved in solving puzzles, judgment, and motor function; the parietal lobes aid in body positioning, and sensation; the temporal lobes function in hearing and memory; and the occipital lobes house the brain's visual processing system. The limbic lobes, comprising the nuclei, the hippocampus, the amygdala, the septal nucleus, and insula (Rajmohan and Mohandas, 2007), are responsible for behavioral and emotional responses.

The brain, particularly the developing brain, is highly sensitive to chemicals and environmental pollutants (Lanphear 2015; Atiba et al. 2016). The adverse effects of these chemicals or free radicals generated through oxidative stress, especially at the formative stages of the brain and other neural elements responsible for memory and intelligence, may lead to dysfunction later in life (Atiba et al. 2021). The hippocampus, which plays a significant role in learning and memory, defines memory as bits of experiences that have low variance across space and time (Fuhs and Touretzky, 2007; Zucker and Ranganath, 2015b; Ekstrom and Ranganath, 2018).

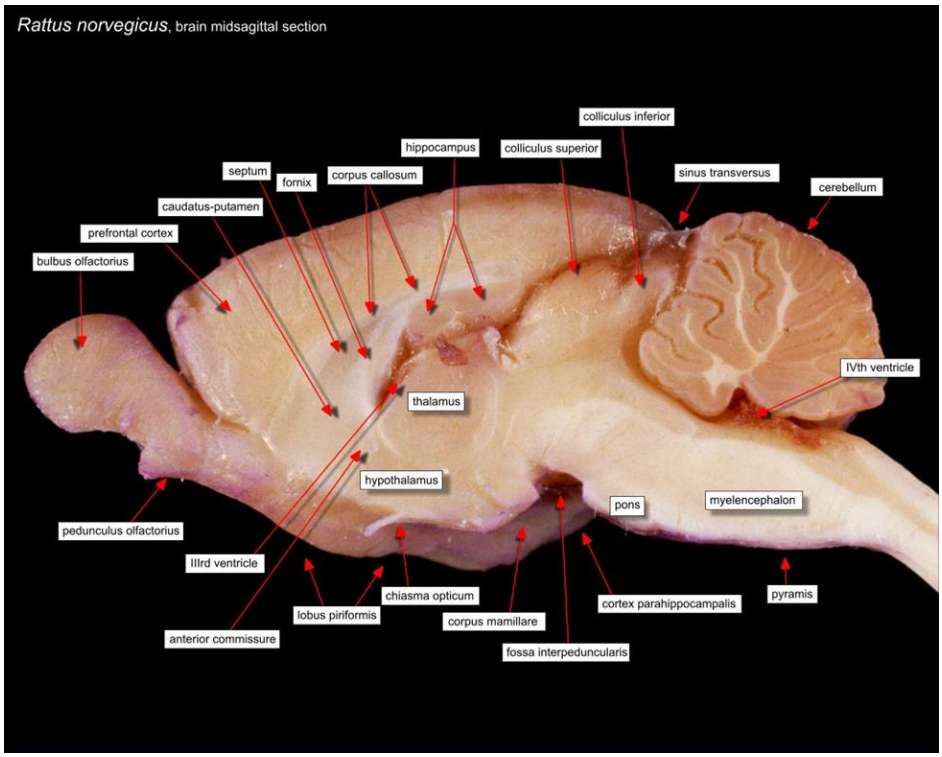



**Figure 2.3** Mid sagittal section of the brain of a rat (©www.anatomie-amsterdam.nl/sub_sites/anatomie zenuwwerking/123_neuro/html_pages/details/details_rat_brain_lb.htm) accessed on 13/07/2024

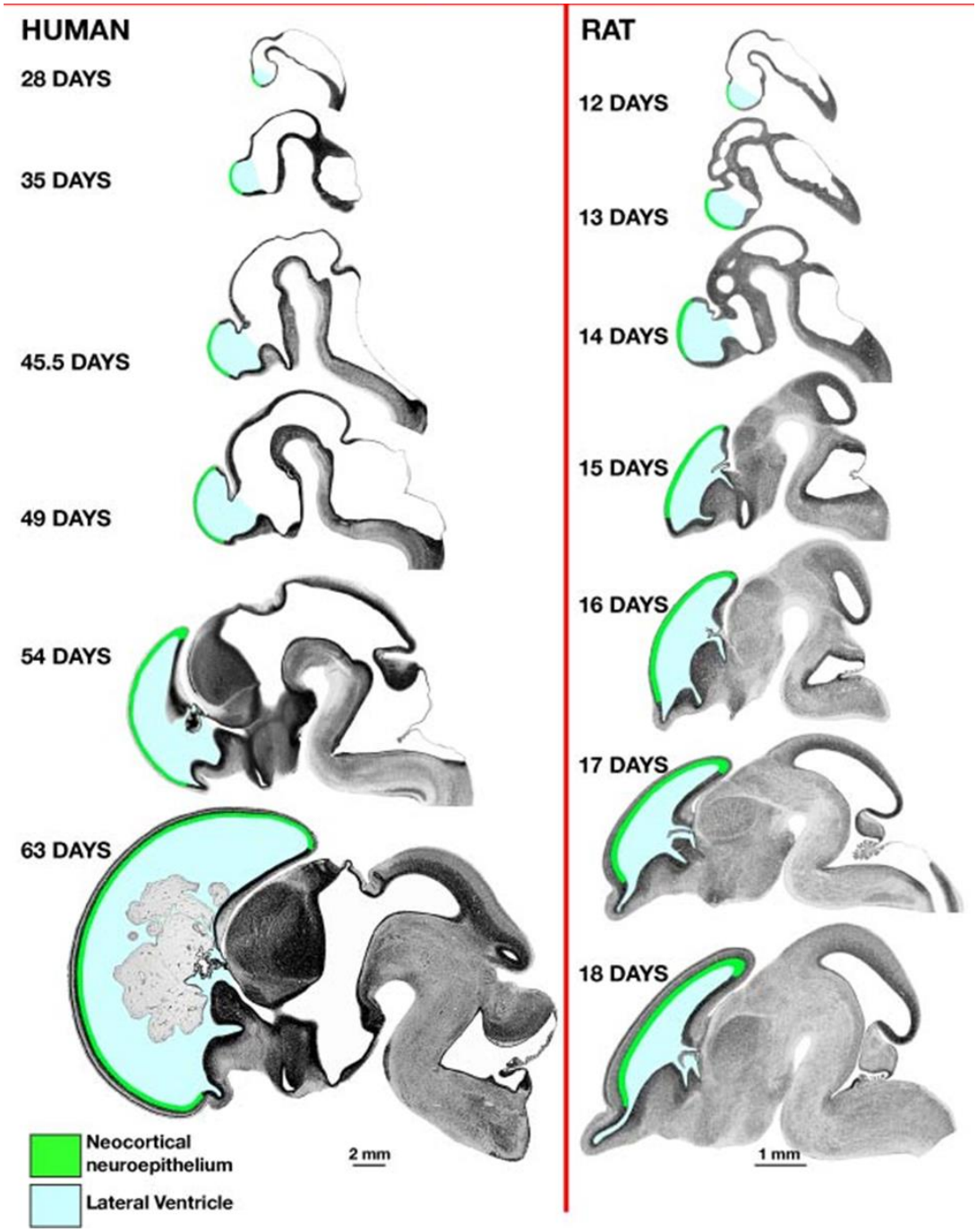



**Figure 2.4:** Brains of human and rat embryos at several matched stages of development. (© NeuronDevelopment.org)

### 2.3.2 Cytoarchitecture of the Hippocampus

The hippocampus, a temporal extension of the cerebral cortex (figure 2.4), can be externally identified as a layer of densely packed neurons that curl into a seahorse-shaped structure on the edge of the temporal lobe. As such, it is classified as a component of the limbic lobe, with 'limbic' meaning border (Gilbert and Brushfield, 2009). Although it is located subcortically, it is not entirely considered a subcortical structure. The hippocampus is part of the hippocampal formation and has two major parts, the hippocampal proper and the dentate gyrus. In adult humans, the volume of the hippocampus on each side of the brain is approximately 3-3.5 $cm^3$, compared to 320-420 $cm^3$ for the volume of the neocortex. This makes the hippocampus 100 times smaller in volume than the cerebral cortex (Gilbert and Brushfield, 2009; Anand and Dhikav, 2012).

One of the few brain regions where neurogenesis occurs even in adults is the hippocampus (Bonfanti, 2016). It receives information from the cerebral cortex and is thought to be involved in Alzheimer's disease (Frisoni et al., 2010). The hippocampus is organized in layers of different cells, making it the most extensively studied part of the brain for neurophysiology (Anand and Dhikav, 2012). Initially described as "too little," neurogenesis in the brain is now thought to be functionally important. It has been observed that the neurons produced integrate into the mainstream neurons, proving to be functionally important (Dhikav and Anand, 2011; Knapman et al., 2012).

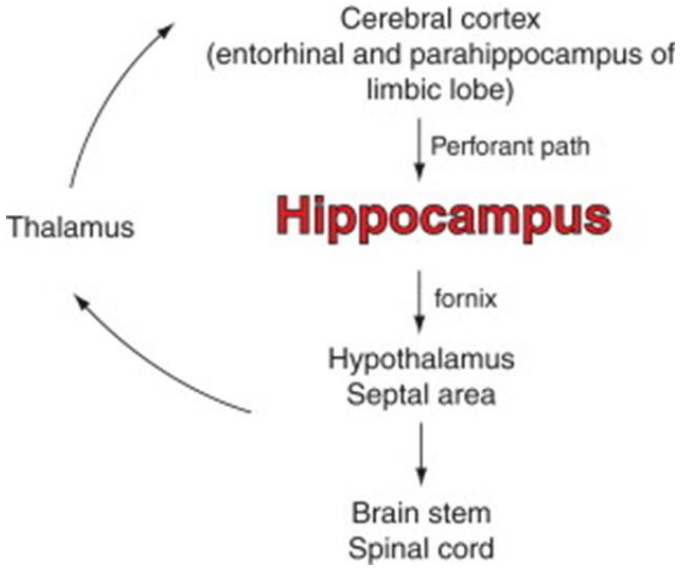



**Figure 2.5**: Schematic of the major communication pathways between hippocampus and other brain areas *Source: with permission Comphensive toxicology 2010*

### 2.3.3 Hippocampal Formation

The hippocampus, a component of the hippocampal formation, is made up of the Cornu ammonis (hippocampus proper) and the dentate gyrus (Anand and Dhikav, 2012). Some anatomists further divide the hippocampus into Cornu ammonis (hippocampus proper), dentate gyrus (DG), subiculum, and entorhinal area (Anand and Dhikav, 2012). The hippocampus has a head, body, and tail, with the head being the most expanded and the tail being the thinnest and most curved. The hippocampus is a laminar structure that receives sensory information mostly from the entorhinal cortex (Schultz and Engelhardt, 2014).

Histologically, the hippocampus proper (Cornu ammonis) is divided into CA1, CA2, CA3, and CA4 (Per et al., 2007). The Cornu ammonis (hippocampus proper) and the dentate gyrus are separated by the hippocampal sulcus and curve into each other. The subiculum, located opposite CA1, connects the hippocampus to the entorhinal cortex in the ventricle. The hippocampus is protected by the choroid plexus. The posterior cerebral artery, which has three branches: anterior, middle, and posterior, supplies the hippocampus. The anterior choroidal artery also contributes to the supply (uncal branch).

The Dentate Gyrus (DG), located deep within the hippocampus and surrounded by Cornu ammonis (CAs), is critical as an information processor from the entorhinal cortex (EC) to CA3. Cells in the DG discharge at a low frequency and provide low-intensity stimulation of CA3, which is thought to be a cost-effective storage process (Ohm, 2007). The DG is made up of three layers in its cortical region with a distinct U or V shape and is like the hippocampus structure but not divided into regions (Anand and Dhikav, 2012). The first layer is made of a cell-sparse molecular layer which the hilus is separated by the second layer. The second layer is the dense layer of granule neurons, with their dendrites extended into the third layer called the overlying molecular layer (Amaral et al., 2007).

### 2.3.4 Functions of the Hippocampus

The hippocampus aids in memory, learning, navigation, and spatial perception. The hippocampus has the ability to integrate information from all sensory modalities, which may be conferred by the highly convergent-divergent organization of its connections (Per et al., 2007). Connections between the hippocampus and the neocortex are critical for conscious knowledge awareness

(Morgado-Bernal, 2011). The hippocampus enables the encoding of memories and the retrieval of experiences from the frontal lobe.

There are two major learning and memory loop pathways: polysynaptic and direct. The hippocampus receives afferent connections from the parietal, temporal, and occipital areas via the entorhinal cortex and then to the dentate gyrus to CA3 to CA1 to subiculum to alveus to fimbria to fornix to mammillothalamic tract to anterior thalamus to posterior cingulate to retrosplenial cortex via the polysynaptic pathway. It receives input from the temporal association cortex via the perirhinal and entorhinal areas to CA1 via the direct intra-hippocampal pathway (Morgado-Bernal, 2011; Anand and Dhikav, 2012). The hippocampus is now known to be important not only in learning and memory but also in spatial navigation (Stella et al., 2012), hypothalamic function regulation (Koehl and Abrous, 2011), and emotional behavior (Toyoda et al., 2011).

### 2.3.5 Blood supply to the Hippocampus

The hippocampus, a component of the hippocampal formation, receives its blood supply from the branching of the posterior cerebral artery, the anterior choroidal artery, and numerous internal hippocampal arterioles arising from both vessels (Rusinek et al., 2011). The arterial supply to the hippocampus is through the hippocampal arteries (HA) (figure:2.5), which are branches of the longitudinal hippocampal artery, a branch of the posterior cerebral artery. The remaining branches of the longitudinal hippocampal artery supply the rest of the hippocampal formation (Coyle, 1976).

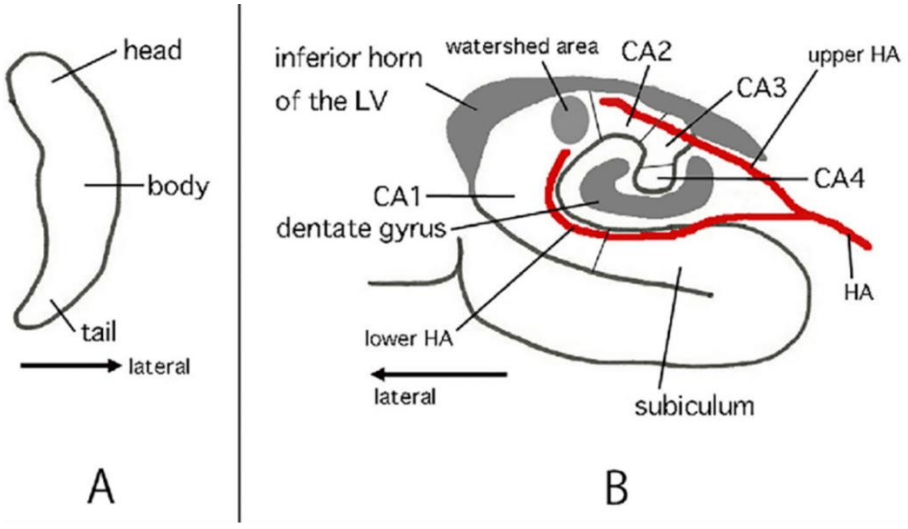



**Figure 2.6**: Blood supply of the hippocampus.
Source: Ogawa et al., 2018

### 2.3.6 Clinical conditions associated with Hippocampus.

Several clinical conditions are associated with human hippocampus:

1. **Alzheimer's Disease**

Brain atrophy in the hippocampal region is one of the most consistent features of Alzheimer's disease (Dhikav and Anand, 2011). It is the first brain region to be affected and is the most severely affected. A theory known as 'hippocampo-cortical-dissociation' proposes that early damage to the hippocampus causes a 'dissociation' between the hippocampus and the cerebral cortex, resulting in failure to register information emanating from the hippocampus. All patients with Alzheimer's disease (AD) have some degree of hippocampal atrophy. A few neurotransmitter changes occur in Alzheimer's disease brains, including noradrenergic, serotonergic, and glutaminergic regions, which correspond to neuron loss in the hippocampal region (Frisoni et al., 2010; Dhikav and Anand, 2011).

2. **Hypertension**

Hypertension and other risk factors are increasingly being considered as potential causes of hippocampal atrophy (Dhikav and Anand, 2007; Fiford et al., 2020). Possible causes include vascular insult, which causes ischemia, hypoperfusion, and neuron hypoxia. Several studies have found that blood pressure is elevated in many Alzheimer's patients' decades before the disease manifests itself (Bast, 2011; Dhikav and Anand, 2011; Anand et al., 2015).

3. **Epilepsy**

In the event of death and medically refractory temporal lobe epilepsy, up to 50% to 75% of patients with epilepsy may have hippocampal sclerosis upon postmortem examination (Anand and Dhikav, 2012; Jardim et al., 2016). However, it is unclear whether epilepsy is caused by hippocampal sclerosis or if repeated seizures damage the hippocampus (Dhikav and Anand, 2011; Caciuc et al., 2016). Hippocampal sclerosis in epilepsy may be caused by uncontrolled local hippocampal inflammation and blood-brain barrier damage (Yang et. al. 2010).

### 4. Schizophrenia

One of the most consistent findings in MRI of schizophrenic patients is a reduction in hippocampal volume (Bast, 2011). There have also been functional and biochemical abnormalities discovered. Though the pathophysiology of schizophrenia was initially focused primarily on the prefrontal cortex, the hippocampus has been considered in the last 20 years or so. There is now compelling evidence that anatomical and functional abnormalities in the hippocampus of schizophrenic patients are caused by neuronal disturbances. MRI, PET, and MRS studies of schizophrenia-related hippocampus disturbances have yielded evidence (Dhikav and Anand, 2011). The volume reduction in the hippocampus of schizophrenia patients is modest and not as severe as that seen in Alzheimer's disease patients. Nonetheless, biochemical, and functional disturbances provide reliable evidence of the hippocampus's involvement in the pathophysiology of schizophrenia (Bast, 2011; Dhikav and Anand, 2011).

### 2.3.7 Behavioural hippocampus tests.

Cognitive evaluations have been an integral part of psychopharmacology and psychological research. Learning can be evaluated with repeated training trials to measure acquisition, and memory can be repeatedly assessed across time to evaluate retention (Moser, 2011). Therefore, the hippocampus could be evaluated in spatial learning, memory, and exploratory behaviour (Mcdaniel and Moser, 1993; Calton and Taube, 2009; Moser, 2011). Some of the cognitive tests to assess hippocampal function are cliff avoidance, surface righting, spontaneous movements, open field, object recognition and location, and radial-arm maze (Antunes and Biala, 2012; Mustapha et al., 2014; Nguyen et al., 2017; Delcourt et al., 2018).

# CHAPTER THREE

# MATERIALS AND METHODS

## 3.1 ANIMAL STUDY

### 3.1.1 Collection of kolanut and preparation of aqueous extract

Fifty-five kg of fresh red (commonly consumed) kolanut fruits were bought from kolanut plantation, Ode Remo, Shagamu, Ogun State, Nigeria. This was taken to the Botanist at the Federal Research Institute of Nigeria for identification and authentication, and an herbarium index number (HIN) was obtained, 109605. Kolanut fruits were washed thoroughly to remove dirt, cut into small cubes and room dried until a constant weight was achieved (28 kg). The dried kolanut fruits were grounded with a miller and mixed with distilled water. The soaked kolanut was vortexed every one hour for 6 hours and the supernatant filtered first with cloth sieve, then with Whatman filter paper 1 (cat. No. 1001. 125). The filtrate was concentrated using a rotary evaporator (BUCHI Labortechnik AG: CH-9230 Flawil Switzerland), freeze dried (LTE Lyotrap plus by LTE scientific LTD. Great Britain) and stored in airtight container in the refrigerator at 4°C. The refrigeration helped in keeping its consistency without fermenting till it was ready for the experiment.

### 3.1.2 Experimental animals

Twelve (12) sexually matured female *Sprague Dawley* rats aged 11-12 weeks, weighing approximately 200-300g (and a total of 72 Sprague Dawley pups) were used for the study. The female rats mated by matured *Sprague Dawley* male rats (at the ratio of 1 female to 1 male overnight) and mating noted by vaginal plugs with confirmation sperm smear. This day was taken as pregnancy day 1 (McDonnell-Dowling et al., 2017). The 12 dams gave the needed minimum pups of 72 (calculated by power of study (Charan and Kantharia, 2013) and the pups were sacrificed at different PNDs; 0, 7, 21, 56, 70 (Appendix I).

All procedures on animal handling were conformed to the acceptable guidelines on the ethical use of animals in research as approved by the Animal Research Ethics Committee of the University of the Witwatersrand with ethical clearance certificate number: 2020/06/02/C.

### 3.1.3 Animal housing

The animals were obtained through the Central Animal Sciences (CAS) and housed in CAS building, University of the Witwatersrand, Johannesburg, Republic of South Africa. The dams were kept under sterile conditions in individual Perspex cages of 43mm long, 220mm wide and

200mm height allowing free movement of the rats. After parturition, the pups remained with their biologic dams until they were weaned at PND 21, and thereafter sorted into different individual cages according to sex. Standard animal house conditions were maintained (temperature: $25^0$C, light approximately 12 hours' light dark cycle).

### 3.1.4 Animal treatment group

The 12 pregnant dams were randomly divided into two groups with 6 animals each:

**Group 1 (Control):** The pregnant dams received plain gelatine cubes via oral route from first day of pregnancy till parturition.

**Group 2 (Treated):** The pregnant dams received 400 mg/kg body weight of aqueous crude extract of kolanut (Atiba et al., 2021) infused in gelatine cubes via oral route from first day of pregnancy till parturition.

Also, the following subgroups were achieved based on different termination timelines.

**Sub-group 1(PND 0):** The group helped to determine the immediate intrauterine effects on the developing brain.

**Sub-group 2 (PND 7):** This group was used to determine the effects of kolanut at neonatal period.

**Sub-group 3 (PND 21):** This group was used to determine the extent of kolanut impact after weaning.

**Sub-group 4 (PND 56):** This group helped to determine effect of kolanut exposure during intrauterine life in adulthood.

The experimental design was further explained in (appendix I) in the flow chart. The sacrifice at different developmental stages was to determine the progress of exposure effect, stage at which specific effect of the exposure occurred and impact of prenatal exposure in adult life.

### 3.1.5 Oral gelatine cubes

In a preliminary study (Atiba et al., 2021), the LD50 was established and the 400 mg/kg dose was found to be adequate for observable alteration in the Wistar rats strain. The control dams received only plain gelatine cubes (Zhang et al., 2012; Dhawan et al., 2018). The drug was administered through oro-gastric route, which mimicked the natural route of administration to pregnant women (Turner et al., 2011). After administration, the animals were allowed to continue normal Altromin rodent breeder diets (Lab Chef: Johannesburg, South Africa) and water *ad libitum*.

### 3.1.6 Food and water intake

The dams were fed normal rat chow and drank water supplied by the animal unit of the University of the Witwatersrand. From the first day, starting with 500 g and 500 ml of water, measurement of the amount of food eaten and water drank were done and topped it to 500 g and 500 ml respectively. This process was repeated every 3 days until parturition.

### 3.1.7 Body weight

The body weights of both the dams and the pups were measured throughout the study using meter weighing scale (Clover South Africa, WP003). The dams' body weights were measured every 3 days till parturition while the pups' body weights were measured every week till PND 70.

### 3.1.8 Behavioural tests

Some of the cognitive tests to assess hippocampal function were cliff avoidance, surface righting, spontaneous movements, open field, object recognition and location, and radial-arm maze (Antunes and Biala, 2012; Mustapha et al., 2014; Nguyen et al., 2017; Delcourt et al., 2018). On PND 4, 5, 6, and 7, 10 animals from each group underwent cliff avoidance, surface righting and spontaneous movements; while open field, novel object recognition and location and radial arm maze were only done on PNDs 21 and 56 to measure kolanut effects on short- and long-term memory. All tests were done between the hours of 9:00 am and 3:00 pm (Fujimoto et al., 2015) and the animals were brought into the test room 30 minutes before the actual tests in order to acclimatize before the tests (Denninger et al., 2018).

#### 3.1.8.1. Cliff avoidance

This test was done to assess developmental reflex and memory in neonates. The snout and forelimbs of the pups were placed over the edge of the tabletop, with shavings of soft bedding placed 15 cm under the edge, and the maximum time scored was 20 secs. The time it took each pup to turn from the edge were recorded and scored; 0 =no response or fall over the edge within 20 secs, 1 =attempted/avoided backwards within 20 secs, 2 =avoided with a successful turn (Tanaka, 2012; Nguyen et al., 2017).

#### 3.1.8.2. Surface righting

This test was also used to assess the developmental reflex and memories in the neonates, through the repetition of the test for 4 days. The decrease in the time taken to perform the task were used to assess learning and memory. The pups were placed in the supine position with the four paws

facing upwards. The time started immediately after letting go of the pup. Righting was achieved once the pup flipped and all four paws were on the surface. The maximum time allocated was 15 minutes. This task was scored as follows; 0=for laying on the back throughout the allocated time, 1=laying on either left or right side throughout the allocated time, 2=successfully placed the four paws on the surface within the allocated time (Nguyen et al., 2017).

#### 3.1.8.3. Spontaneous movements

This test was used to assess the locomotor activities in neonatal pups in the glass container of 10 cm in diameter and 10 cm in height. Different measurements were done within the speculated time of 2 minutes. This includes locomotion (general translocation of the body of at least 1 cm in the glass container), immobility (no visible movement of the animal when placed with all the four paws on the floor of the glass container), head rising (number of head rising up and forward), face washing (forepaws moving back and forth from the ears to the snouts and occasionally to the mouth), wall climbing (placements of forelimbs on the wall of the container), pivoting (locomotor activity involving the front limbs resulting in lateral oriented movements) and curling (a vigorous rolling of the animal while on its back or side and drawing the head closer to the hump). The frequency and duration of each behavioural pattern were measured (Tartaglione et al., 2020).

#### 3.1.8.4. Open field

This test was used to assess locomotor activity, involving exploratory movements and anxiety. Each animal was placed in a 120 cm × 120 cm box with black background for 5 minutes. The open field box was cleaned with 70% ethanol and allowed to dry between tests. The distance travelled by each animal and mean velocity were recorded and analyzed with ANY-maze behavioral tracking software (Tartaglione et al., 2020). Presence of urine and fecal bolus were noted.

#### 3.1.8.5. Novel object recognition and Location (NOR/NOL)

NOR/L has been used to test the effects of various pharmacological treatments and brain damage (Goulart et al., 2010). These tests are useful to study short-term, intermediate-term and long-term memories through manipulation of the retention interval (Antunes and Biala, 2012; Khazen et al., 2019). The NOR and NOL tasks were used to evaluate the rodents' ability to recognize a novel object and location in the environment without any positive or negative reinforcement (Ennaceur and Delacour, 1988; Gerstein et al., 2013). In this study, the open field box was used as the

environment and the animals completed three stages of the tests. These two tests were done in three days; Day 1 signified habituation stage, in which each animal was introduced into the environment by placing them facing the walls of the corner (release corner) and allowed to roam freely for a period of 5 min and this was done twice on the same day, making the habituation period to be 10 min per animal. Day 2 was the training stage. This was done exactly 24 hrs after the habituation stage, but two similar objects (plastic leggo) were placed at the opposite sides (represented by A & B) of the open field box at 2.5 cm from the corners of the box. Each animal was allowed to roam freely and explore the two similar objects for a period of 10 min. At day 3 (24 hrs after training), test stage, each animal was tested for spatial memory by measuring reference for the object in a novel location versus familiar location (NOL) using the same objects while NOR was tested by replacing one of the familiar objects with a novel (bottle) object and the reference for the novel object versus the familiar object were determined. The following parameters were measured; total amount of time spent exploring (when its head was oriented toward the object within 1cm or the nose/whisker touching the object) the novel and familiar object (NOR) and at novel location (NOL); relative exploration time (novelty index) was calculated using:

$$\frac{\text{Time spent exploring object in novel location}}{\text{Time spent exploring both objects in total.}} \times 100$$

Also, discrimination index was calculated using (tn-tf)/(tn+tf):

tn = amount of time rats explored novel object; tf = amount of time rats explored the familiar object.

Also, the path taken by each animal was recorded too.

**3.1.8.6. Radial arm maze (RAM)**

Spatial memory is highly important to humans and species survival. RAM is one of the most suitable devices for measuring cognitive research to understand exploratory behaviour (Olton and Samuelson, 1976); spatial learning and memory (Brown and Giumetti, 2006), social learning and decisions making (Brown et al., 2009; Delcourt et al., 2018), underlying brain structures (Crusio and Schwegler, 2005) and neurotoxicology (Walsh and Chrobak, 1987; Levin, 2015). RAM is mostly used in isolated experimental animals such as rodents, pigs, rabbits, hedgehogs, dogs, bird

species and reptiles (Lipp et al., 2001; Pleskacheva, 2009; Macpherson and Roberts, 2010; Mueller-Paul et al., 2012; Delcourt et al., 2018). The apparatus consisted of eight horizontal arms (57 cmx11 cm), placed radially away from a central region above the floor, and doors (20 cm high) placed at the entrance of each arm. The animals were deprived of food during the dark cycle and given the baits daily for three days before the test began in order to stimulate them to eat the baits during tests (Dale and Roberts, 1986; Timberlake and White, 1990; De Luca et al., 2016). The test consisted of three stages; habituation, in which each animal was placed at the centre stage and allowed to roam freely for a period of 5 min within the maze for three consecutive days with daily reinforcer (but reduced on the last day of habituation) scattered at the centre and arms of the maze and the habituation stage ended when all the arms have been visited; the training stage and test stages were done on the same day for four consecutive days. During the training stage, 4 arms were blocked, and the remaining four arms were opened and baited, and the rats allowed to freely explore for 5 minutes to retrieve the baits. After the 5 minutes of training the animals were retrieved and kept in their own cage for 5 minutes while the arms were wiped with 70 % alcohol and dried. The blocked arms were now opened and baited, and each animal was brought to the centre and tested for another 5 minutes. Number of errors (working and reference), correct choices and entries into different arms were recorded (Tarragon et al., 2012; Delcourt et al., 2018).

### 3.1.9. TERMINATION OF RATS AND TISSUE PREPARATION

The animals were weighed and terminated at different PNDs with an intramuscular lethal dose of pentobarbital at 240 mg/ml (Zatroch et al., 2017) and graded based on the age of the animals. Intra-cardiac perfusion of the animals with phosphate buffer saline was done after the termination. The brains and other organs were harvested, weighed, and put in different fixatives for histology and immunohistochemistry, some were placed in -80$^{o}$C for biochemical analyses while the hippocampal tissues were placed in RNAlater, a non-toxic tissue reagent that preserves the quality and quantity of RNA for molecular analyses.

### 3.1.10. HISTOLOGICAL EVALUATION

#### 3.1.10.1. Nissl staining histological technique.

The brain hemispheres were sectioned sagittal with a cryostat and collected in 1:5 series in phosphate buffered solution (PBS). The sections were mounted onto 0.5 % chrome alum gelatin coated slides and dried overnight. The dried sections were placed in solution of 50 % alcohol and

50 % chloroform overnight. These slides were rehydrated in alcohol (100 %-5 minutes (2 changes), 95 %-2 minutes, 70 %-2 minutes, 50 %-2minutes) then placed in filtered cresyl violet stain (1minute) and washed in distilled water (1minute). The slides were dehydrated in ascending alcohol (50 %-2 minutes, 70 %-90 secs, 95 %-2 minutes, 100 %-5 minutes), these were cleared in two changes of xylene at 5 minutes each. Mountant, Entellen® new (Merck KGaA, Germany, 2019) were applied and cover slipped. They were checked for bubbles under a light microscope and left to dry overnight and stored in slide boxes.

**3.1.10.2. Golgi-cox staining histological technique.**

A Golgi-cox staining method adapted from the protocols used by Bayrom-Weston et al. (2016) was used for this study. The Golgi–cox solution consists of two solutions as follows:

Solution A: 100 ml of 5% potassium dichromate solution (Sigma-Aldrich, no. P5271, USA) stirred into warm deionized water, with100 ml 5% mercuric chloride (Sigma-Aldrich, no. M136, India) stirred into hot deionized water. Solution B: 200 ml of dH2O and 80 ml of 5 % potassium chromate (Sigma-Aldrich, no. 216615, USA) stirred into cold deionized water. Solution A was then slowly poured into solution B with constant stirring. A red-yellow precipitate was formed. The Golgi–Cox solution was stored in the dark for 72 hours. and filtered before use.

**3.1.10.2.1. Tissue fixation**

The tissues were fixed in 4% paraformaldehyde (4% PFA) (Fischer Scientific, Loughborough, UK) for 24 hrs. at room temperature. They were transferred to Golgi-cox solution for 14 days in the dark with the solution changed daily. After sufficient impregnation, on the 14th day, the solution drained, and excess fluid removed with blotting paper and transferred to $+4^0$C of 25% sucrose in 0.1 M Phosphate Buffered Saline (PBS), then stored in the dark until they sink. They were transferred into antifreeze, wrapped with foil and stored at $-20^0$C until ready for sectioning.

**3.1.10.2.2. Sectioning**

The brain tissues were replaced in 25 % sucrose two days before sectioning. The brains were cut into 80 µm coronal sections using a freezing-stage sledge microtome. The cut sections were collected in a 1:6 series in Tris-buffered saline (TBS) solution at $+4^0$C. Sections were mounted onto gelatine-coated slides and left to air dry overnight at room temperature in the dark. Sections were washed with dH2O for 5 minutes and transferred into 20% ammonium hydroxide with dH2O (ACES, no. 38499, Johannesburg, SA) for 10 minutes. The sections were washed in dH2O for a further 5 minutes, passed through ascending grades of alcohol (70%, 95% and 100%), placed in xylene for 40 minutes, followed by cover slipping with Entellen® new (Merck KGaA,

Germany, 2019).

### 3.1.11. IMMUNOHISTOCHEMISTRY

#### 3.1.11.1. Single immunohistochemistry for Ki-67 and DCX

The brain tissues were fixed in 4% PFA (Fischer Scientific, Loughborough, UK) for 24 hours. at $+4^0$C. These were placed in 30% sucrose for two days before sectioning. The brains were cut into 50 µm sagittal sections using a freezing-stage sledge microtome. The cut sections were collected in a 1:5 series in phosphate buffer saline (PBS) solution of 24 wells-plate at $+4^0$C. Sections from B1-B6 of each plate were washed thrice for 10 minutes in phosphate buffer (PB) under a gentle vortex at room temperature before being transferred into an endogenous peroxidase inhibition solution (49.2% Methanol, 49.2% PB and 1.66% $H_2O_2$) for 30 minutes. The sections were then washed thrice for 10 minutes and treated with a blocking solution (0.25% Triton X-100 in 0.1M PB, 3 % normal goat serum, 2 % bovine serum albumin (BSA)) for 2 hours at room temperature under gentle shaking. Each section was incubated in 500 ul of primary antibody (Ki-67 (Ab 15580/Merck (MAB 4190)/DCX (Ab 8723 MAB 17066)) prepared in blocking buffer solution at +4 ºC under gentle shaking for 48 hours. After the incubation, the sections were placed on the bench to reach room temperature, followed by a 3 times 10 minutes wash in 0.1 M PB under gentle shaking at room temperature. Sections were then incubated with (biotinylated goat anti-rabbit polyclonal) secondary antibodies in 0.1 M PB supplemented with 3 % normal serum, 2% BSA for 2 hours at room temperature under gentle shaking. After that, sections were washed 3 times for 10 minutes in 0.1 M of PB under gentle shaking at room temperature. Thirty minutes before the time of use, Avidin Biotin Complex (ABC) reagent in PB (40ul A + 40ul B in 5 ml of PB (0.1M) with 3% Normal serum and 2% BSA) were prepared and applied to the brain sections for 1 hour at room temperature under gentle shaking. Sections were then washed in PB 3 times for 10 minutes each under gentle shaking at room temperature. The sections were then incubated in DAB chromogen for 5minutes at room temperature under gentle shaking. 700ul of DAB were placed per well and allowed to stand for 5 minutes at room temperature. Then 3ul of 30% $H_2O_2$ per 1ml of DAB solution were added to the well. The brain sections were placed in PB as soon as the color developed. Sections were then washed in PB 3 times for 10 minutes each under gentle shaking at room temperature, then mounted on 0.5% gelatine coated slides and left to dry for two days. Dehydration was done in ascending alcohol (70%-2hrs, 95%-5minutes, and two changes of 100%-5 minutes each) and cleared in two changes of xylene for 5 minutes each. The slides were mounted with Entellen® new (Merck KGaA, Germany, 2019), cover slipped and left to dry.

### 3.1.12. BIOCHEMICAL ASSAYS (TBARS, SOD, GPX, GSH, BDNF, ACTH)

Biochemical assays carried out were Protein concentration determination, Lipid peroxidation (LPO), Superoxide dismutase (SOD), Reduced glutathione (GSH) and Glutathione peroxidase (GPX). Activities were ran using the spectraMax plate reader (Molecular Devices, CA, USA). Brain derived neurotropic factor (BDNF) and Acetylcholine (ACTH) activity were run by an ELISA kit. The brain tissues used for these assays were homogenized in 0.01M of PBS.

#### 3.1.12.1. Protein Determination

Protein concentrations of the pup brains were performed following manufacturer's protocol, BIO-RAD (Farbstoff-Konzentrat; Cat No. 500-0006 (Heidemannstralse, 164.80939 München) and method (Bradford, 1976).

#### 3.1.12.2. Assessment of Lipid Peroxidation (LPO)

Lipid peroxidation was determined by measuring the formation of thiobarbituric acid reactive substances (TBARS) present in the test sample according to the method of Varshney and Kale (1990).

**Principle**

This method is based on the reaction between 2-thiobarbituric acid (TBA) and MDA: a product of lipid peroxide during peroxidation. On heating in acidic pH, the product is a pink complex which absorbs maximally at 532 nm, and which is extractable into organic solvents such as butanol. Malondialdehyde (MDA) is often used to calibrate this test and thus the results are expressed as the amount of the free MDA produced.

**Preparation of reagents**

1. **30% Trichloroacetic acid (TCA)**

   6g of TCA ($CCl_3COOH$) was dissolved in distilled water and made up to 20 ml with the same.

2. **0.1 M Hydrochloric acid (HCl)**

   17.33µl of concentrated HCl (36.5-38%) was added to distilled water and the volume made up to 20ml with the same.

3. **0.75% Thiobarbituric acid (TBA)**

   0.1125 g of TBA was dissolved in 0.1 M HCl and made up to 15 ml with the same. Dissolution was aided by stirring in a hot water bath (50˚C).

**4. 0.15 M Tris-KCl buffer (pH 7.4)**

0.6708 g of KCl and 1.0908 g of Tris base were dissolved in 55 ml of distilled water, the pH was adjusted to 7.4 with HCl and the volume made up to 60 ml with the same.

**Procedure**

An aliquot of 100µl of the brain homogenate was mixed with 400µl of Tris-KCl buffer to which 125µl of 30% TCA was added. Then 125µl of 0.75% TBA was added and placed in a water bath for 45 minutes at 80$^{o}$C. This was then cooled in ice to room temperature and centrifuged at 3000 rpm for 10 min. The clear supernatant was collected, and absorbance measured against a reference blank of distilled water at 540nm using spectra-max plate reader.

**Calculation**

The MDA level was calculated using an extinction coefficient of 0.156 $\mu M^{-1}cm^{-1}$ (Adam-Vizi and Seregi, 1982).

$$\text{Lipid peroxidation (nmole MDA/mg protein)} = \frac{\text{Absorbance} \times \text{volume of mixture}}{E_{532nm} \times \text{volume of sample} \times \text{mg protein/ml}}$$

=mmol/mg protein

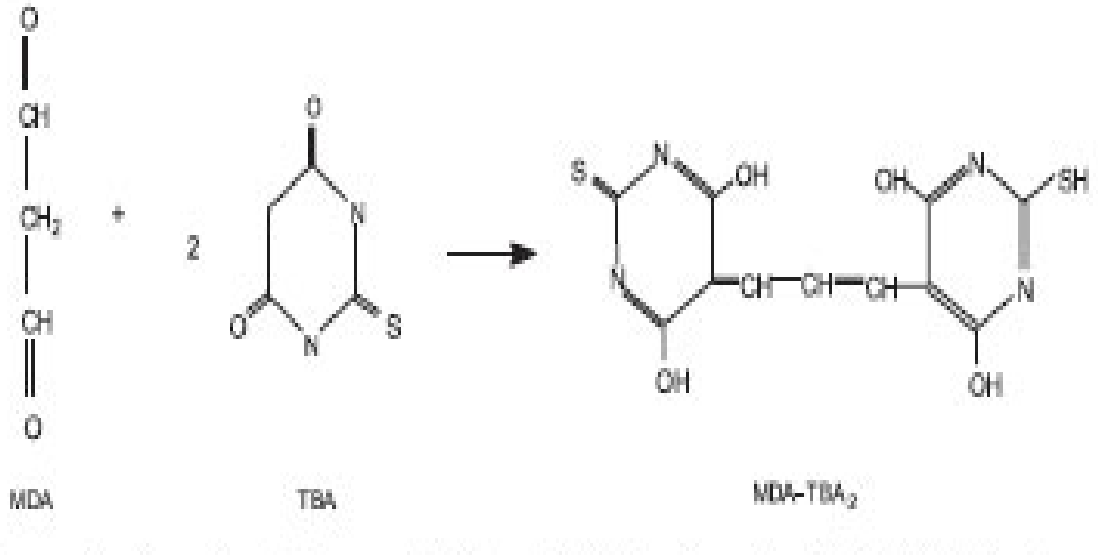



**Figure 3. 1**. Structure of TBA+MDA ⟹ MDA-TBA (pink colored complex)

### 3.1.12.3. Determination of Superoxide Dismutase (SOD) Activity

The brain SOD activity was determined by modification of the methods of Murthy et. al., 2002 and Beauchamp and Fridovich (1971) which was based on the reduction of Nitro Blue Tetrazolium (NBT) to water insoluble blue formazan.

Preparation of reagents

1. 10% brain homogenate

   1g of brain tissue in 10ml of 0.01 M PBS

2. 0.01 M PBS

   1ml of 0.1 M of PBS was added to 9ml of distilled water and pH to 7.4.

3. 50mM Sodium carbonate

   0.53 g of sodium carbonate was dissolved in 80 ml of distilled water and made up to 100ml of the same.

4. 24 µM of Nitro Blue Tetrazolium (NBT)

   0.045 g of NBT was dissolved in 8 ml of distilled water and made up to 10 ml of the same.

5. 0.1mM EDTA

   0.037 g of EDTA was dissolved in 900 ml of distilled water and made up to 1L of the same.

6. 1mM Hydroxylamine hydrochloride

   0.0695 g of hydroxylamine hydrochloride was dissolved in 1L of distilled water.

**Procedure**

1 ml of 50 mM sodium carbonate was added to 0.4 ml of 24 µM of NBT and 0.2 mL of 0.1 mM of EDTA. Then 0.5 ml of brain homogenate was also added to the solution. The reaction was initiated by adding 0.4 ml of 1 mM hydroxylamine hydrochloride to the solution. The absorbance was read at 560 nm at zero and five mins at $25^0$C. This was done concurrently for the control (all the reagents without brain homogenate). The unit of SOD was expressed as the amount of enzyme needed to inhibit the reduction of NBT by 50%. The specific activity was expressed in terms of units per milligram of proteins.

### 3.1.12.4. Determination of GPX activity

Glutathione peroxidase (GPX) activity was determined based on some modifications of Rotruck *et al.* (1973) method.

**Principle**

Glutathione peroxidase is allowed to bind $H_2O_2$ to glutathione for a particular period after which the reaction is stopped. The remaining glutathione is reacted with Ellman's reagent and the remaining measured for enzyme activity.

**Preparation of reagents**

1. 10 mM sodium azide

3.25g of sodium azide was dissolved in 50 ml of distilled water.

2. 4 mM Reduced glutathione

12.3 mg of GSH was dissolved in 10 ml of 0.1 M phosphate buffer, pH 7.4.

3. 2.5 mM Hydrogen peroxide

14 µl of 30% hydrogen peroxide was added to distilled water and the volume made up to 50 ml with distilled water.

4. 10% Trichloroacetic acid

   2 g of TCA was dissolved in distilled water and the volume made up to 20 ml with the same.

5. 0.3 M Dipotassium hydrogen orthophosphate

   4.11g of $K_2HPO_4.3H_2O$ was dissolved in distilled water and the volume made up to 60 ml with same.

6. Ellman's reagent (DTNB)

   19.8 mg of DTNB was dissolved in 50 ml of 0.1 M phosphate buffer, pH 7.4.

7. 0.1 M, pH 7.4 Phosphate buffer

   1.399 g of dipotassium hydrogen phosphate trihydrate and 0.527 g of potassium dihydrogen phosphate were dissolved in 90 ml of distilled water, the pH adjusted to 7.4 and the volume made up to 100 ml with distilled water.

**Procedure**

To 50 µl of phosphate buffer in a test tube was added 10 µl of $NaN_3$, 20 µl of GSH, 10 µl of $H_2O_2$ and 50 µl of brain homogenate (added last). The reaction mixture was incubated for 3 minutes at

37˚C after which 50 µl of TCA was added and the final mixture centrifuged at 3,000 rpm for 5 minutes. To 50 µl of the supernatants, 100 µl of $K_2HPO_4$ and 50 µl of DTNB were added and the absorbance read against a reagent blank of 50 µl distilled water, 100 µl of $K_2HPO_4$ and 50 µl of DTNB at 450 nm in a micro plate using a plate reader.

**Calculation**

GSH consumed = initial GSH amount (129.39 µg) – GSH remaining

GPX activity = GSH consumed/mg protein

= µg GSH/mg protein

**3.1.12.5. Determination of Acetylcholine (ACH) in brain samples**

This was carried out using ELISA kit (Elabscience® Catalog No: E-EL-0081, USA).

**Principle**

This ELISA kit used a competitive-ELISA principle. The micro plate provided in the kit was precoated with ACH. The ACH in the samples and standard competed with the fixed amount of ACH on the solid phase supporter for sites on the Biotinylated Detection Ab specific to ACH.

**Reagents Preparation**

The kit and samples were brought out from the fridge 20 minutes before the procedure to allow them to be at room temperature.

**Wash Buffer**: 30 ml of concentrated wash buffer was diluted with 720 ml of deionized water to make 750 ml of wash buffer.

**Standard working solution**: The standard was first centrifuged at 10,000xg for 1 minute. 1 ml of reference standard and sample diluent was added to the standard and allowed to stand for 10 minutes, mixed thoroughly by pipette giving a working solution of 1000 pg/ml. Then a serial dilution gradient was made as follows; 1,000, 500, 250, 125, 62.5, 31.25, 15.63, 0 pg/ml.

**Biotinylated detection antibody working solution**: Concentrated biotinylated detection Ab was centrifuged at 800xg for 1 minute and diluted to 100x concentrated biotinylated Ab to 1x working solution with biotinylated detection Ab diluent (1:99). The calculated total volume needed was 5,000 µl. 50 µl of secondary Ab was added to 4950 µl of diluent biotinylated solution.

**Horseradish Peroxidase (HRP) conjugate working solution:** Concentrated HRP Conjugate was centrifuged at 800xg for 1 minute, then dilute the 100x concentrated HRP Conjugate to 1x

working solution with HRP conjugate diluent (1:99). 110 µl of concentrated HRP was added to 10,890 µl of HRP diluent to make the total volume needed.

**Procedure**

The samples were centrifuged at 10,000 rpm for 10 minutes. Add 50 µl of sample to their designated well (in duplicates) and immediately add 100 µl of biotinylated detection antibody working solution to each well. The plate was covered with a sealer that was provided alongside with the kit. The plate was incubated for 45 minutes at 37ºC. Afterwards, the solution was decanted from each well and 350 µl of wash buffer was added to each well, left to soak for 1 minute and decanted, left to dry on an absorbent paper. The wash was repeated three times using a microplate washer with excess conjugate and unbound sample/standard washed from the plate. 100 µl Avidin conjugated to Horseradish Peroxidase (HRP) were added to each microplate covered with a new sealer and incubated for 30 minutes at 37ºC. The solution was decanted and washed five times, then a 90 µl TMB substrate reagent was added to each well covered with a new sealer and incubated for 15 minutes at 37ºC. The enzyme-substrate reaction was terminated by the addition of 50 µl of stop solution. The plate was protected from light, the microplate reader (Anthos 2010) was preheated 15 minutes before measurement of the optical density and the color change was measured at a wavelength of 450 nm. The concentration of ACH in the samples was then determined by comparing the optical density of the samples to the standard curve.

### 3.1.12.6. Determination of Brain-derived Neurotrophic Factor (BDNF) in brain samples

This was carried out using ELISA kit (Elabscience® Catalog No: E-EL-R1235, USA).

**Principle**

This ELISA kit used a sandwich-ELISA technique. The micro-ELISA plate provided in the kit was precoated with an antibody specific to rat BDNF.

**Reagents Preparation**

The kit and samples were brought out from the fridge 20 minutes before use to allow them to be at room temperature.

**Wash Buffer**: 30 ml of concentrated wash buffer was diluted with 720 ml of deionized water to make 750 ml of wash buffer.

**Standard working solution**: The standard was first centrifuged at 10,000xg for 1 minute. 1 ml of reference standard and sample diluent was added to the standard and allowed to stand for 10

minutes, mixed thoroughly by pipette giving a working solution of 1,000 pg/ml. Then a serial dilution gradient was made as follows; 2,000, 1,000, 500, 250, 125, 62.5, 31.25, 0 pg/ml.

**Biotinylated detection antibody working solution**: Concentrated biotinylated detection Ab was centrifuged at 800xg for 1 minute and diluted to 100x concentrated biotinylated Ab to 1x working solution with biotinylated detection Ab Diluent (1:99). The calculated total volume needed was 5, 000 µl. 50 µl of secondary Ab was added to 4950 µl of diluent biotinylated solution.

**Horseradish Peroxidase (HRP) conjugate working solution:** Concentrated HRP Conjugate was centrifuged at 800xg for 1 minute then diluted the 100x concentrated HRP Conjugate to 1x working solution with HRP conjugate diluent (1:99). 110 µl of concentrated HRP was added to 10, 890 µl of HRP diluent to make a total volume needed.

**Procedure**

The samples were centrifuged at 10, 000 rpm for 10 minutes. Add 100 µl of standard or sample to their designated well and incubate for 90 minutes at $37^0$C. The liquid was removed completely and 100 µl of biotinylated detection antibody/antigen was added to each well and incubated for 1 hour at $37^0$C. The liquid was aspirated and washed three times with 350 µl of wash buffer. 100 µl of HRP Conjugate was added to the wells and incubated for 30 minutes at $37^0$C. The liquid was aspirated and further washed five times in 350 µl of wash buffer. A 90 µl of substrate reagent was added and incubated for 15 minutes at $37^o$C. The enzyme-substrate reaction was terminated by the addition of 50 µl of stop solution. The plate was preheated 15 minutes before measurement of the optical density and the color change was measured at a wavelength of 450 nm. The concentration of BDNF in the samples was then determined by comparing the optical density of the samples to the standard curve.

### 3.1.13. DETERMINATION OF MEMORY AND EARLY GENES

#### 3.1.13.1. RNA Extraction

RNA extraction was performed using the standard Trizol-chloroform-isopropanol method. The hippocampus tissues were excised at the termination site and kept immediately in 500 ul RNA later. On the day of the extraction, RNA free glass ware and equipment were prepared. Empty Eppendorf tubes were weighed first, then weighed with the hippocampus tissues in them. Three samples were used for each group. The tissues were dissected into smaller pieces with each sterile fine scissor for each specimen. 150 ul of Trizol (Sigma Aldrich, Missouri, USA) were added to each tube and mixed with the tissue. These were homogenised with homogenizer (Tissue Ruptor II, Qiagen, Hilden, Germany) using different tip for each sample. Additional 850 ul of Trizol were

added and mixed by shaking and allowed to stand in Trizol for 5 minutes at room temperature. 200 ul of Chloroform was added to each tube, shook briskly using a vortex for 15 seconds. This was also allowed to stand at room temperature for 3 minutes. This concoction was centrifuged over ice at 14, 000 rpm for 15 minutes. The colourless supernatant was collected while the middle and bottom layers were discarded. 500 ul of Isopropanol was added and allowed to stand at room temperature for 10 minutes. This was centrifuged for 15 minutes at 14, 000 rpm. The supernatant was decanted and added to the pellet. This was centrifuged at 8, 000 rpm for 5 minutes. The isopropanol was removed and allowed to stand for 10 minutes at room temperature. The pellets were dissolved in 70 ul of RNAse free water by repeatedly pipetting. This was placed on a heating block at 60°C for 10 minutes. The concentration and the purity ($A_{260}/A_{280}$) of RNA were determined in each sample with NanoDrop 1,000 version 3.81 Spectrophotometer (Thermo Scientific, Waltham, Ma). The most suitable RNA based on the level of purity and concentration were used for the procedure. The RNA was then stored at -80°C until use.

**3.1.13.2. Reverse Transcription (cDNA synthesis)**

Applied Biosystems high-capacity Reverse Transcription Kit. (Cat # 4368814) was used to reverse-transcribe the extracted RNA. Standard MIQE guidelines were followed. PCR tubes, water and pipette tips were autoclaved at 180 kpa for 30 minutes before use. All reagents were kept on ice throughout the experiment. RNA was diluted using the following equation: C1V1=C2V2. Reactions were set up as follows:

**Table 3.1. RNA Dilution Reaction**

| Component | Volume/reaction µl | NRT control µl | Water control µl |
|---|---|---|---|
| **10 RT Buffer** | 2.0 | 2.0 | 2.0 |
| **25 x DNTP mix (100mM)** | 0.8 | 0.8 | 0.8 |
| **10 RT random primer** | 2.0 | 2.0 | 2.0 |
| **Nuclease free water** | 4.2 | 5.2 | 4.2 |
| **Reverse Transcription enzyme** | 1.0 | - | 1.0 |
| **Total per reaction** | 10 | 10 | 10 |

An equal volume of RNA (10 µl) was added to each reaction. Reverse transcription was run on a PCR machine using the following temperature cycles:

**Table 3.2. PCR Temperature Cycles**

| | **Step 1** | **Step 2** | **Step 3** | **Step 4** |
|---|---|---|---|---|
| **Temperature (°C)** | 25 | 37 | 85 | 4 |
| **Time (min)** | 10 minutes | 59.59 minutes X 2 | 1 minute | ∞ |

### 3.1.13.3. Quantitative PCR

Quantitative PCR reaction was set up as follows and run on QuantStudio™ 5 Real-Time PCR machine, 96-well (Applied Biosystems; ThermoFisher Scientific Inc., Norway). The mRNA level of AQP4 was normalized to GAPDH and β-Actin and was calculated using $2^{-\Delta\Delta Ct}$

**Table 3.3. mRNA level calculation**

| **Components** | **Initial** | **Final** | **Per tube** |
|---|---|---|---|
| **Water** | | | 1.52 |
| **Syber Green** | 2x | 1 | 6µl |
| **Forward Primer** | 10 µmol | 0.2 µmol | 0.24 µmol |
| **Reverse Primer** | 10 µmol | 0.2 µmol | 0.24 µmol |
| **cDNA** | 2.5 ng/µl | 10 ng | 4 µl |
| **Total volume** | | | 12 µl |

The reaction samples, including the inter-plate calibrator, were organized in triplicates. MicroAmp™ 0.1mL 96-wells white plates (Applied Biosystems) were used for the quantitative PCR. The plate was covered with optical adhesive film (Applied Biosystems) and centrifuged using a plate centrifuge prior to placing in the thermocycler. Amplification of CDNA was performed on the StepOne thermocycler (Applied Biosystems).

PCR Cycling Parameters for Power Up SYBER Green Master Mix were as follows:

1. UDG activation at 50°C for 2 minutes
2. Dual –Lock DNA polymerase at 95°C for 2 minutes (x 40 cycles)
3. Denature at 95°C for 15 seconds
4. Anneal/extend at 60°C for 1 minute

### 3.1.13.4. Genes and Primers

The primers for genes of interest were designed online using Eurofins genomics tool (https://www.eurofinsgenomics.com) and analyzed on IDT OligoAnalyzer tool

(https://eu.idtdna.com/calc/analyzer) available online. The primers were purchased from Inqaba Biotechnical Industry, Pretoria, South Africa.

The primer sequences of the genes of interest and house-keeping genes were as follows:

**Rattus Norvegicus *c-fos***

Forward primer 5'ACAGGGTGCTTATGGGAAGGA3'

Reverse primer 5'CTTGGCCTCCCTGCATAGGT3'

**Rattus Norvegicus *c-jun***

Forward primer 5'ACCAGAGGTCATTCTCGGCA3'

Reverse primer 5'ACCTGGGAACAAAACACCACC3'

**Rattus Norvegicus *CREB1***

Forward primer 5'AGGTAATAGTGTAAGATGTTAGAATAAGG3'

Reverse primer 5'CAACCTTAAATCTCTCTCCCCTAA3'

**Rattus Norvegicus *CREB2***

Forward primer 5'TTTGACGCGGTGTTTACGTG3'

Reverse primer 5'AGACGCCATAACAACCCCAG3'

**Rattus Norvegicus *Dlg3***

Forward primer 5'CACTGGTTGGGAAGGGGTTG3'

Reverse primer 5'CCCCTTCTCTTCCTGTAGGTT3'

**Rattus Norvegicus *Dlg4***

Forward primer 5'TCCCTTCCCACCCTTCTTATTT3'

Reverse primer 5'TACAGCGGGTACTCCTTGAGA3'

**Rattus Norvegicus *GAPDH***

Forward primer 5'AAGAAGGTGGTGAAGCAGG3'

Reverse primer 5'CAAAGGTGGAAGAATGGGAG3'

**Rattus Norvegicus *ACTB***

Forward primer 5'AACCTTCTTGCAGCTCCTC3'

Reverse primer 5'ACCCATACCCACCATCACAC3'

### 3.1.14. IMAGES

The histological images were scanned and captured using Olympus microscope (VS200 ASW 3.4.1) and analyzed by OlyVIA 3-4-1 26606.

### 3.1.15. DATA ANALYSIS

All data were tested for normality using the Shapiro-Wilks test. When data was found to be normally distributed, ANOVA was used for comparisons and when not normally distributed Kruskal-Wallis was used. All data were computed and compared using ANOVA followed by Bonferroni *post hoc* test or Kruskal-Wallis followed by Dunn's *post hoc* test. All analyses were performed using Prism 9 software (GraphPad software, CA). A significance level of $p<0.05$ was used.

### 3.1.16 FUNDING

The main funding for this research was through the Consortium for Advanced Research Training in Africa (CARTA) fellowship with supplemented funding from the Tertiary Education Trust Fund (TETFUND), Nigeria.

### 3.1.17 ENCOUNTERED PROBLEM

The encountered problem during this research includes delay in the timeline due to unnecessarily delayed visa (8 months), COVID-19 pandemic, inability of the dams to litter required pups and relative delay in getting the required chemicals, reagents, primers, and antibodies needed for this research due to inflation caused by COVID-19.

### 3.1.18 OUTCOME

At the end of this research, four articles are expected to be written and published in reputable journals:

- Prevalence and consumption pattern of kolanut among pregnant women in Ibadan metropolis. Published.
- Dynamic changes in the hippocampal memory index and biochemical indices in *Sprague Dawley* rats exposed to intrauterine kolanut. Published.
- Kolanut consumption in pregnancy and postnatal toxicosis: suppression of neurogenesis in hippocampal dentate gyrus of adolescent rat. Under review.
- Prenatal kolanut consumption provokes immediate and later-life hippocampal morphological aberration and decrease in arborization and synaptic density in *Sprague Dawley* rat. In preparation.

  However, as at the time of submission of the thesis, two have been published and two

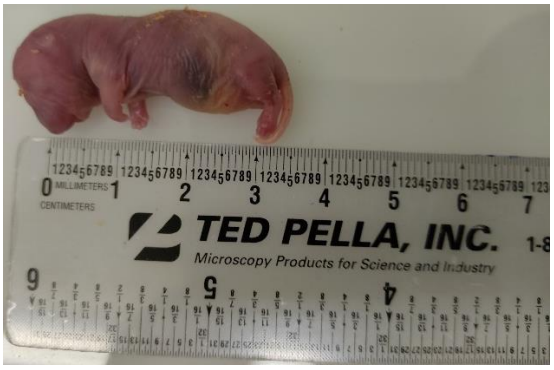
submitted. Dynamic changes in the hippocampal memory index and biochemical indices in *Sprague Dawley* rats exposed to intrauterine kolanut was presented at an international conference of Society of Neuroscientists of Africa (SONA) and Prenatal kolanut consumption provokes immediate and later-life hippocampal morphological aberration and decrease in arborization and synaptic density in *Sprague Dawley* rat would be presented in October 2024 at Society for Neuroscientists (SFN) conference in Chicago.

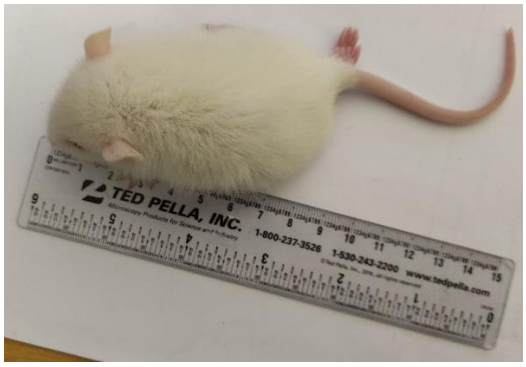
# CHAPTER FOUR

# RESULTS

## 4.1 General observations of the effect of kolanut consumption in dams and pups

The dams in the kolanut treated groups were more active and slightly aggressive compared with the control dams in the first week of kolanut treatment. At the second week post-birth, there was a marked reduction in body weight, amount of food pellets eaten in kolanut-treated dams but increase in the volume of water intake. It was also observed that two out of six (33.3 %) treated dams did not get pregnant and one (16.7 %) had stillbirth of 4 pups (figure 4.1a). Also, one of the pups (4.0 %) from the treated group had paralysis a few days after birth (figure 4.1b). The specific roles of prenatal kolanut consumption as observed in these conditions are yet to be determined.




**a.** **b.**

**Figure 4. 1: A**. shows one of the still births from kolanut-fed dams; **B.** shows the paralytic pup.

## 4.2 Gross morphometry of the dams following exposure to pre-natal kolanut consumption.

### 4.2.1 Effect of pre-natal kolanut consumption on body weight in the dams

The average body weight for 6 control and 6 kolanut treated dams were 306.8 ±7.548 g and 294.2 g respectively as presented in figure 4.2.1. There was a steady increase in body weight of the control dams while the treated weights were not constant. Although there was a difference in the body weight, it was not quite significant with a mean effect of treatment ($F_{(1,80)} = 2.788$; $p = 0.0989$). The mean effect of age throughout the parturition period was significant ($F_{(7,80)} = 14.43$; $p < 0.0001$). There was no significant difference in the interaction between treatment and age ($F_{(7,80)} = 1.586$; $p = 0.1517$)].

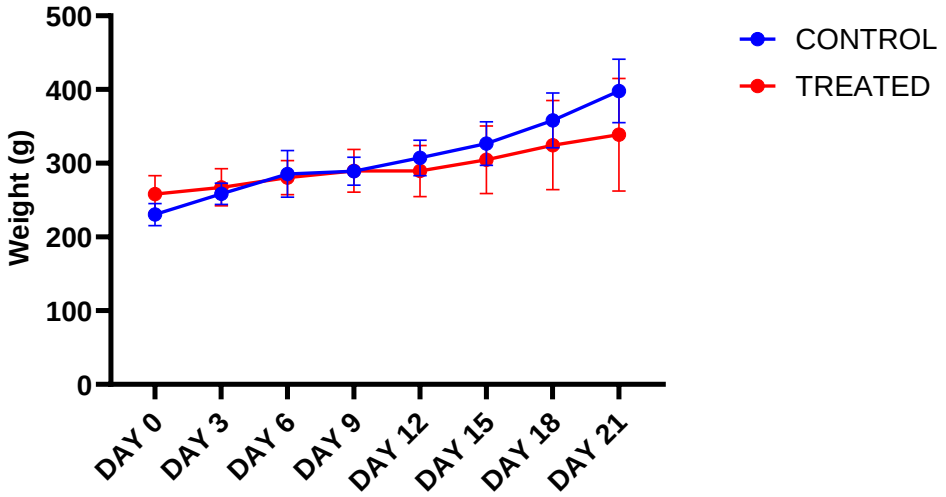



**Figure 4.2.1:** Effect of prenatal kolanut consumption on body weight in the dams. Data are represented as mean ± S.E.M.; significant differences are shown in P-value, *** against control.

### i. Effect of prenatal kolanut consumption on body weight in the pups

The average body weight for 10 control and 10 kolanut treated pups were 133.0 ±2.164 g and 125.5 g respectively as presented in figure 4.2.2. There was a steady increase in body weight of both the control and treated pups with the control pups having a higher body weight throughout the timelines than the treated pups. Although, there was body weight difference in the treatment effect across age groups, but the interaction was not significant [mean effects of treatment ($F_{(1, 308)} = 11.92$; $p = 0.0006$); mean effect of age ($F_{(10, 308)} = 785.4$; $p < 0.0001$); interaction between treatment and age ($F_{(10, 308)} = 1.310$; $p = 0.2241$)].

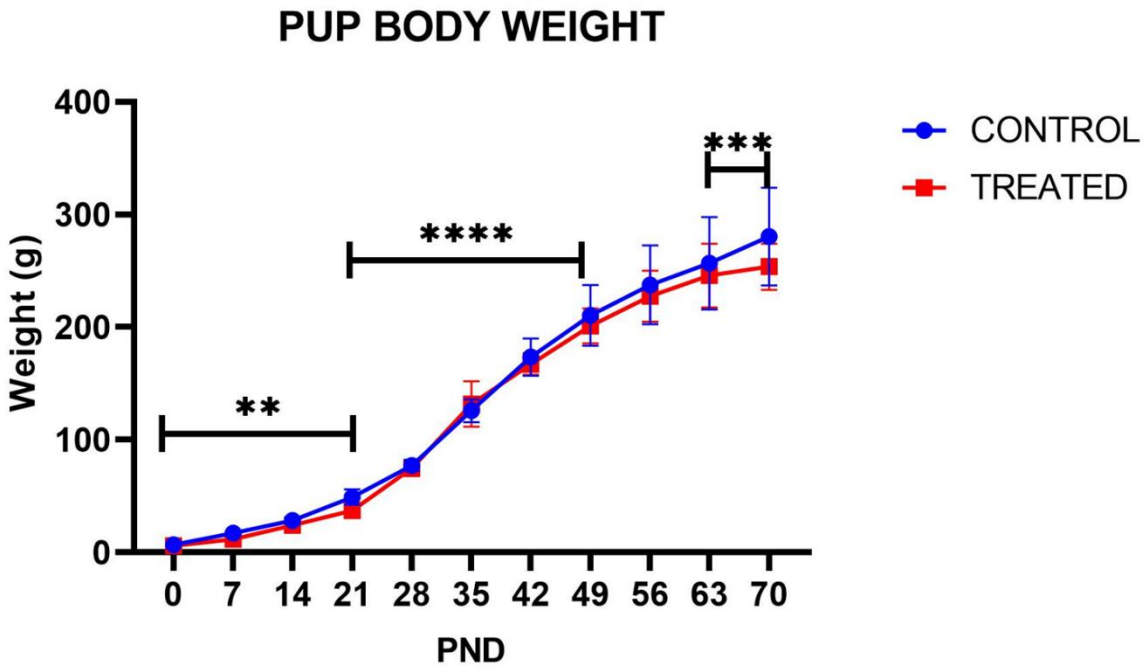



**Figure 4.2.2:** Effect of prenatal kolanut consumption on body weight in the pups. Data are represented as mean ± S.E.M.; significant differences are shown in P-value, */*** and **** against control.

### 4.2.3 Effect of prenatal kolanut consumption on brain weight in the dam

The mean brain weight for the 6 control and kolanut 6 treated dams were 2.00 ± 0.158 g and 2.15 g respectively as shown in figure 4.2.3. The treated dams had a significant [$F_{(18.96, 5,5)}$; p = 0.0058) higher brain weight when compared to the control dams.

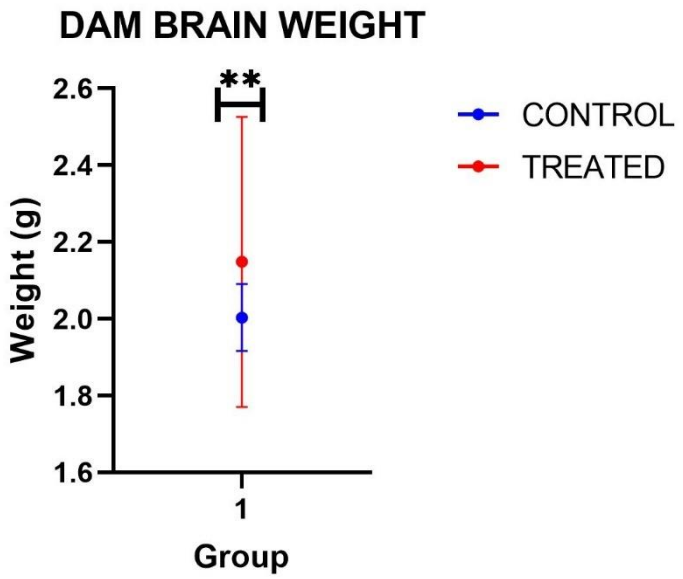



**Figure 4.2.3:** Effect of prenatal kolanut consumption on brain weight of the dams. Data are represented as mean ± S.E.M.; significant differences are shown in P-value, ** against control.

### 4.2.4 Effect of prenatal kolanut consumption on brain weight in the pups

The average brain weight for 10 control and 10 kolanut treated pups were 1.20 ± 0.016 g and 1.29 g respectively as presented in figure 4.2.4. There was a significant steady increase in brain weight of both the control and treated pups with the treated pups significantly evident from PND 56 till PND 70. There was a significant difference in the treatment effect across age groups [mean effects of treatment ($F_{(1, 90)} = 34.77$; $p < 0.0001$); mean effect of age ($F_{(4, 90)} = 1650$; $p < 0.0001$) interaction between treatment and age ($F_{(4, 90)} = 11.55$; $p < 0.0001$)].

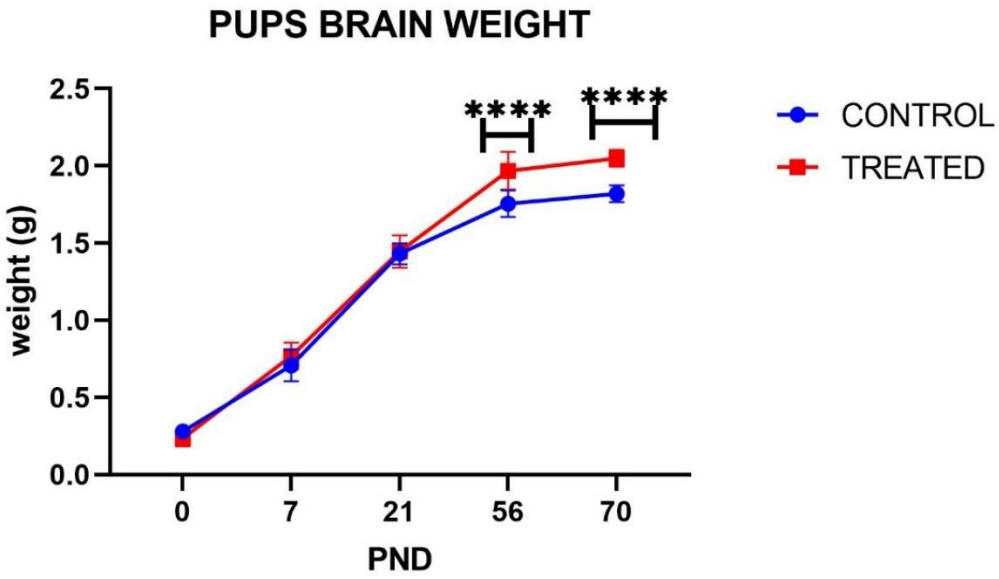



**Figure 4.2.4:** Effect of prenatal kolanut consumption on brain weight of the pups. Data are represented as mean ± S.E.M.; significant differences are shown in P-value, **** against control.

### 4.2.5 Effect of prenatal kolanut consumption on the level of food intake in pregnancy

The average food consumed by the 6 control and 6 kolanut treated dams were 119.6 ±2.559 g and 113.1 g respectively as presented in figure 4.2.5. There was a significant steady increase in the food consumed by both the control and treated dams until day 15 when there was a significant decrease in the amount of food consumed by the treated dams. The difference between food consumed by the treated dams and the control increased with age reaching a maximum difference at 24 days after commencement of the experiment [mean effects of treatment ($F_{(1, 90)} = 6.550$; $p = 0.0122$); mean effect of age ($F_{(8, 90)} = 1411$; $p < 0.0001$); interaction between treatment and age ($F_{(8, 90)} = 2.969$; $p = 0.0054$)].

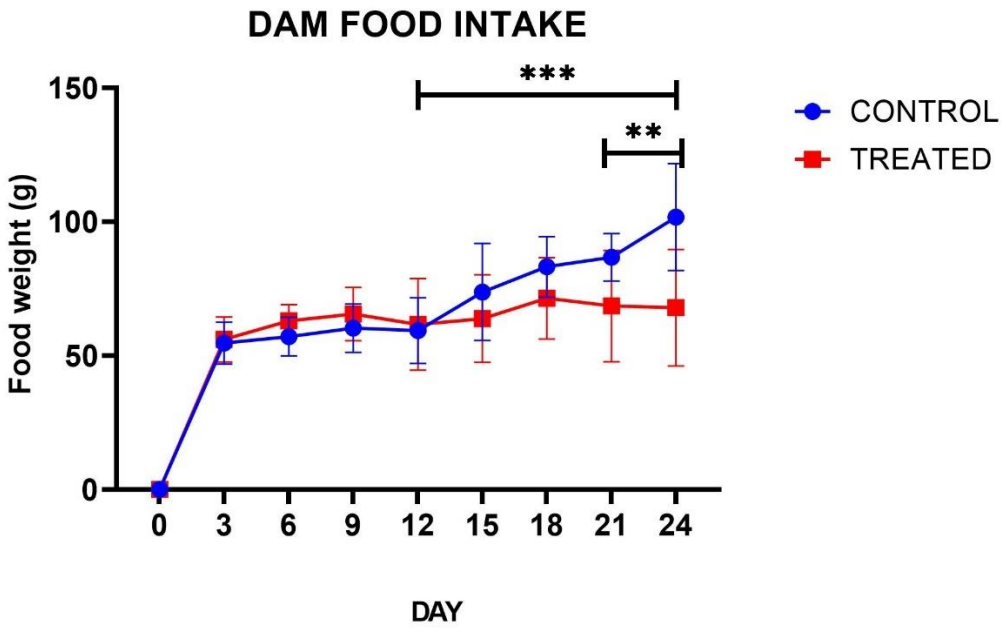



**Figure 4.2.5:** Effect of prenatal kolanut consumption on the level of food intake in pregnancy. Data are represented as mean ± S.E.M.; significant differences are shown in P-value, ** and *** against control.

### 4.2.6 Effect of prenatal kolanut consumption on the volume of water intake in pregnancy

The average water consumed by the 6 control and 6 kolanut treated dams were 174.6 ± 5.822 ml and 195.5 ml respectively as presented in figure 4.2.6. There was a significant steady increase in the volume of water consumed by the treated dams compared to the control dams throughout the duration of the experiment except on the 24$^{th}$ day [mean effects of treatment ($F_{(1, 90)} = 12.87$; $p = 0.0005$); mean effect of age ($F_{(8, 90)} = 186.6$; $p < 0.0001$) interaction between treatment and age ($F_{(8, 90)} = 2.448$; $p = 0.0191$)].

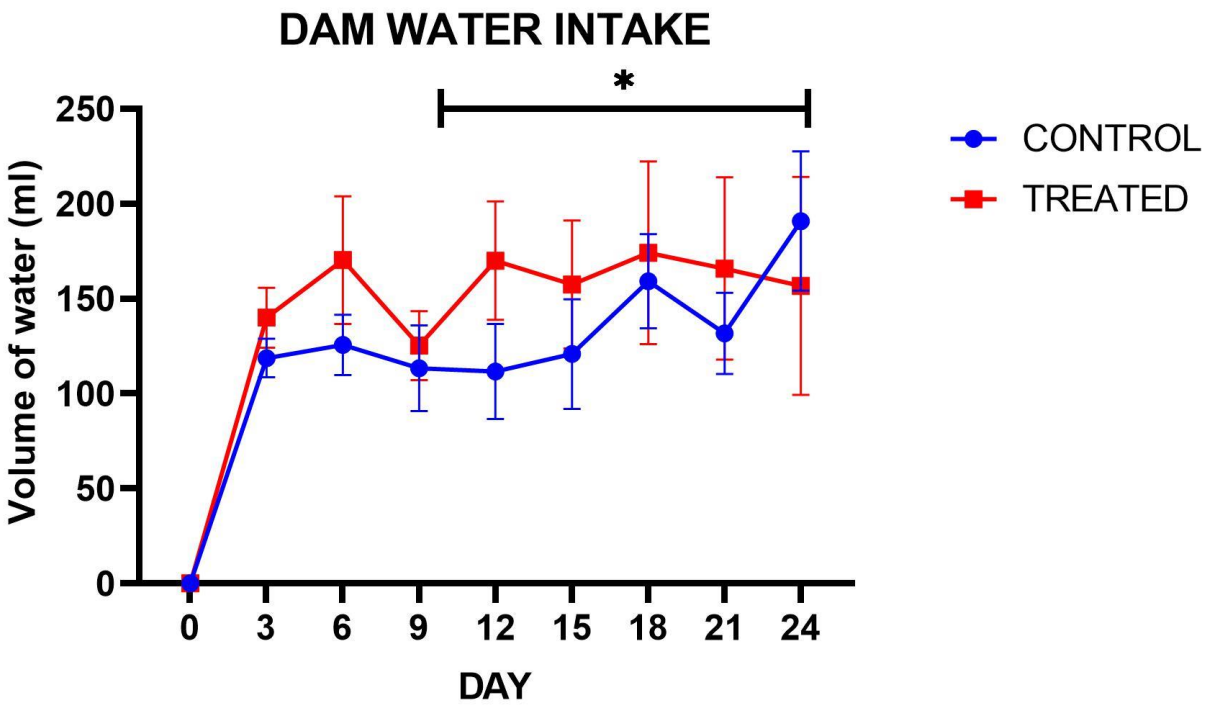



**Figure 4.2.6:** Effect of prenatal kolanut consumption on the volume of water intake in pregnancy. Data are represented as mean ± S.E.M.; significant differences are shown in P-value, * against control.

## 4.3 Results of behavioral test in the pups following exposure to prenatal kolanut consumption

### 4.3.1. Effect of prenatal kolanut consumption on cliff avoidance test in pups after birth

In each of the behavioral tests, 10 pups were used for the control and 10 pups for kolanut treated groups.

As shown in figure 4.3.1, the cliff avoidance (CA) was used to check the learning and memory of the pups prenatally exposed to kolanut extract compared to the control. The cliff avoidance of the prenatally exposed pups took a longer time than the control groups at all ages. The CA was extremely significant at different treatment across all age with no significant difference at interaction between the treatment and the age [mean effect of treatment ($F_{(1,72)=43.03}$; $p < 0.0001$), mean effect of age ($F_{(3,72)=0.8647}$; $p = 0.4635$), mean effect interaction between age and treatment ($F_{(3,72)=1.954}$; $p = 0.1286$)].

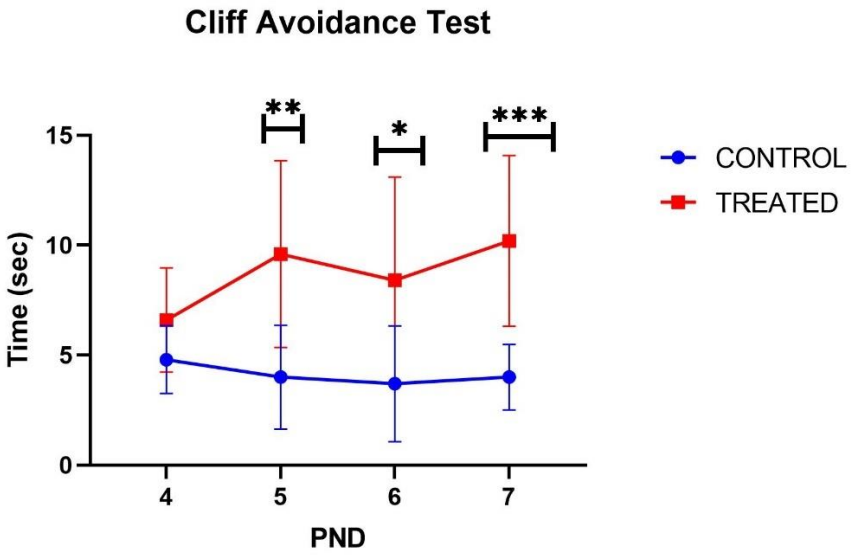



**Figure 4.3.1**: Effects of kolanut exposure on cliff avoidance test. The diagram shows the cliff avoidance in secs in pups from kolanut-treated dams from PND 4, 5, 6 and 7. Data is represented by mean ± SEM with 10 pups in each group. P values are */**/*** indicates significant differences as compared to control.

**4.3.2. Effect of prenatal kolanut consumption on surface righting test in pups after birth**

As shown in figure 4.3.2, the latency of surface righting test (SRT) was significantly different at different treatments at PND 5 [mean effect of treatment ($F_{(1,72)} = 21.33$; $p < 0.0001$); mean effect of age ($F_{(3;72)} = 1.551$); $p = 0.2087$); interaction between age and treatment was also not significant ($F_{(3;72)} = 1.8992$); $p = 0.4459$)]. However, the latency of SRT for the control groups gradually decreases as the age increases.

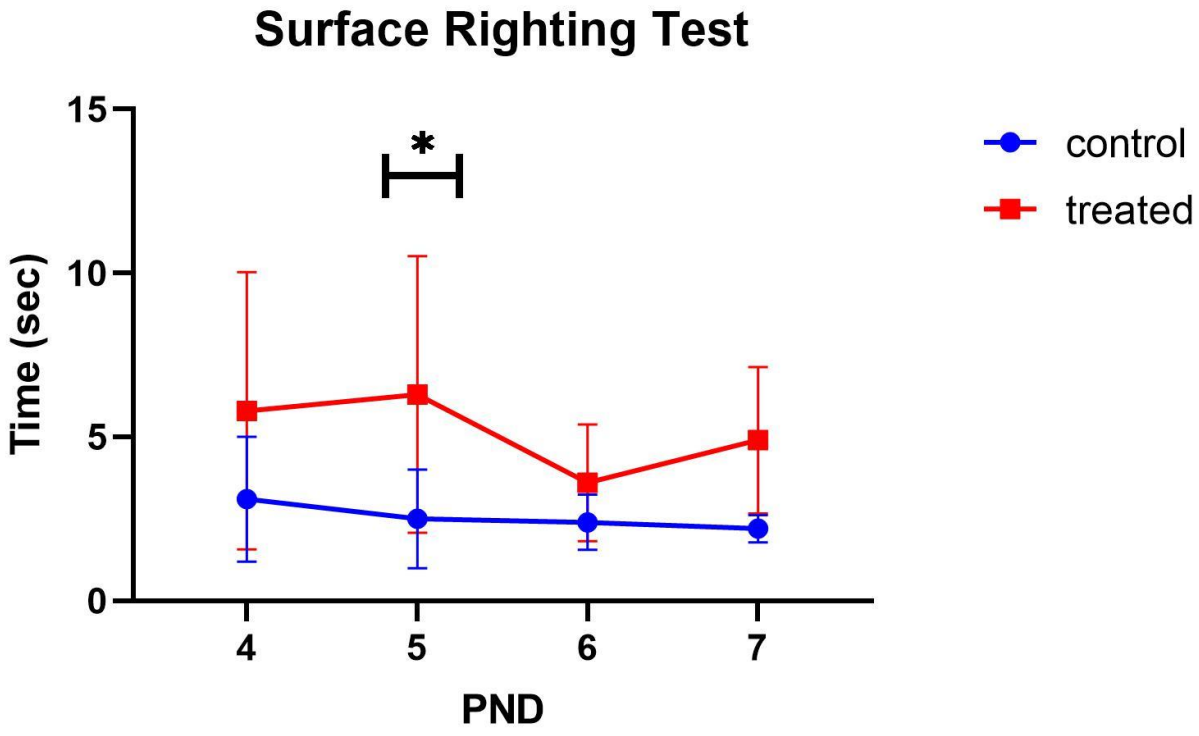



**Figure 4.3.2:** Effects of kolanut exposure on surface righting test. The diagram shows the surface righting test in secs in pups from kolanut-treated dams from PND 4, 5, 6 and 7. Data is represented by mean ± SEM with 10 pups in each group. * indicates significant differences as compared to control.

### 4.3.3. Effect of prenatal kolanut consumption on spontaneous movement in pups after birth

#### 4.3.3.1 Head Rising

As presented in figure 4.3.3.1, the frequency of head rising was significantly different at different treatments [(mean effect of treatment ($F_{(1,72)} = 10.05$; $p = 0.0022$) but not significant at the mean effect of age ($F_{(3,72)} = 1.789$; $p = 0.1569$), however, there was a significant difference in interaction between age and treatment effect ($F_{(3,72)} = 2.956$; $p = 0.0381$)]. Also, the latency of head rising was significantly different across age group [(mean effect of age ($F_{(3,72)} = 3.317$; $p = 0.0246$) but not significant at different treatment, mean effect of treatment ($F_{(1,72)} = 0.03978$; $p = 0.88425$), however, there was a significant difference in interaction between age and treatment ($F_{(3,72)} = 2.801$; $p = 0.0460$)].

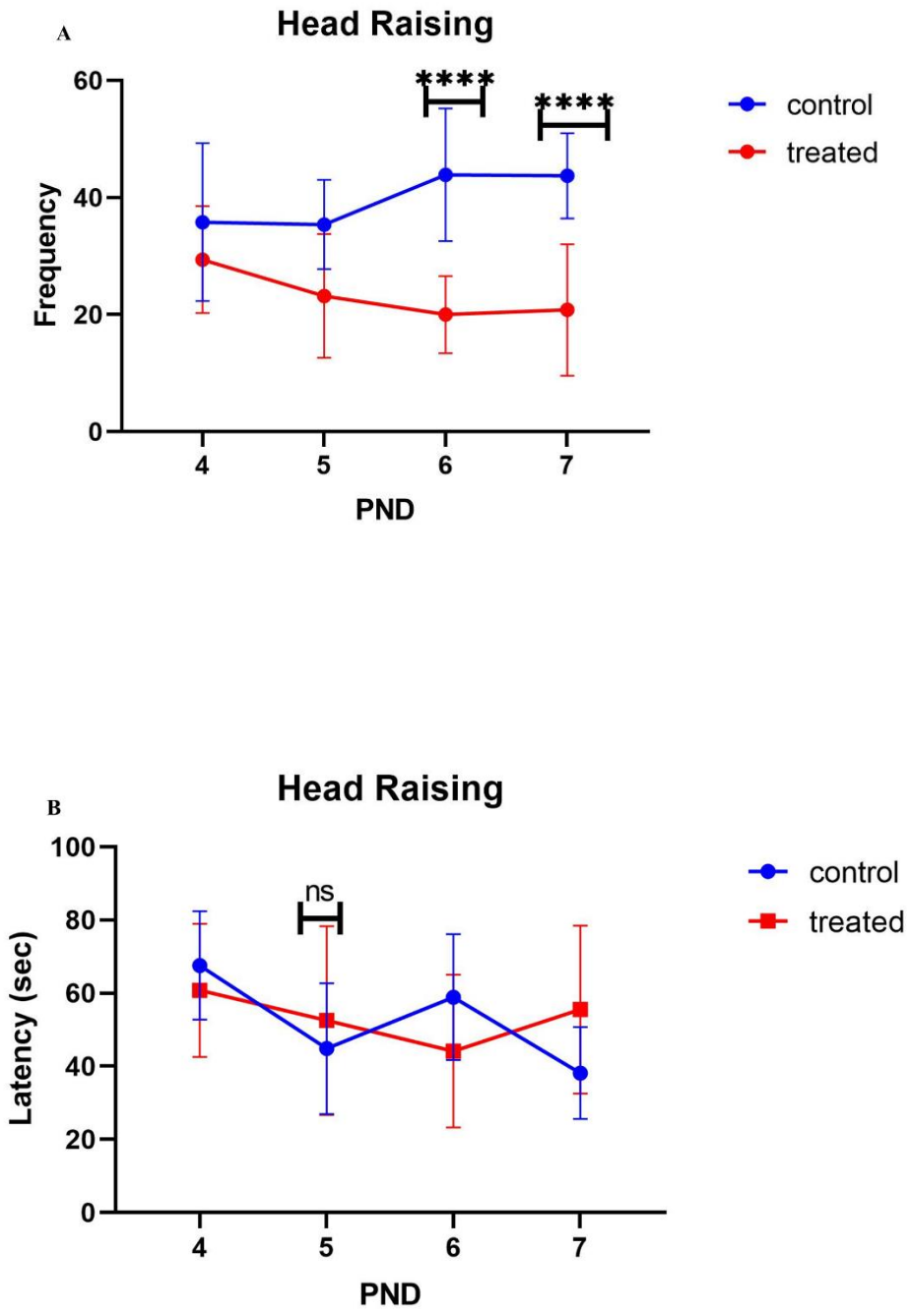



**Figure 4.3.3.1:** Effects of kolanut exposure on head rising test. The diagrams show the frequency (A) and the time (B) it took to raise the head (secs) in the given time slot in pups from kolanut-treated dams from PND 4, 5, 6 and 7. Data is represented by mean ± SEM with 10 pups in each group. **** indicates significant differences as compared to control.

#### 4.3.3.2. Pivoting

As represented in figure 4.3.3.2a-b, there was a significant difference in the treatment effects given across the age groups on frequency of pivoting [mean effects of treatment ($F_{(3,72)} = 22.81$; $p < 0.0001$); mean effect of age ($F_{(3,72)} = 8.193$; $p<0.0001$); also, a significant difference in the interaction between treatment and age ($F_{(3,72)} = 8.013$; $p= 0.0001$)]. The number of pivoting times for the control increases every day with the highest on PND 7 while those of treated group decreases as the days increased (figure 4.3.3.2a). There was also a significant difference in the treatment effects across the age groups [mean effects of treatment ($F_{(1,72)} = 12.88$; $p=0.0006$); mean effect of age ($F_{(3,72)} = 8.193$; $p=0.0075$) and a significant difference in the interaction between treatment and age ($F_{(3,72)} = 8.013$; $p= 0.0083$)] in the amount of time taken pivoting (figure 4.3.3.2b).

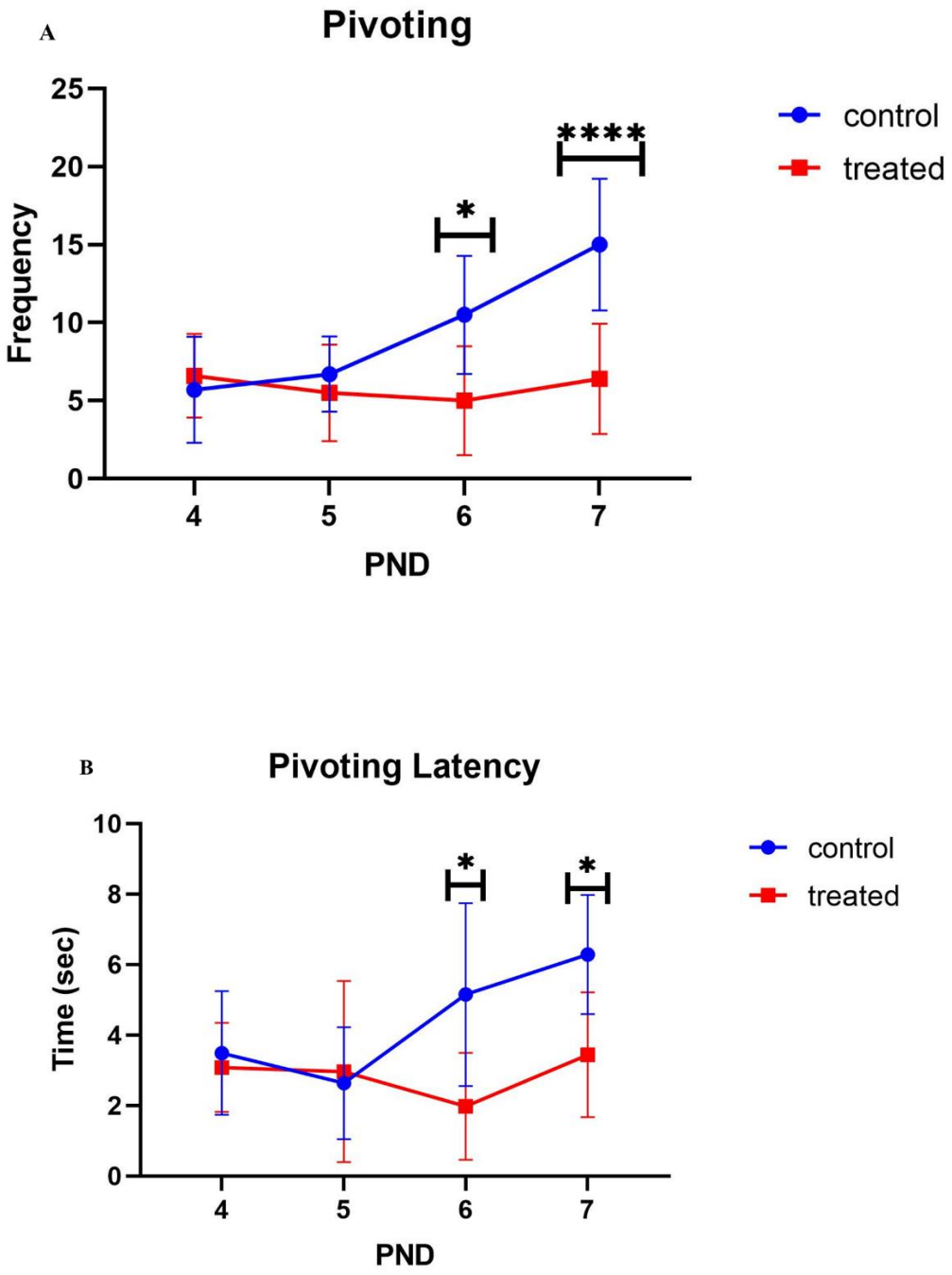



**Figure 4.3.3.2:** Effects of kolanut exposure on frequency (A) and latency (B) on pivoting. The diagrams show the frequency and latency on pivoting in frequency in secs in pups from kolanut-treated dams from PND 4, 5, 6 and 7. Data is represented by mean ± SEM with 10 pups in each group. * and **** indicates significant differences as compared to control.

#### 4.3.3.3 Locomotion

There was a significant difference in the treatment effects as indicated by the amount of movement done but not significant across age group [mean effect of treatment ($F_{(1,72)} = 10.12$; $p<0.0022$), mean effect of age ($F_{(3;72)} = 1.883$); $p=0.1401$), while the interaction between treatment effects and age was significant ($F_{(3,72)} = 4.872$; $p=0.0038$)] as PND 7 (figure 4.3.3.3).

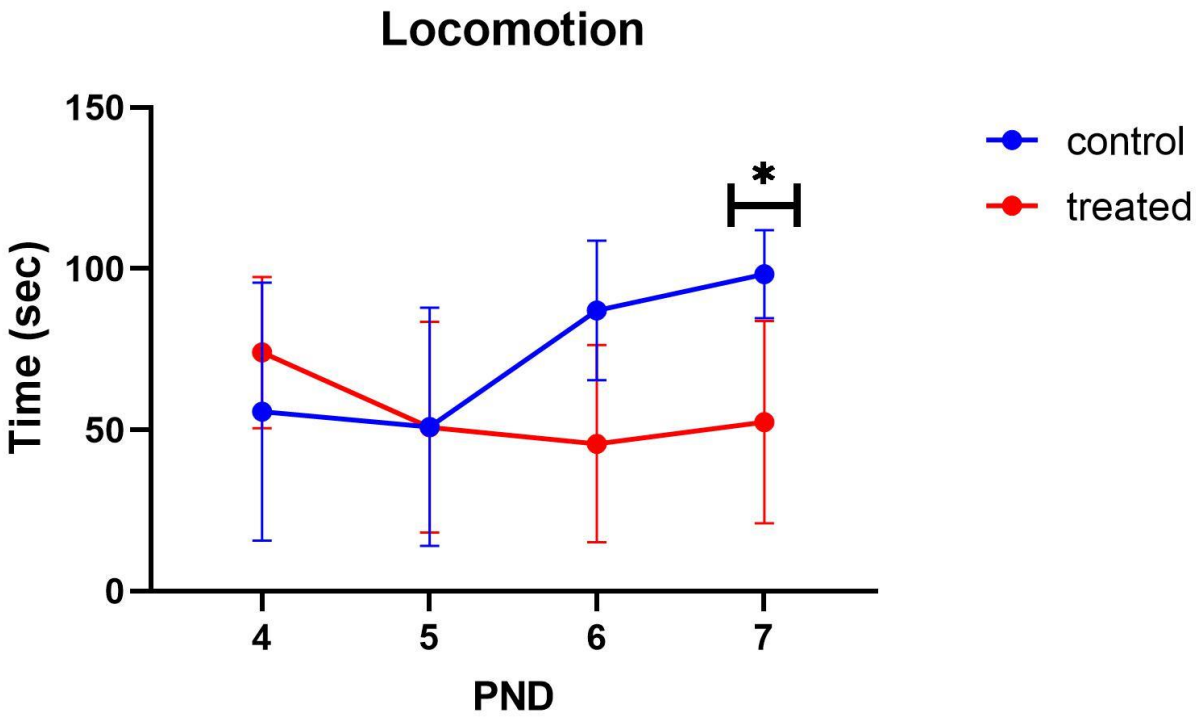



**Figure 4.3.3.3:** Effects of kolanut exposure on locomotion on spontaneous movement test. The diagram shows the locomotion time in secs in pups from kolanut-treated dams from PND 4, 5, 6 and 7. Data is represented by mean ± SEM with 10 pups in each group. * indicates significant differences as compared to control.

### 4.3.4 Effect of prenatal kolanut consumption on open field test in pups after birth

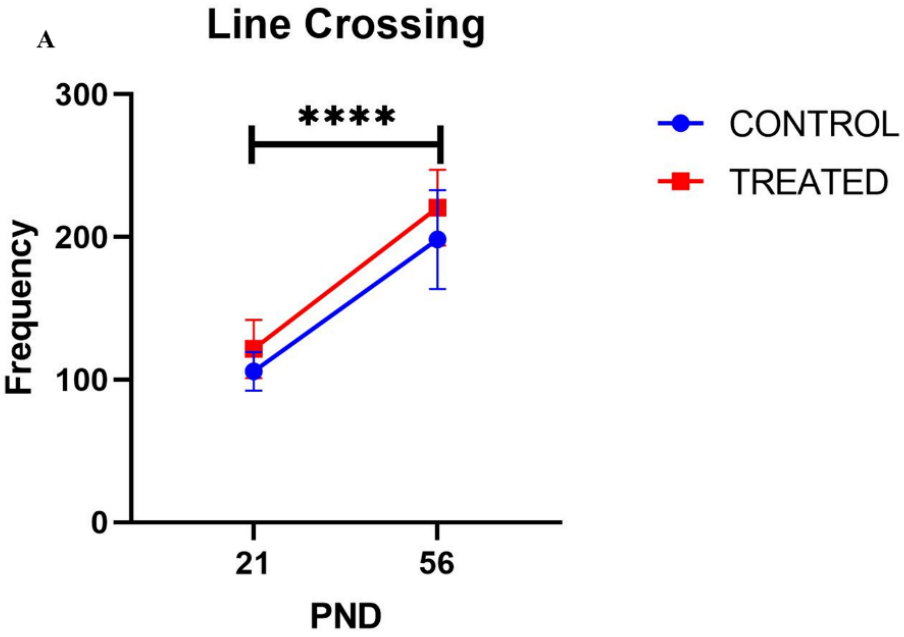
#### 4.3.4.1 Line crossings, corner time, distance covered and mean velocity.

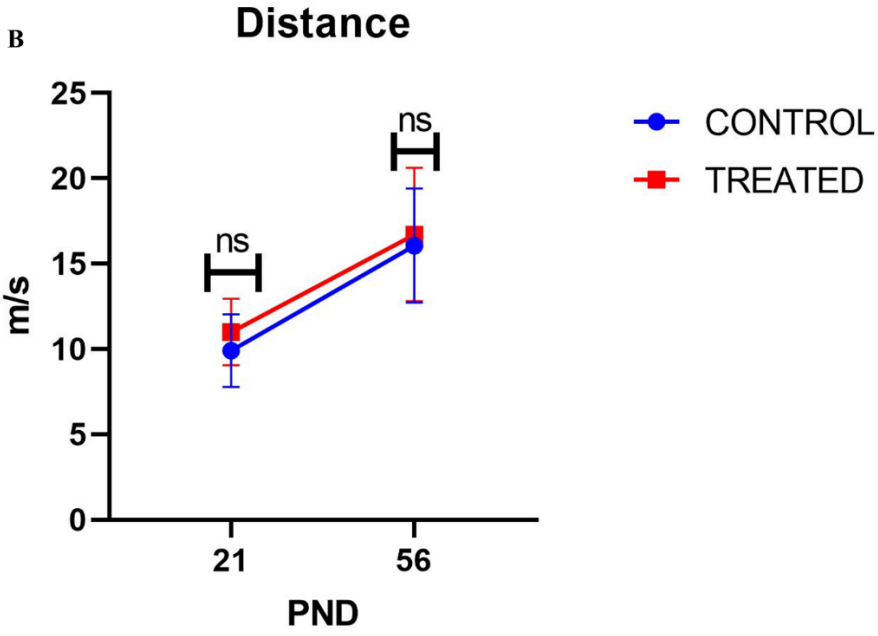
As presented in figure 4.3.4.1 (A), the pups were tested in the open field apparatus on different tests at PND 21 and 56. A significant difference was observed in the treatment effects across all age groups as indicated by the frequency of line crossings [(mean effects of treatment ($F_{(1,36)} = 5.777$; $p=0.0215$), mean effect of age ($F_{(1,36)} = 146.9$; $p<0.0001$)], however, no significant difference in the interaction of the treatment and the age [($F_{(1,36)} = 0.1743$; $p= 0.6788$)] when compared to their respective control groups.

There was a significant difference in the time spent at the corner box in the open field test across the age groups [mean effect of age ($F_{(1,36)} = 14.95$; $p=0.0004$) but not significant in the treatments effects, mean effects of treatment ($F_{(1,36)} = 1.118$; $p=0.2975$) nor in the interaction between the treatment and the age, mean effect of interaction ($F_{(1,36)} = 2.137$; $p= 0.1525$)] when compared to the control groups respectively. However, the amount of time spent at corners reduced with increased age in the control groups (figure 4.3.4.1 B).

Also, there was a highly significant difference in the distance covered by the pups across the age groups [(mean effect of age ($F_{(1,36)} = 40.51$; $p<0.0001$) while the treatment effects was not significant, (mean effects of treatment ($F_{(1,36)} = 0.8567$; $p=0.3608$) neither the interaction between the treatment and age was significant ($F_{(1,36)} = 0.05829$; $p= 0.8106$)] (figure 4.3.4.1 C).

Moreover, there was a highly significant difference in the velocity at which the distance was covered at all ages [(mean effect of age ($F_{(1,36)} = 21.88$; $p<0.0001$), but the treatment effect was not significant, (mean effects of treatment ($F_{(1,36)} = 0.2176$; $p=0.6437$) and no significant difference in the interaction between the age groups and the treatment given ($F_{(1,36)} = 0.2702$; $p= 0.6064$)] was also observed when compared to the control groups (figure 4.3.4.1 D).


A
Line Crossing
300
200
100
0
Frequency
****
21
56
PND
CONTROL
TREATED


B
Distance
25
20
15
10
5
0
m/s
ns
ns
21
56
PND
CONTROL
TREATED

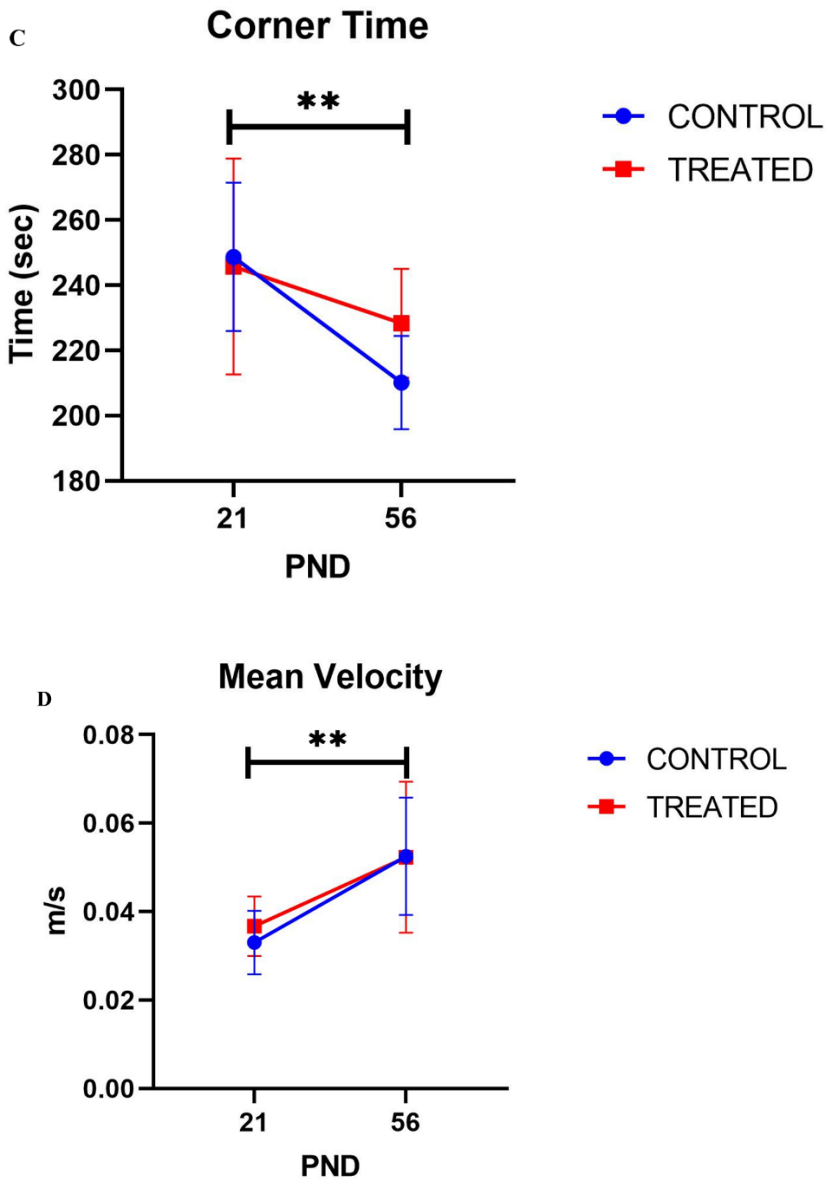



**Figure 4.3.4.1**: Effects of kolanut exposure on the amount of movement in open field test as seen in line-crossing (**A**), distance (**B**), corner time (**C**), and mean velocity (**D**) by the animals. The diagram shows the results of number of times the animals crossed both vertical and horizontal lines (**A**), the distance covered (**B**), time spent at each corner of the box (**C**), and the rate of movement in the given time (**D**) of animals from kolanut-treated dam between PND 21 and 56. Data is represented by mean ± SE with 10 animals in each group. */**, and **** indicate significant differences as compared to control.

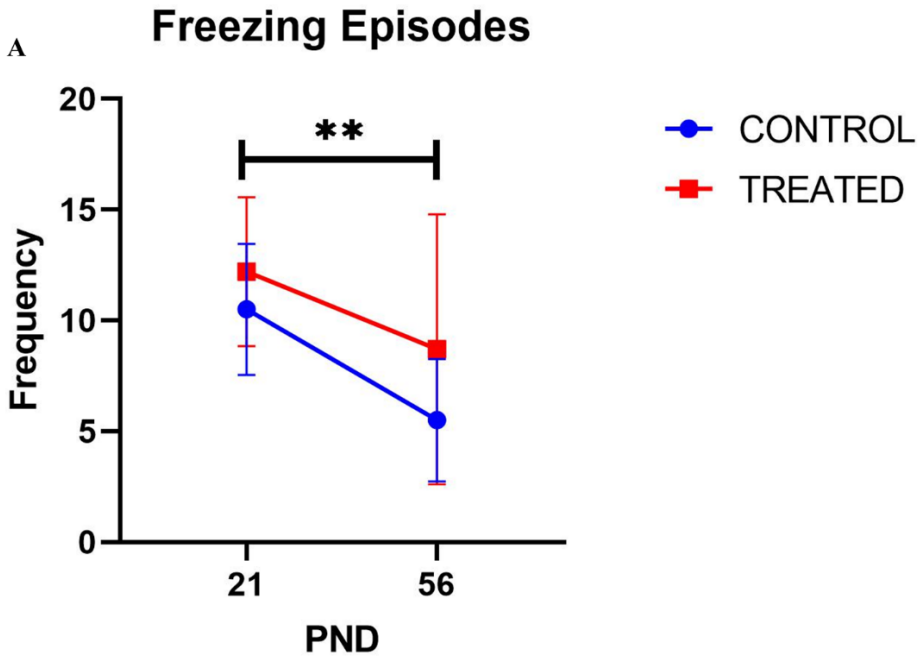
### 4.3.4.2 Frequencies of freezing episodes, grooming and rearing.

There was a significant difference in the frequency of freezing episodes across age groups [mean effect of age ($F_{(1,36)} = 11.16$; $p=0.0027$), the treatment effects was not significant, mean effects of treatment ($F_{(1,36)} = 3.708$; $p=0.062$) as well as no significant difference in the interaction between age and treatment ($F_{(1,36)} = 0.3475$; $p= 0.5592$)] was shown (figure 4.3.4.2 A).

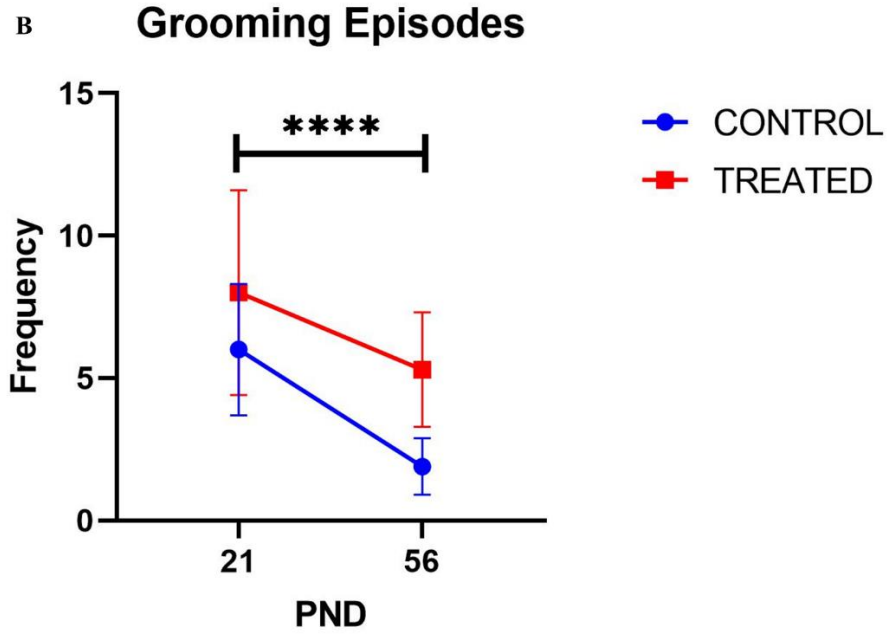
The frequency of grooming at different treatment effects was significantly different across the age groups [(mean effect of treatment ($F_{(1,36)} = 12.56$; $p=0.0011$), as well as the mean effect of age ($F_{(1,36)} = 19.91$; $p<0.0001$) while the interaction between age and treatment was not significant ($F_{(1,36)} = 3.269$; $p=0.8440$)] (figure 4.3.4.2 B).

As presented in figure 4.3.4.2 C, the rearing behavior was used to access the level of memory in the animals especially after the initial contact with the location. The rearing in the control reduced significantly at different treatments effects [mean effect of treatment ($F_{(1,36)} = 15.28$; $p=0.0004$) mean effects of age ($F_{(1,36)} = 0.02096$; $p=0.8857$) and the interaction between age and treatment was highly significant ($F_{(1,36)} = 15.28$; $p= 0.0004$)] when compared to the controls. This showed that the control animals remembered the environment and were less fidgeting than the treated animals.

A


Freezing Episodes
**
Frequency
20
15
10
5
0
21
56
PND
CONTROL
TREATED

B


Grooming Episodes
****
Frequency
15
10
5
0
21
56
PND
CONTROL
TREATED

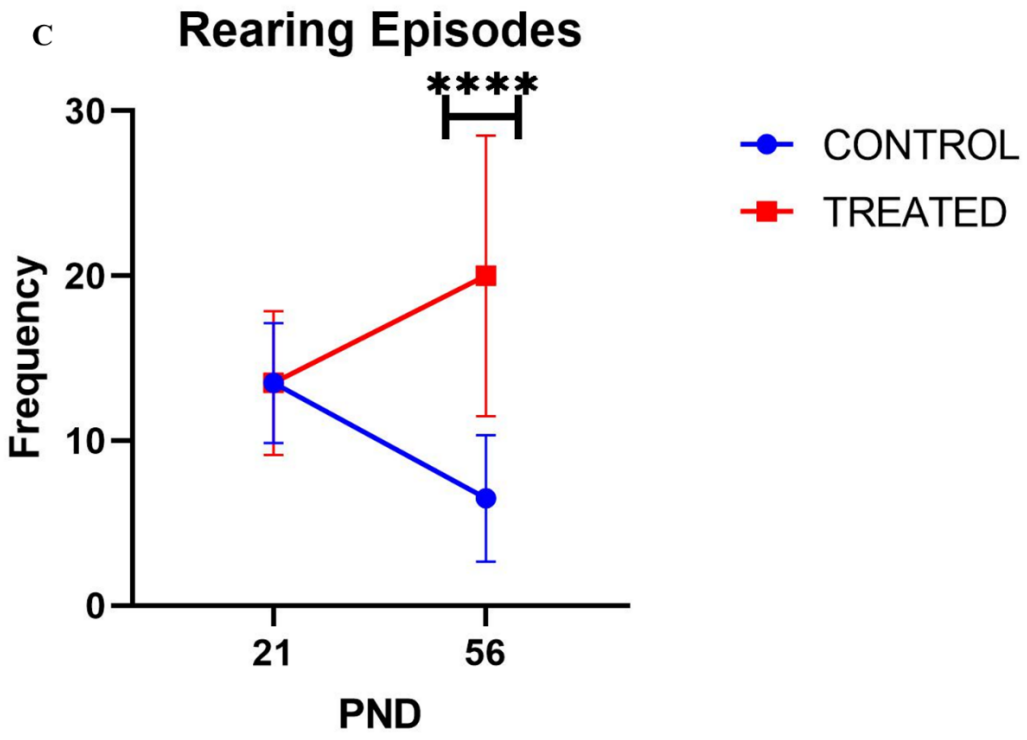



**Figure 4.3.4.2**: Effects of kolanut exposure on the animals from open field test on freezing (**A**), grooming (**B**), rearing (**C**). The diagram shows the results of number of freezing episodes (**A**), number of grooming times (**B**), and number of times the animals reared (**C**) from kolanut-treated dam between PND 21 and 56. Data is represented by mean ± SE with 10 animals in each group. */**** indicates significant differences as compared to control.

### 4.3.4.3 Fecal bolus and urination

As presented in figure 4.3.4.3a-b, the number of fecal boli (A) was significantly different at different treatment effects and across age groups [(mean effect of treatment ($F_{(1,36)} = 21.30$; $p<0.0001$); mean effect of age ($F_{(1,36)} = 0.04401$; $p=0.8350$) but the interaction of treatment across the age group was not significant ($F_{(1,36)} = 0.04401$; $p=0.8350$)] when compared to the control groups (figure 4.3.4.3a). However, the frequency of urination (B) was also significantly different at PND 56 [(mean effect of treatment ($F_{(1,36)} = 17.29$; $p=0.0002$); mean effect of age ($F_{(1,36)} = 0.1429$; $p=0.7077$) and interaction between age and treatment ($F_{(1,36)} = 0.1429$; $p=0.7077$)] (fig. 4.3.4.3b).

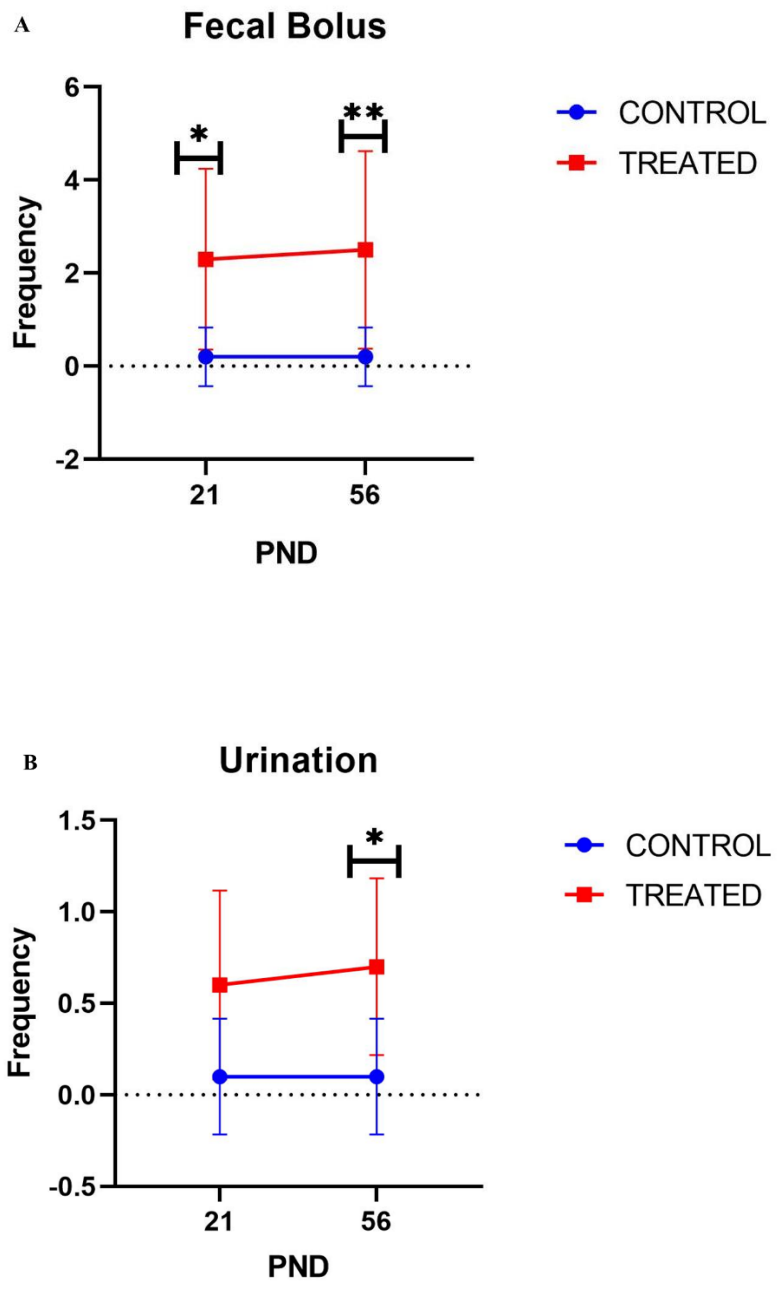



**Figure 4.3.4.3:** Effects of kolanut exposure on frequency of fecal bolus (**A**) and urination (**B**). The diagrams show the number of fecal bolus and urination passed during open field test by animals from kolanut-treated dam between PND 21 and 56. Data is represented by mean ± SEM with 10 animals in each group. */** indicates significant differences as compared to control.

### 4.3.5 Effect of prenatal kolanut consumption on novel object recognition test in pups after birth

The NOR was conducted on PND 21 and 56 pups. For PND 21 (Table 4.1), four out of ten animals in the control (1-10) animals had positive difference score and discrimination ratio greater than 0.5. Whereas 60.0 % (11-20) of animals in the treated group had positive difference score and discrimination ratio. On PND 56 (Table 4.2), 90.0 % of control animals had both positive difference score and discrimination ratio, while 70.0 % treated animals had positive score and discrimination ratio. The time spent with the old object was significant across age groups and interaction between treatment and age was also significant [mean effect of age ($F_{(1,36)} = 2.944$; $p<0.0001$), interaction ($F_{(1,36)} = 4.353$; $p = 0.0441$) and mean effects of treatment ($F_{(1,36)} = 2.944$; $p = 0.0948$)]. The time spent with the new object was significant across the age groups while treatment and interaction were not significant [mean effect of age ($F_{(1,36)} = 90.22$; $p<0.0001$) mean effects of treatment ($F_{(1,36)} = 2.241$; $p=0.1431$) and interaction ($F_{(1,36)} = 4.353$; $p= 0.1959$)]. The treatment was not significant across the age group on novelty index, neither was the interaction between treatment and age was significant [mean effect of age ($F_{(1,36)} = 3.281$; $p=0.0784$) mean effects of treatment ($F_{(1,36)} = 0.3041$; $p=0.5847$) and interaction ($F_{(1,36)} = 0.04545$; $p= 0.8324$)] when compared to their respective control groups.

**Table 4.1:** Novel object recognition at PND 21 showing difference scores and discrimination ratios.

| S/N | Familiar object time (tf) (sec) | Novel object time (tn) (sec) | Total time tn+tf (sec) | Difference score (tn-tf) (sec) | Discrimination ratio (tn/total time) (sec) |
|---|---|---|---|---|---|
| 1 | 17.2 | 21.8 | 39.0 | 4.60* | 0.56** |
| 2 | 37.6 | 23.4 | 61.0 | -14.20 | 0.38 |
| 3 | 36.6 | 25.4 | 62.0 | -11.20 | 0.41 |
| 4 | 38.3 | 27.9 | 66.2 | -10.40 | 0.42 |
| 5 | 19.2 | 43.3 | 62.5 | 24.10* | 0.69** |
| 6 | 26.2 | 24.3 | 50.3 | -1.90 | 0.48 |
| 7 | 16.0 | 7.9 | 23.9 | -8.10 | 0.33 |
| 8 | 27.5 | 24.1 | 51.6 | -3.40 | 0.47 |
| 9 | 0.4 | 10.1 | 10.5 | 9.70* | 0.96** |
| 10 | 28.0 | 29.2 | 57.2 | 1.20* | 0.51** |
| 11 | 5.5 | 8.1 | 13.6 | 2.60* | 0.60** |
| 12 | 13.8 | 31.5 | 45.3 | 17.70* | 0.70** |
| 13 | 47.8 | 30.2 | 78.0 | -17.60 | 0.39 |
| 14 | 30.0 | 5.5 | 35.5 | -24.50 | 0.15 |
| 15 | 15.3 | 31.8 | 47.1 | 16.50* | 0.68** |
| 16 | 24.4 | 44.9 | 69.3 | 20.50* | 0.65** |
| 17 | 32.5 | 22.3 | 54.8 | -10.20 | 0.41 |
| 18 | 14.0 | 8.5 | 22.5 | -5.50 | 0.38 |
| 19 | 28.8 | 32.0 | 60.8 | 3.20* | 0.53** |
| 20 | 20.4 | 23.0 | 43.4 | 2.60* | 0.53** |

1-10 = control pups; 11-20 = treated pups
tn = time spent on familiar object; tf = time spent on the novel object
*= Positive difference score = Object Recognition
**= Discrimination >0.5 = Object Recognition

**Table 4.2:** Novel Object Recognition PND 56 showing difference scores and discrimination ratios.

| S/N | Familiar object time (tf) (sec) | Novel object time (tn) (sec) | Total time tn+tf (sec) | Difference score (tn-tf) (sec) | Discrimination ratio (tn/total time) (sec) |
|---|---|---|---|---|---|
| 1 | 32.1 | 52.8 | 84.9 | 20.70* | 0.62** |
| 2 | 29.4 | 51.7 | 81.1 | 22.30* | 0.64** |
| 3 | 38.5 | 44.0 | 82.5 | 5.50* | 0.53** |
| 4 | 45.3 | 66.1 | 111.4 | 20.80* | 0.59** |
| 5 | 49.0 | 44.8 | 93.8 | -4.20 | 0.48 |
| 6 | 44.5 | 71.9 | 116.4 | 27.40* | 0.62** |
| 7 | 35.8 | 54.0 | 89.8 | 18.20* | 0.60** |
| 8 | 40.4 | 67.4 | 107.8 | 27.00* | 0.63** |
| 9 | 22.6 | 75.7 | 98.3 | 53.10* | 0.77** |
| 10 | 37.5 | 48.8 | 86.3 | 11.30* | 0.57** |
| 11 | 78.5 | 81.8 | 160.3 | 3.30* | 0.51** |
| 12 | 49.4 | 48.6 | 98.0 | -0.80 | 0.50 |
| 13 | 49.6 | 76.8 | 126.4 | 27.20* | 0.61** |
| 14 | 46.2 | 50.3 | 96.5 | 4.10* | 0.52** |
| 15 | 35.2 | 74.8 | 110.0 | 39.60* | 0.68** |
| 16 | 64.5 | 62.6 | 127.1 | -1.9 | 0.49 |
| 17 | 36.3 | 67.8 | 104.1 | 31.50* | 0.65** |
| 18 | 48.5 | 55.8 | 104.3 | 7.30* | 0.53** |
| 19 | 37.4 | 110.3 | 147.7 | 72.90* | 0.75** |
| 20 | 78.3 | 62.8 | 141.1 | -15.50 | 0.45 |

1-10 = control pups; 11-20 = treated pups
tn = time spent on familiar object; tf = time spent on the novel object
*= Positive difference score = Object Recognition
**= Discrimination >0.5 = Object Recognition

### 4.3.6 Effect of prenatal kolanut consumption on novel object location test in pups after birth

The NOL was done in PND 21 and 56 animals. On PND 21 (Table 4.3), 30.0 % of the control (1-10) pups had positive difference score and discrimination ratio of greater than 0.5 and 10.0 % of (11-20) pups in the treated group had positive difference score and discrimination ratio. On PND 56 (Table 4.4), 40.0 % of the control pups had both positive difference score and discrimination ratio while only 10.0 % of the treated pups had positive score and discrimination ratio. The time spent at the old location was significant across age groups while treatment and interaction were not significant [mean effect of age ($F_{(1,36)} = 9.566$; $p = 0.0038$) mean effects of treatment ($F_{(1,36)} = 1.960$; $p = 0.1701$) and interaction ($F_{(1,36)} = 0.3093$; $p = 0.5816$)]. The time spent with the new object was significant across the age groups while treatment and interaction were not significant [mean effect of age ($F_{(1,36)} = 88.44$; $p < 0.0001$) mean effects of treatment ($F_{(1,36)} = 1.092$; $p = 0.3030$) and interaction ($F_{(1,36)} = 0.7331$; $p = 0.3975$)]. The novelty index was not significant across the age and treatment groups, and interaction was not also significant [mean effect of age ($F_{(1,36)} = 0.7776$; $p = 0.3837$) mean effects of treatment ($F_{(1,36)} = 1.608$; $p = 0.2129$) and interaction ($F_{(1,36)} = 0.1169$; $p = 0.7344$)].

**Table 4.3:** Novel Object Location PND 21 showing difference scores and discrimination ratios.

| S/N | Familiar object time (tf) (sec) | Novel object time (tn) (sec) | Total time tn+tf (sec) | Difference score (tn-tf) (sec) | Discrimination ratio (tn/total time) (sec) |
|---|---|---|---|---|---|
| 1 | 26.5 | 8.8 | 35.3 | -17.70 | 0.25 |
| 2 | 20.5 | 29.2 | 49.7 | 8.70* | 0.59** |
| 3 | 17.9 | 24.6 | 42.5 | 6.70* | 0.58** |
| 4 | 65.8 | 23.1 | 88.9 | -42.70 | 0.26 |
| 5 | 42.0 | 23.0 | 65.0 | -19.00 | 0.35 |
| 6 | 7.9 | 9.8 | 14.7 | 1.90* | 0.67** |
| 7 | 19.4 | 14.7 | 34.1 | -4.70 | 0.43 |
| 8 | 24.8 | 13.7 | 38.5 | -11.10 | 0.36 |
| 9 | 21.1 | 15.8 | 36.9 | -5.30 | 0.43 |
| 10 | 16.8 | 7.1 | 23.9 | -9.70 | 0.30 |
| 11 | 13.6 | 10.2 | 23.8 | -3.40 | 0.43 |
| 12 | 17.2 | 12.4 | 29.6 | -4.80 | 0.42 |
| 13 | 26.3 | 25.7 | 52.0 | -0.60 | 0.49 |
| 14 | 166.7 | 16.8 | 183.5 | -149.90 | 0.09 |
| 15 | 30.6 | 14.3 | 44.9 | -16.30 | 0.32 |
| 16 | 16.2 | 5.8 | 22.0 | -10.40 | 0.26 |
| 17 | 20.7 | 3.7 | 24.4 | -17.00 | 0.15 |
| 18 | 0.4 | 1.8 | 2.2 | 1.40* | 0.82** |
| 19 | 24.1 | 10.3 | 34.4 | -13.80 | 0.30 |
| 20 | 26.1 | 12.4 | 38.5 | -13.70 | 0.32 |

1-10 = control pups; 11-20 = treated pups
tn = time spent on familiar location; tf = time spent on the novel location
*= Positive difference score = Object Location
**= Discrimination >0.5 = Object Location

**Table 4.4:** Novel Object Location PND 56 showing difference scores and discrimination ratios

| S/N | Familiar object time (tf) (sec) | Novel object time (tn) (sec) | Total time tn+tf (sec) | Difference score (tn-tf) (sec) | Discrimination ratio (tn/total time) (sec) |
|---|---|---|---|---|---|
| 1 | 57.2 | 61.4 | 118.6 | 4.20* | 0.52** |
| 2 | 66.3 | 33.3 | 99.6 | -33.00 | 0.33 |
| 3 | 63.8 | 37.5 | 101.3 | -26.30 | 0.37 |
| 4 | 72.8 | 56.5 | 129.3 | -16.30 | 0.44 |
| 5 | 52.0 | 40.6 | 92.6 | -11.40 | 0.44 |
| 6 | 45.6 | 28.5 | 74.1 | -17.10 | 0.38 |
| 7 | 49.9 | 25.1 | 75.0 | -24.80 | 0.33 |
| 8 | 33.5 | 41.9 | 75.4 | 8.40* | 0.56** |
| 9 | 35.2 | 36.0 | 71.2 | 0.80* | 0.51** |
| 10 | 24.5 | 47.1 | 71.6 | 22.60* | 0.66** |
| 11 | 47.2 | 33.8 | 81.0 | -13.40 | 0.42 |
| 12 | 61.2 | 28.2 | 89.4 | -33.00 | 0.32 |
| 13 | 59.0 | 49.0 | 108.0 | -10.00 | 0.45 |
| 14 | 51.3 | 37.8 | 89.1 | -13.50 | 0.42 |
| 15 | 41.9 | 44.9 | 86.8 | 3.00* | 0.52** |
| 16 | 131.7 | 38.0 | 169.7 | -93.70 | 0.22 |
| 17 | 77.9 | 49.7 | 127.6 | -28.20 | 0.39 |
| 18 | 72.5 | 31.5 | 104.0 | -41.00 | 0.30 |
| 19 | 95.7 | 52.7 | 148.4 | -43.00 | 0.36 |
| 20 | 46.0 | 36.0 | 82.0 | -10.00 | 0.44 |

1-10 = control pups; 11-20 = treated pups
tn = time spent on familiar location; tf = time spent on the novel location
*= Positive difference score = Object Location
**= Discrimination >0.5 = Object Location

### 4.3.7 Effect of prenatal kolanut consumption on radial arm maze test in pups after birth

RAM was conducted on the pups on both PND 21 and PND 56. The times taken to complete the tasks were evaluated. RAM task for PND 21 was significantly different as compared to control, mean effects of treatment ($F_{[1,180] = 107.3}$; $p < 0.0001$), mean effects of age ($F_{[9,180] = 1.071}$; $p = 0.3864$), and interaction ($F_{[9,180] = 4.048}$; $p < 0.0001$) (figure 4.3.7A). Likewise, the time taken for the completion of the task on PND 56 was significantly higher than control at different treatment groups across the age group, mean effects of treatment ($F_{[1,180] = 90.20}$; $p < 0.0001$), age ($F_{[9,180] = 0.7403}$; $p = 0.0250$), and interaction ($F_{[9,180] = 0.6823}$; $p = 0.7243$) (figure 4.3.7B).

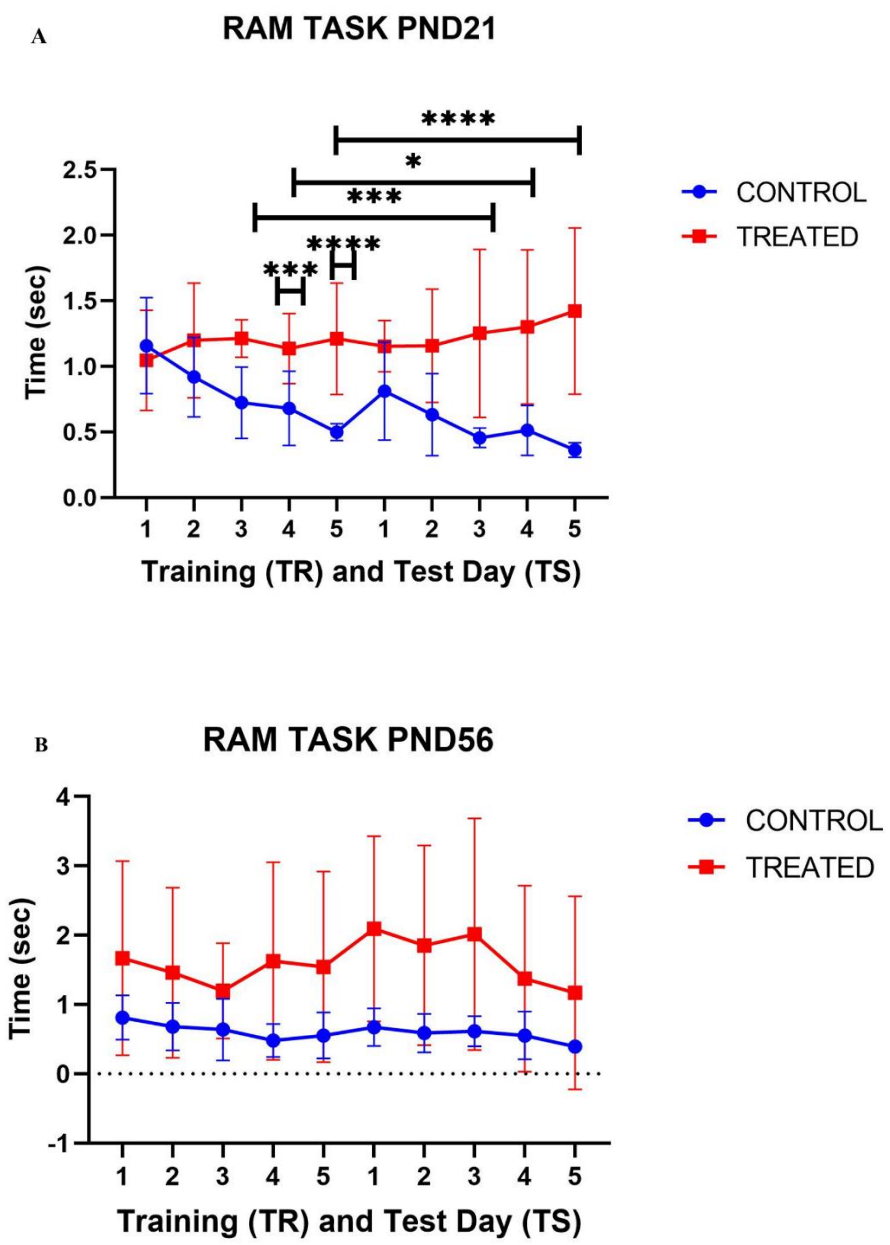



**Figure 4.3.7**: Effects of kola nut exposure on the pups as seen in radial arm maze test on PND 21 (A) and PND 56 (B). The diagram shows the time taken to complete the task in the pups from the kolanut-treated dams. The data are represented by mean ± SE, with 10 animals in each group. The values */**/*** and **** indicate significant differences between the two groups.

## 4.4 Histological evaluation in the brain of pups following exposure to prenatal kolanut consumption.

Golgi-cox is a special type of staining technique that enables the visualization of a whole neuron and its entirely structure alongside, the cell soma, axon, dendrites and spines. It also helps to quantify the aborization of the dendrites, thus suggesting the quantity of synapse made by the neuron. Four brains from each group and for each timeline were used for this special stain.

### 4.4.1. Effect of prenatal kolanut consumption on hippocampal morphology after birth

Golgi-Cox analysis of hippocampal neurons shows the pyknotic and inflamed soma of the kolanut treated brains compared to the control that exhibit a relatively normal pyramidal neuron in PND 0, with increase in the synapses in the control than the kolanut treated animal PND 7 (figure 4.4.1.1). Increase in arborization of hippocampal neurons was observed in the control brains as compared to loss of dendritic abors in the kolanut treated in the dentate gyrus (DG) and CA 3 of PND 21 (figure 4.4.1.2.). There was loss of spines, presence of discontinuation of the dendrites (constriction) and fragmentation of the dendrites in the kolanut treated brains as compared to the control of PND 21 and PND 56 (figure 4.4.1.3.).

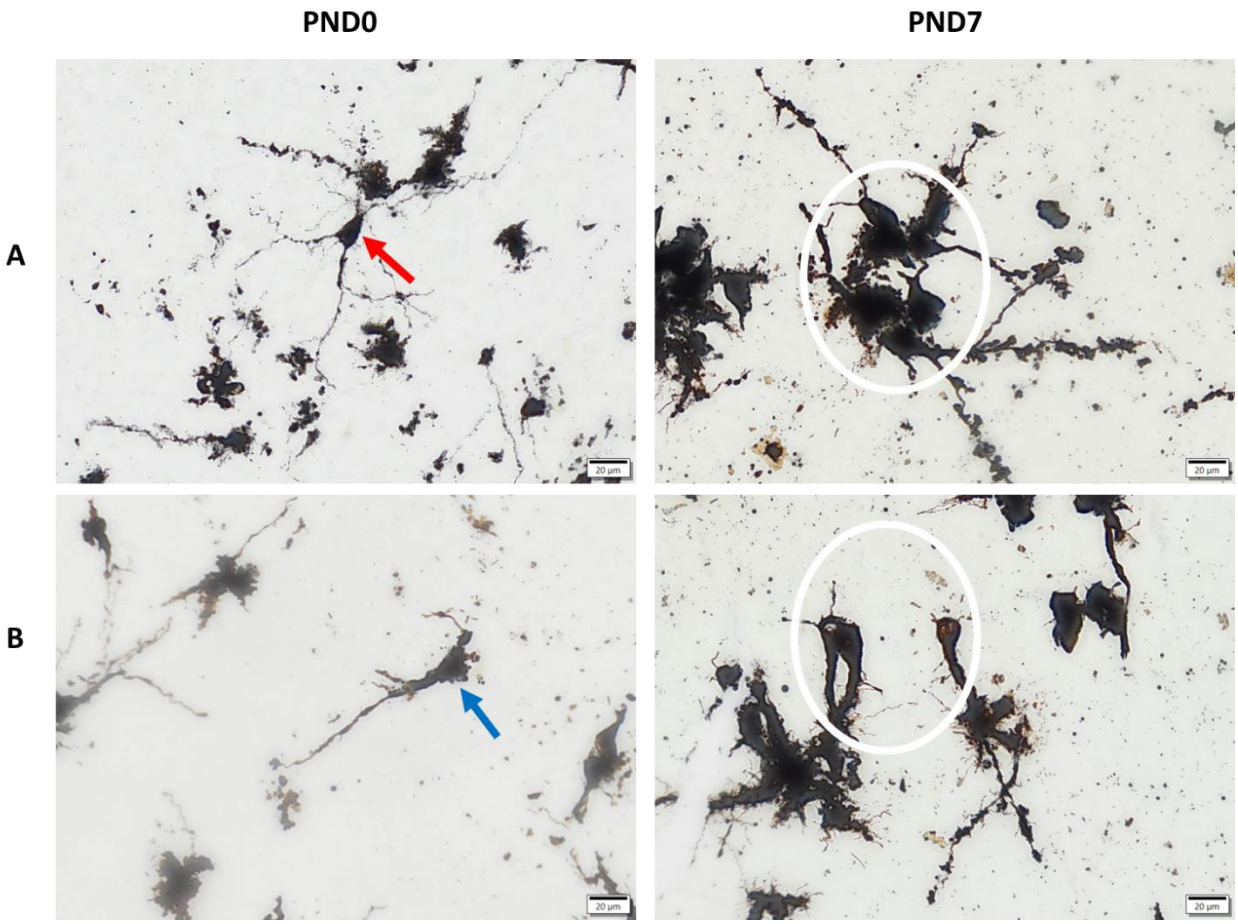



**Figure 4.4.1.1.** Golgi-Cox analysis of hippocampal neurons. A–B. (A) Brains from control group of PNDs 0 and 7 and (B) kolanut treated animal of PNDs 0 and 7. (A) PND 0 shows the relatively normal pyramidal neuron (red arrow) while (B) PND 0 shows pyknotic neuron (blue arrow). (A) PND 7 shows the higher synaptic activity with increase in synaptic density (white circle) while (B) PND 7 shows reduced synaptic activity and synaptic density (white circle).

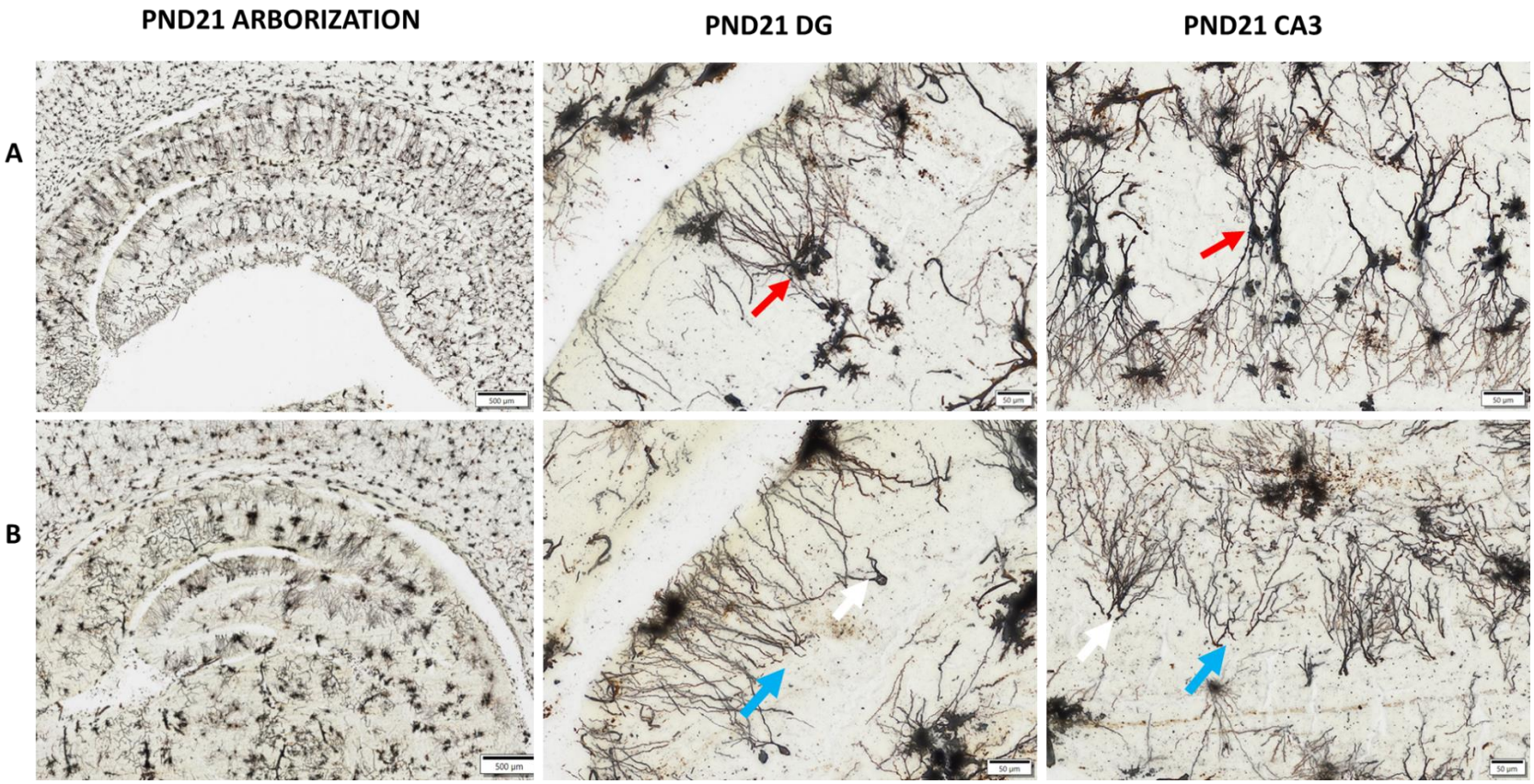



**Figure 4.4.1.2.** Golgi-Cox analysis of hippocampal neurons showing the aborization of the neurons across different regions of PND 21 brain. The plate row B shows a diffuse loss of dendritic arbors (B PND21 DG vs B PND21 CA3; blue arrows), loss of soma (white arrow). Relatively normal arborization with synapses (red arrow) Scale bars: PND21 Arborization A & B 500 mm while the rest is 50 mm.

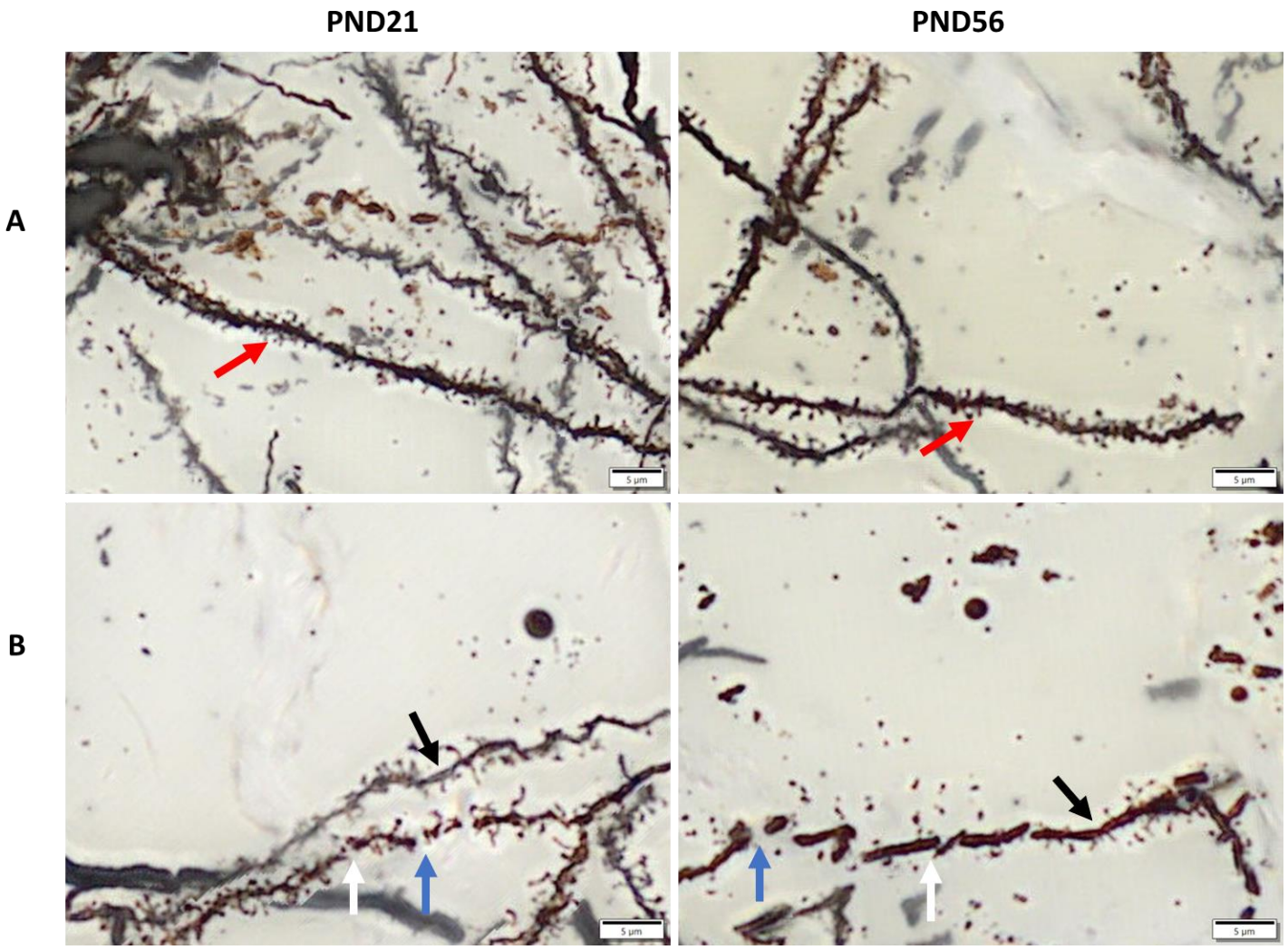



**Figure 4.4.1.3.** Golgi-Cox analysis of hippocampal neurons of PNDs 21 and 56. Row A indicates the control brain while row B indicates treated brain. Red arrow indicates the abundance of spines on the control dendrites while black arrow indicates loss and reduction of spines on the treated dendrites. White arrows pointing at the constricted dendritic spines and blue arrows at the dendritic fragmentations. Scale bars: 5 mm.

### 4.4.2. Effect of prenatal kolanut consumption on hippocampal density in the soma (cell body) after birth

The hippocampal neurons identified by their somata (cell body) size were measured and quantified in the different regions of the hippocampus as presented in figure 4.4.2. The neuron density and soma size in the kolanut-treated group was compared to control immediately after birth (PND 0, 7, 21, 56, 70). As shown in figure 4.4.2. (A), there were significant differences observed in the different hippocampal region relative to the control. The cell body (soma) area in the CA1-3 and DG of the hippocampus was significantly different at different treatments; CA1 [mean effect interaction: ($F_{(3,56)} = 20.14$; $p < 0.0001$), mean effect of treatment: ($F_{(1,56)} = 41.00$; $p < 0.0001$) and Age × treatment ($F_{(3,56)} = 45.69$; $p < 0.0001$)], CA2 [mean effect interaction: ($F(3,56) = 27.40$; $p < 0.0001$), mean effect of treatment: ($F_{(1,56)} = 75.42$; $p < 0.0001$) and Age × treatment ($F_{(3,56)} = 56.99$; $p < 0.0001$)], CA3 [mean effect interaction: ($F_{(3,56)} = 11.40$; $p < 0.0001$), mean effect of treatment: ($F_{(1,56)} = 191.81$; $p < 0.0001$) and Age × treatment ($F_{(3,56)} = 176.74$; $p < 0.0001$)] and DG [mean effect interaction: ($F_{(3,56)} = 9.19$; $p < 0.0001$), mean effect of treatment: ($F_{(1,56)} = 93.51$; $p < 0.0001$) and Age × treatment ($F_{(3,56)} = 221.27$; $p < 0.0001$)]. However, the different treatment effect within the kolanut-treated brains was compared. There was significant decrease in CA1-3 and DG soma size of the hippocampus in kolanut-treated brains at PNDs 21, 56, 70 when compared to the kolanut-treated brains at PND 7 (figure 4.4.2. B-E).

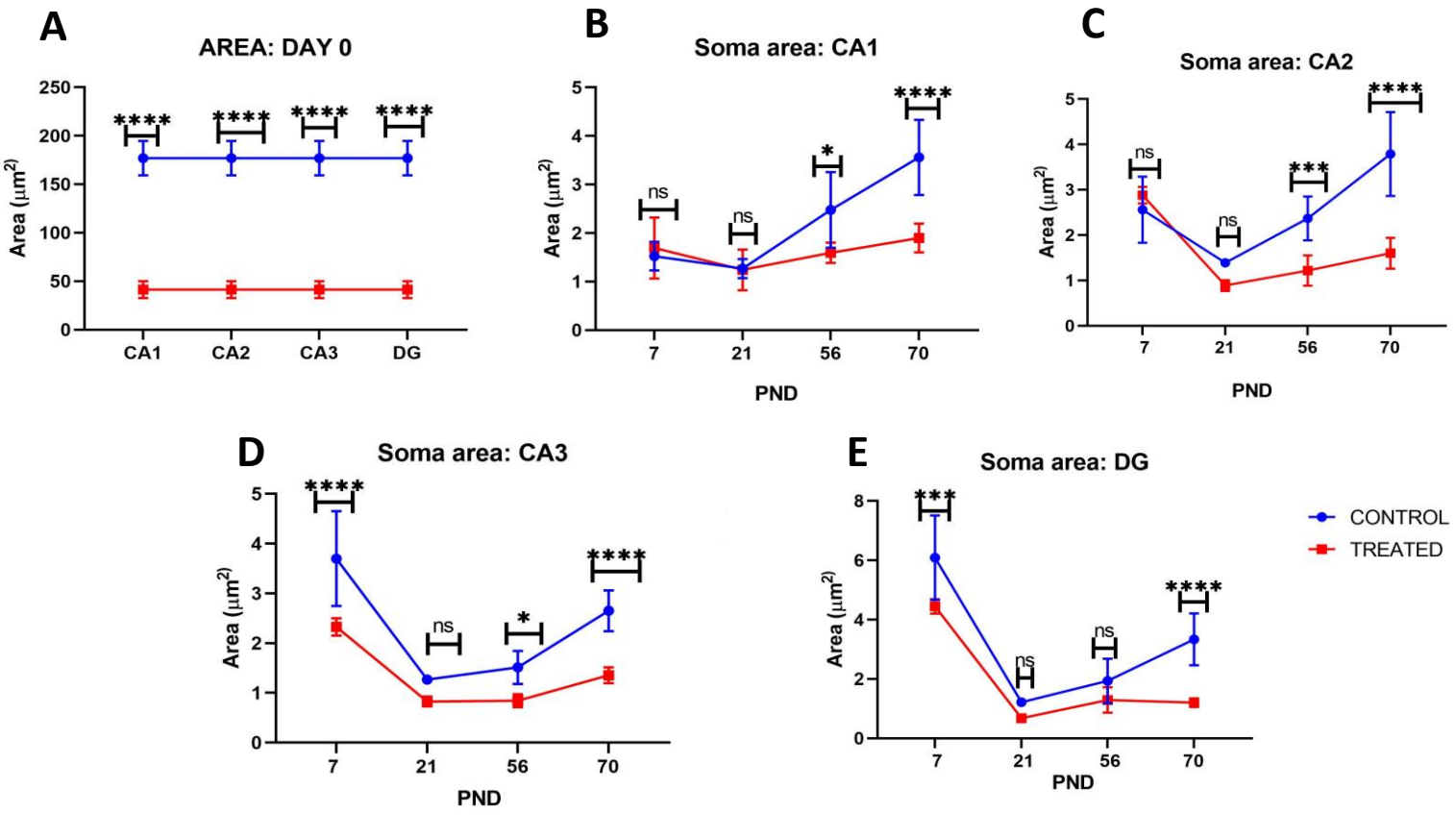



**Figure 4.4.2.**: Effect of prenatal kolanut consumption on hippocampal cell body area (µm2) PND 0 (**A**), CA1 density PND 7-70 (**B**), CA2 density PND 7-70 (**C**), CA3 density PND 7-70 (**D**), DG density (**E**) after birth. Data are represented as mean ± S.E.M.; significant differences are shown in P-value, */***/**** against control.

### 4.4.3 Effect of prenatal kolanut consumption on hippocampal diameter of soma (cell body) in rat after birth

As shown in figure 4.4.3., the diameter of the cell body (soma) in the CA1-3 and DG were significantly different at different treatments; CA1 [mean effect interaction: ($F(3,56) = 3.45$; $p = 0.0224$), mean effect of treatment: ($F(1,56) = 119.60$; $p < 0.0001$) and Age × treatment ($F(3,56) = 35.43$; $p < 0.0001$)], CA2 [mean effect interaction: ($F(3,56) = 2.12$; $p = 0.1077$), mean effect of treatment: ($F(1,56) = 127.34$; $p < 0.0001$) and Age × treatment ($F(3,56) = 44.24$; $p < 0.0001$)], CA3 [mean effect interaction: ($F(3,56) = 35.00$; $p < 0.0001$), mean effect of treatment: ($F(1,56) = 68.42$; $p < 0.0001$) and Age × treatment ($F(3,56) = 42.88$; $p < 0.0001$)] and DG [mean effect interaction: ($F(3,56) = 90.94$; $p < 0.0001$), mean effect of treatment: ($F(1,56) = 478.12$; $p < 0.0001$) and Age × treatment ($F(3,56) = 262.26$; $p < 0.0001$)] relative to the control (fig. 4.4.3.1). Similarly, the different treatment effect within the kolanut-treated brains showed significant differences in the diameter of the soma at PND 21 (CA1 and DG), PND 56 (CA3), and PND 70 (CA1-3 and DG) when compared to the kolanut-treated brains at PND 7 (figure 4.4.3.). Also, there was a similar significant difference in the diameter of the soma at PND 56 (CA3) relative to the kolanut-treated brains at PND 21(figure 4.4.3.).

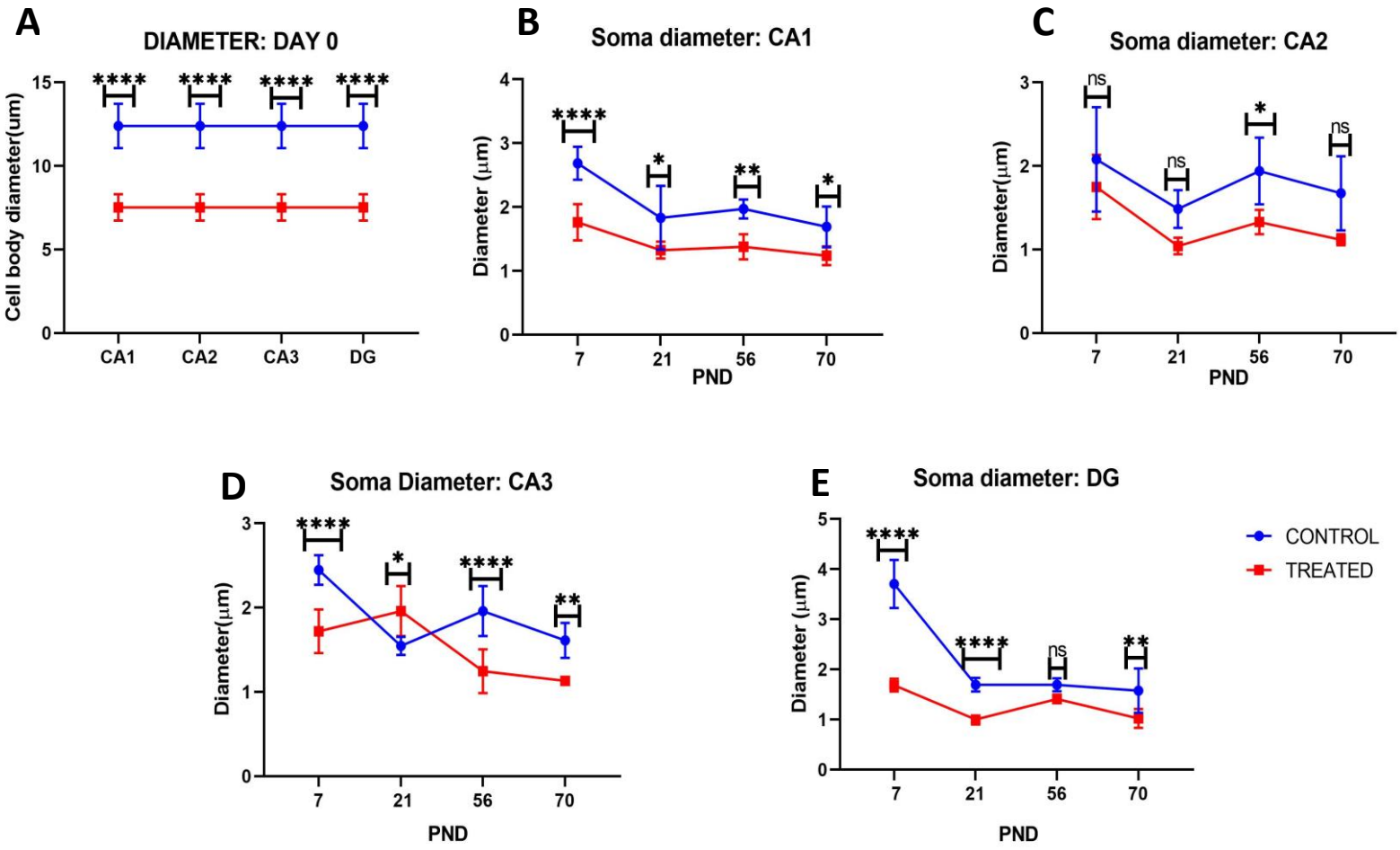



**Figure 4.4.3.:** Effect of prenatal kolanut consumption on hippocampal cell body diameter PND0 (**A**), CA1 soma diameter PND 7-70 (**B**), CA2 soma diameter PND 7-70 (**C**), CA3 soma diameter PND 7-70 (**D**), DG soma diameter PND 7-70 (**E**) in rat after birth. Data are represented as mean ± S.E.M.; significant differences are shown in P-value, */**/**** against control.

### 4.4.4 Effect of prenatal kolanut consumption on hippocampal soma (cell body) counts in the rat after birth.

The total cell body (soma) count estimated in the hippocampal regions of the kolanut-treated brains showed significant differences relative to the control at the different treatment as indicated by CA1 [mean effect interaction: ($F(3,56) = 1475.22$; $p < 0.0001$), mean effect of treatment: ($F(1,56) = 773.21$; $p < 0.0001$) and Age × treatment ($F(3,56) = 2156.82$; $p < 0.0001$)], CA2 [mean effect interaction: ($F(3,56) = 2012.40$; $p < 0.0001$), mean effect of treatment: ($F(1,56) = 1540.97$; $p < 0.0001$) and Age × treatment ($F(3,56) = 1770.55$; $p < 0.0001$)], CA3 [mean effect interaction: ($F(3,56) = 324.99$; $p < 0.0001$), mean effect of treatment: ($F(1,56) = 688.68$; $p < 0.0001$) and Age × treatment ($F(3,56) = 502.90$; $p < 0.0001$)] and DG [mean effect interaction: ($F(3,56) = 1017.27$; $p < 0.0001$), mean effect of treatment: ($F(1,56) = 168.00$; $p < 0.0001$) and Age × treatment ($F(3,56) = 580.90$; $p < 0.0001$)] (figure 4.4.4.). Significant differences were further observed within the kolanut-treated brains as indicated by decrease CA1-3 soma size but not in the DG at PNDs 21, 56, 70 when compared to the kolanut-treated brains at PND 7 (figure 4.4.4.).

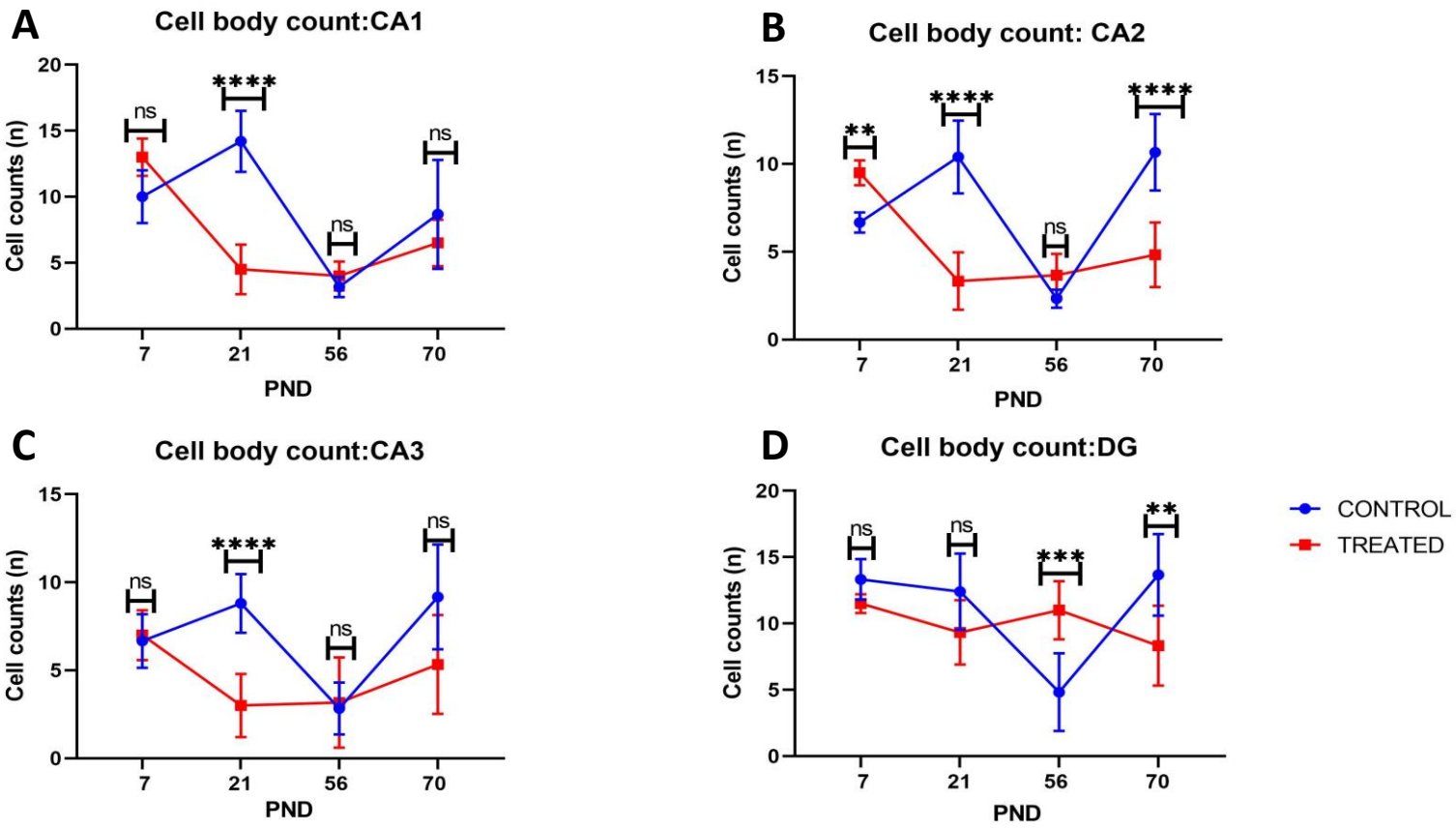



**Figure 4.4.4.:** Effect of prenatal kolanut consumption on hippocampal soma counts in CA1 (**A**), CA2 (**B**), CA3 (**C**), DG (**D**) in rat after birth at PND 7-70. Data are represented as mean ± S.E.M.; significant differences are shown in P-value, */**/***/**** against control.

### 4.4.5 Effect of prenatal kolanut consumption on hippocampal dendritic spine length in rat after birth

The hippocampal pyramidal neurons located in the sensorimotor cortex were assessed for any structural changes. The complete apical and basal dendritic spine length of the CA1, CA2, CA3 and DG was observed to decrease following exposure to prenatal kolanut treatment relative to the control.

#### 4.4.5.1 CA1 hippocampal region

The prenatal kolanut treatment significantly alters the CA1 dendritic length when compared to the control as indicated by: primary branch length [mean effect interaction: ($F(3,56) = 77.89$; $p < 0.0001$), mean effect of treatment: ($F(1,56) = 201.88$; $p < 0.0001$) and Age × treatment ($F(3,56) = 481.67$; $p < 0.0001$)], secondary branch length [mean effect interaction: ($F(3,56) = 213.25$; $p < 0.0001$), mean effect of treatment: ($F(1,56) = 1610.82$; $p < 0.0001$) and Age × treatment ($F(3,56) = 1848.14$; $p < 0.0001$)], tertiary branch length [mean effect interaction: ($F(3,56) = 198.44$; $p < 0.0001$), mean effect of treatment: ($F(1,56) = 148.12$; $p < 0.0001$) and Age × treatment ($F(3,56) = 415.96$; $p < 0.0001$)], quaternary branch length [mean effect interaction: ($F(2,42) = 40.90$; $p < 0.0001$), mean effect of treatment: ($F(1,42) = 135.13$; $p < 0.0001$) and Age × treatment ($F(2,42) = 133.25$; $p < 0.0001$) and axonal branch length [mean effect interaction: ($F(3,56) = 274.24$; $p < 0.0001$), mean effect of treatment: ($F(1,56) = 155.28$; $p < 0.0001$) and Age × treatment ($F(3,56) = 187.50$; $p < 0.0001$)] (figure 4.4.5.). Comparison within the kolanut-treated brains relative to the ages showed that there was significant difference in the primary, secondary, tertiary and axonal branch length when compared to the PND 7. Meanwhile, the results showed further that there was significant difference in the primary, secondary, tertiary, quaternary and axonal branch length when compared to the PND 21 and 56 (figure 4.4.5.1). The axonal branch length at PND 70 when compared to the PND 7, PND 21 and 56 increased markedly but decreased relative to PND 7 (figure 4.4.5.1).

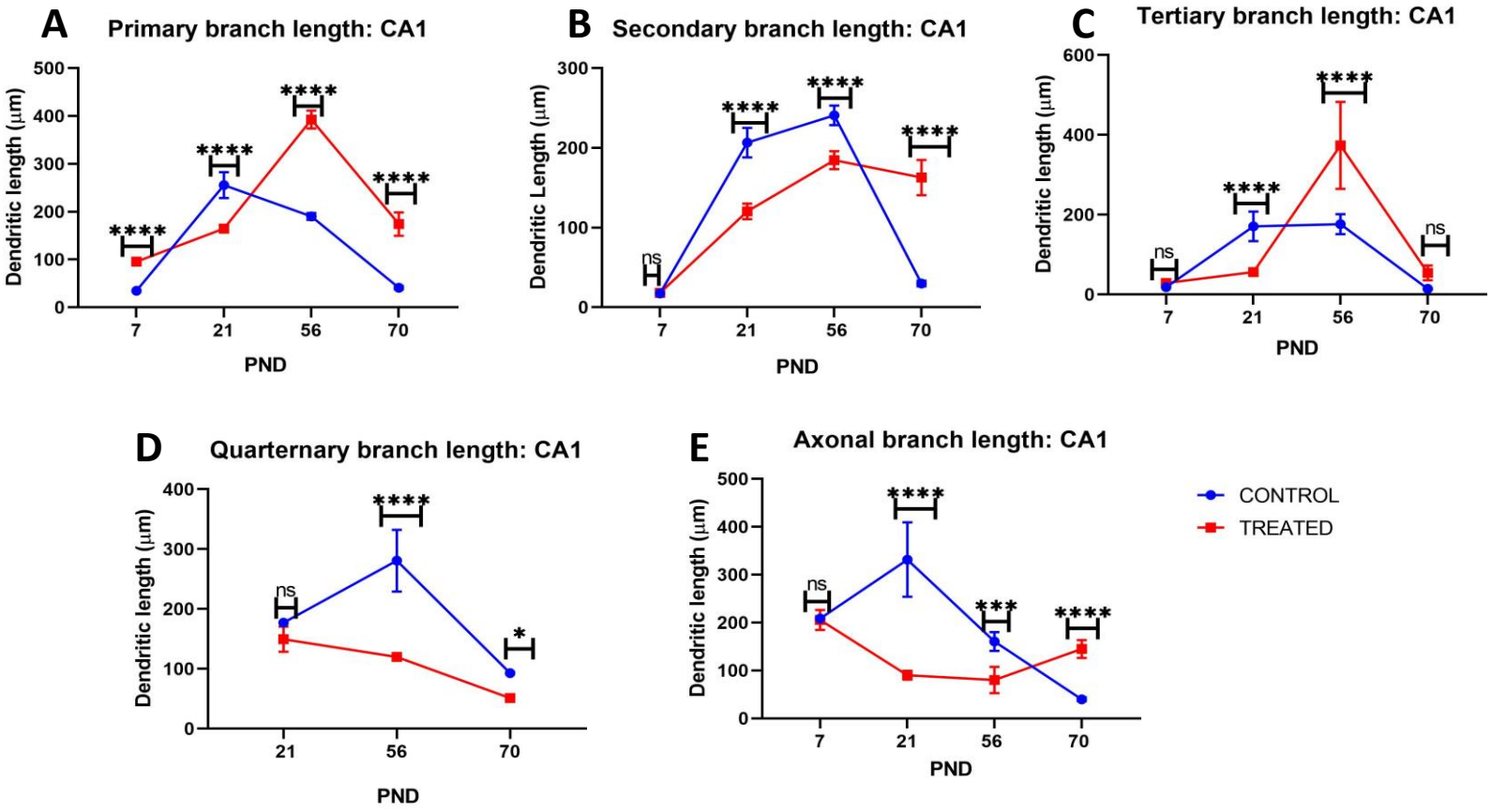



**Figure 4.4.5.1:** Effect of prenatal kolanut consumption on hippocampal dendritic primary branch of the dendritic spine length of CA1 (**A**), secondary branch (**B**), tertiary branch (**C**), quaternary branch (**D**), axonal branch (**E**) in rat after birth from PND 7-70. Data are represented as mean ± S.E.M.; significant differences are shown in P-value, */***/**** against control.

#### 4.4.5.2 CA2 hippocampal region

As shown in Figure 4.4.5.2.1-5, prenatal exposure to kolanut consumption significantly affected the CA2 dendritic length when compared to the control: primary branch length [mean effect interaction: ($F(3,56) = 200.58$; $p < 0.0001$), mean effect of treatment: ($F(1,56) = 904.37$; $p < 0.0001$) and Age × treatment ($F(3,56) = 1291.20$; $p < 0.0001$)], secondary branch length [mean effect interaction: ($F(3,56) = 181.03$; $p < 0.0001$), mean effect of treatment: ($F(1,56) = 2226.85$; $p < 0.0001$) and Age × treatment ($F(3,56) = 1150.28$; $p < 0.0001$)], tertiary branch length [mean effect interaction: ($F(3,56) = 263.21$; $p < 0.0001$), mean effect of treatment: ($F(1,56) = 316.00$; $p < 0.0001$) and Age × treatment ($F(3,56) = 372.76$; $p < 0.0001$)], quaternary branch length [mean effect interaction: ($F(2,42) = 34.02$; $p < 0.0001$), mean effect of treatment: ($F(1,42) = 360.29$; $p < 0.0001$) and Age × treatment ($F(2,42) = 358.95$; $p < 0.0001$) and axonal branch length [mean effect interaction: ($F(3,56) = 160.79$; $p < 0.0001$, mean effect of treatment: ($F(1,56) = 4.53$; $p = 0.0378$) and Age × treatment ($F(3,56) = 194.14$; $p < 0.0001$)] (figure 4.4.5.2 A-E). Similarly, the different treatment effect within the kolanut-treated animals showed significant differences in the dendritic branch length at PND 21 and PND 56 (primary, secondary, and tertiary) and at PND 70 (primary, secondary, quaternary and axonal) when compared to the Kolanut-treated animals at PND 7 (figure 4.4.5.2 A-E). Also, there was a similar significant difference in the dendritic branch length (primary, secondary, tertiary, quaternary and axonal) at PND 56 and PND 70 relative to the kolanut-treated animals at PND 21 and PND 56 (figure 4.4.5.2 A-E).

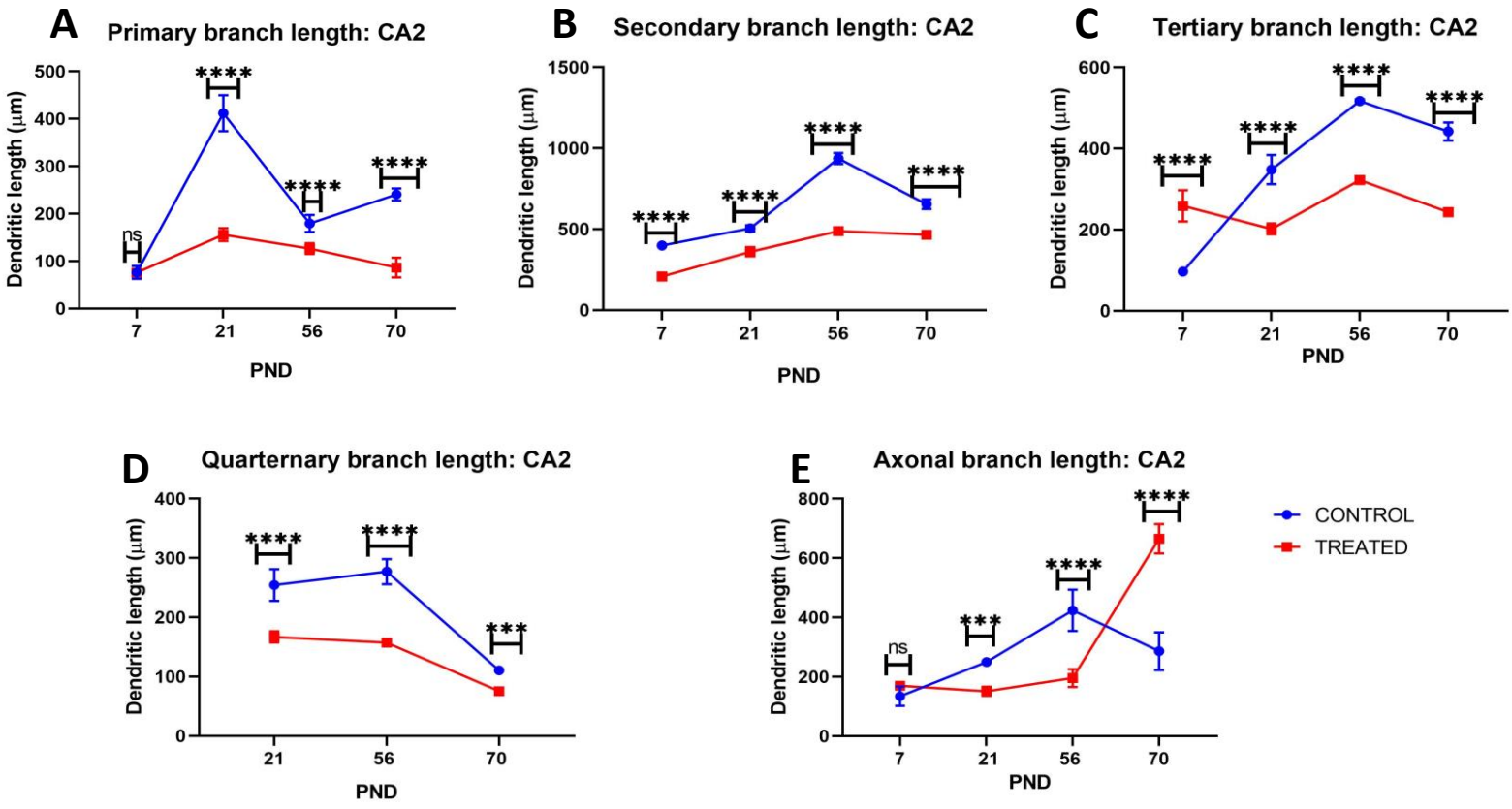



**Figure 4.4.5.2:** Effect of prenatal kolanut consumption on hippocampal dendritic primary branch of the dendritic spine length of CA2 (**A**), secondary branch (**B**), tertiary branch (**C**), quaternary branch (**D**), axonal branch (**E**) in rat after birth from PND 7-70. Data are represented as mean ± S.E.M.; significant differences are shown in P-value, ***/**** against control.

### 4.4.5.3 CA3 hippocampal region

Figure 4.4.5.3 A-E shows the developmental effect of kolanut consumption. The consumption of kolanut affected significantly the CA3 dendritic branch length relative to the control: primary branch length [mean effect interaction: ($F(3,56) = 701.64$; $p < 0.0001$), mean effect of treatment: ($F(1,56) = 4654.99$; $p < 0.0001$) and Age × treatment ($F(3,56) = 934.76$; $p < 0.0001$)] (figure 4.4.5.3 A), secondary branch length [mean effect interaction: ($F(3,56) = 883.69$; $p < 0.0001$), mean effect of treatment: ($F(1,56) = 9278.71$; $p < 0.0001$) and Age × treatment ($F(3,56) = 1605.70$; $p < 0.0001$)] (figure 4.4.5.3 B), tertiary branch length [mean effect interaction: ($F(3,56) = 276.79$; $p < 0.0001$), mean effect of treatment: ($F(1,56) = 3431.94$; $p < 0.0001$) and Age × treatment ($F(3,56) = 536.89$; $p < 0.0001$)] (figure 4.4.5.3 C), quaternary branch length [mean effect interaction: ($F(2,42) = 42.59$; $p < 0.0001$), mean effect of treatment: ($F(1,42) = 2091.77$; $p < 0.0001$) and Age × treatment ($F(2,42) = 784.08$; $p < 0.0001$)] (figure 4.4.5.3 D), and axonal branch length [mean effect interaction: ($F(3,56) = 208.72$; $p < 0.0001$, mean effect of treatment: ($F(1,56) = 234.72$; $p < 0.0001$) and Age × treatment ($F(3,56) = 195.87$; $p < 0.0001$)] (figure 4.4.5.3 E). The treatment effect within the kolanut-treated animals showed further a significant difference in the dendritic branch length at PND 21 (primary, secondary, tertiary, and axonal), PND 56 (primary, secondary, tertiary, quaternary and axonal) and at PND 70 (tertiary, quaternary and axonal) when compared to the kolanut-treated animals at PND 7 (figure 4.4.5.3 A-E). Meanwhile, a similar significant difference was shown in the primary, secondary and tertiary dendritic branch length alone at PND 56 (figure 4.4.5.3 A-C) but seen in the primary, secondary, tertiary, quaternary and axonal branch length at PND 70 relative to the Kolanut-treated animals at PND 21 (figure 4.4.5.3 A-E). Comparing PND 56 and PND 70, a significant difference in PND 70 as indicated by decreased primary, secondary and tertiary dendritic branch length was observed (figure 4.4.5.3 A-C).

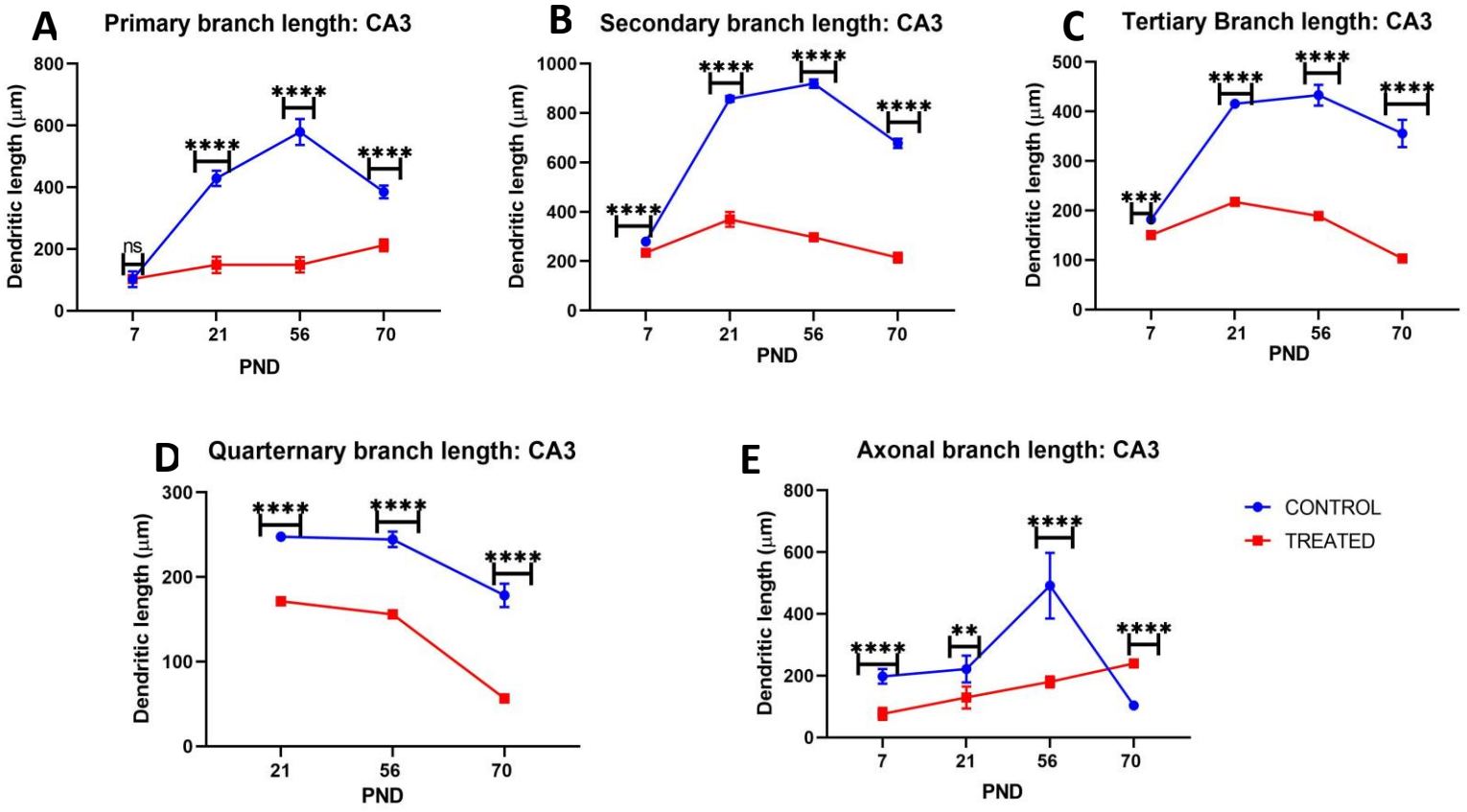



**Figure 4.4.5.3:** Effect of prenatal kolanut consumption on hippocampal dendritic primary branch (**A**), secondary branch (**B**), tertiary branch (**C**), quaternary branch (**D**), axonal branch (**E**) of the dendritic spine length of CA3 in rat after birth from PND 7-70. Data are represented as mean ± S.E.M.; significant differences are shown in P-value, **/***/**** against control.

#### 4.4.5.4 DG hippocampal region

The dendritic branch length in the dentate gyrus (DG) are presented in figure 4.4.5.4 A-E. There was a further significant difference and shorter dendritic branch length of the DG when compared to the control as revealed by primary branch length [mean effect interaction: ($F(3,56) = 2652.61$; $p < 0.0001$), mean effect of treatment: ($F(1,56) = 22819.54$; $p < 0.0001$) and Age × treatment ($F(3,56) = 20585.61$; $p < 0.0001$)] (figure 4.4.5.4 A), secondary branch length [mean effect interaction: ($F(3,56) = 1563.40$; $p < 0.0001$), mean effect of treatment: ($F(1,56) = 17177.98$; $p < 0.0001$) and Age × treatment ($F(3,56) = 676.41$; $p < 0.0001$)] (fig. 4.4.5.4.2), tertiary branch length [mean effect interaction: ($F(3,56) = 673.04$; $p < 0.0001$), mean effect of treatment: ($F(1,56) = 4923.03$; $p < 0.0001$) and Age × treatment ($F(3,56) = 4305.33$; $p < 0.0001$)] (figure 4.4.5.4 C), quaternary branch length [mean effect interaction: ($F(2,42) = 137.38$; $p < 0.0001$), mean effect of treatment: ($F(1,42) = 4105.41$; $p < 0.0001$) and Age × treatment ($F(2,42) = 340.62$; $p < 0.0001$)] (figure 4.4.5.4 D) and axonal branch length [mean effect interaction: ($F(3,56) = 29.72$; $p < 0.0001$, mean effect of treatment: ($F(1,56) = 519.15$; $p < 0.0001$) and Age × treatment ($F(3,56) = 51.47$; $p < 0.0001$)] (figure 4.4.5.4 E). There was a longer length observed in the dendritic branch at PND 21 and PND 56 (primary, secondary and tertiary) and at PND 70 (primary, secondary, quaternary and axonal) when compared to the kolanut-treated brains at PND 7 (figure 4.4.5.4 A-E). Moreover, similar longer length was observed in the dendritic branch at PND56 (primary, secondary, and tertiary) and at PND 70 (primary, secondary, tertiary, quaternary and axonal) relative to the kolanut-treated brains at PND 21 (figure 4.4.5.4 A-E). In addition, the dendritic branch length in PND 70 showed longer length (primary, secondary, tertiary, and axonal) when compared to the kolanut-treated brains at PND 56 (figure 4.4.5.4 A-C, E).

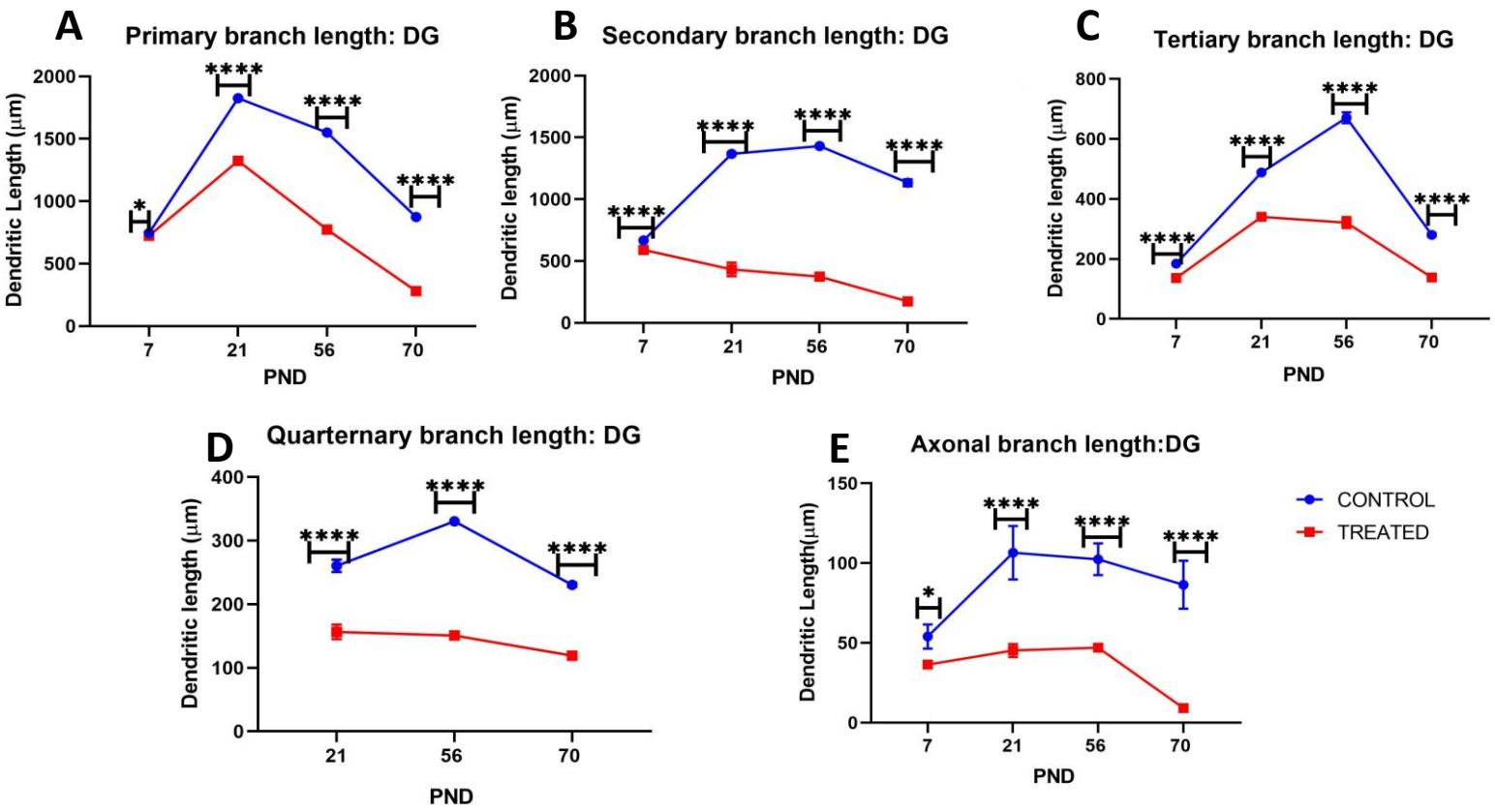



**Figure 4.4.5.4:** Effect of prenatal kolanut consumption on hippocampal dendritic primary branch (**A**), secondary branch (**B**), tertiary branch (**C**), quaternary branch (**D**), axonal branch (**E**) of the dendritic spine length of DG in rat after birth from PND 7-70. Data are represented as mean ± S.E.M.; significant differences are shown in P-value, */**** against control.

#### 4.4.6.1. Effect of prenatal kolanut consumption on mean hippocampal dendritic spine density

The total spine count was estimated in the CA1-3 and DG of animals after prenatal exposure to kolanut. As represented in figure 4.4.6.1.A-D, a significant difference in the mean spine number between the different treatments groups was observed. Results, following analysis showed that there was significant difference in number in the CA1 [mean effect interaction: ($F(3,56) = 56.99$; $p < 0.0001$), mean effect of treatment: ($F(1,56) = 355.84$; $p < 0.0001$) and Age × treatment ($F(3,56) = 270.27$; $p < 0.0001$)] (fig. 4.4.6.1.1), CA2 [mean effect interaction: ($F(3,56) = 39.64$; $p < 0.0001$), mean effect of treatment: ($F(1,56) = 224.69$; $p < 0.0001$) and Age × treatment ($F(3,56) = 152.28$; $p < 0.0001$)] (fig. 4.4.6.1.2), CA3 [mean effect interaction: ($F(3,56) = 101.41$; $p < 0.0001$), mean effect of treatment: ($F(1,56) = 46.30$; $p < 0.0001$) and Age × treatment ($F(3,56) = 78.68$; $p < 0.0001$)] (fig. 4.4.6.1.3) when compared to the control groups, and DG [mean effect interaction: ($F(3,56) = 82.82$; $p < 0.0001$, mean effect of treatment: ($F(1,56) = 315.81$; $p < 0.0001$) and Age × treatment ($F(3,56) = 263.13$; $p < 0.0001$)] at the different PNDs comparative to the control (figure 4.4.6.1 D). The comparative effect of prenatal kolanut exposure was also observed in the kolanut-treated brains with significant different treatment effect in mean spine number at PND 56 (CA1 and CA3) and at PND 70 (CA1 and DG) when compared to the kolanut-treated brains at PND 7 (fig. 4.4.6.1.1 C-D). However, a similar comparative significant different treatment effect in mean spine number at PND 21 and 56 (CA1-2 and DG) and at PND 70 (CA1-2) when compared to the kolanut-treated brains at PND 7 (figure 4.4.6.1 A-B,D). Comparison between PND 56 and 70 to PND 21 showed significant different treatment effect in mean spine number at PND 56 (CA3) and PND 70 (CA3 and DG) when compared to the kolanut-treated brains at PND 21 (figure 4.4.6.1.C-D). In addition to these findings, the mean spine number in PND 70 showed significant decreased treatment effect at the DG hippocampal region when compared to the kolanut-treated brains at PND 56 (figure 4.4.6.1 D).

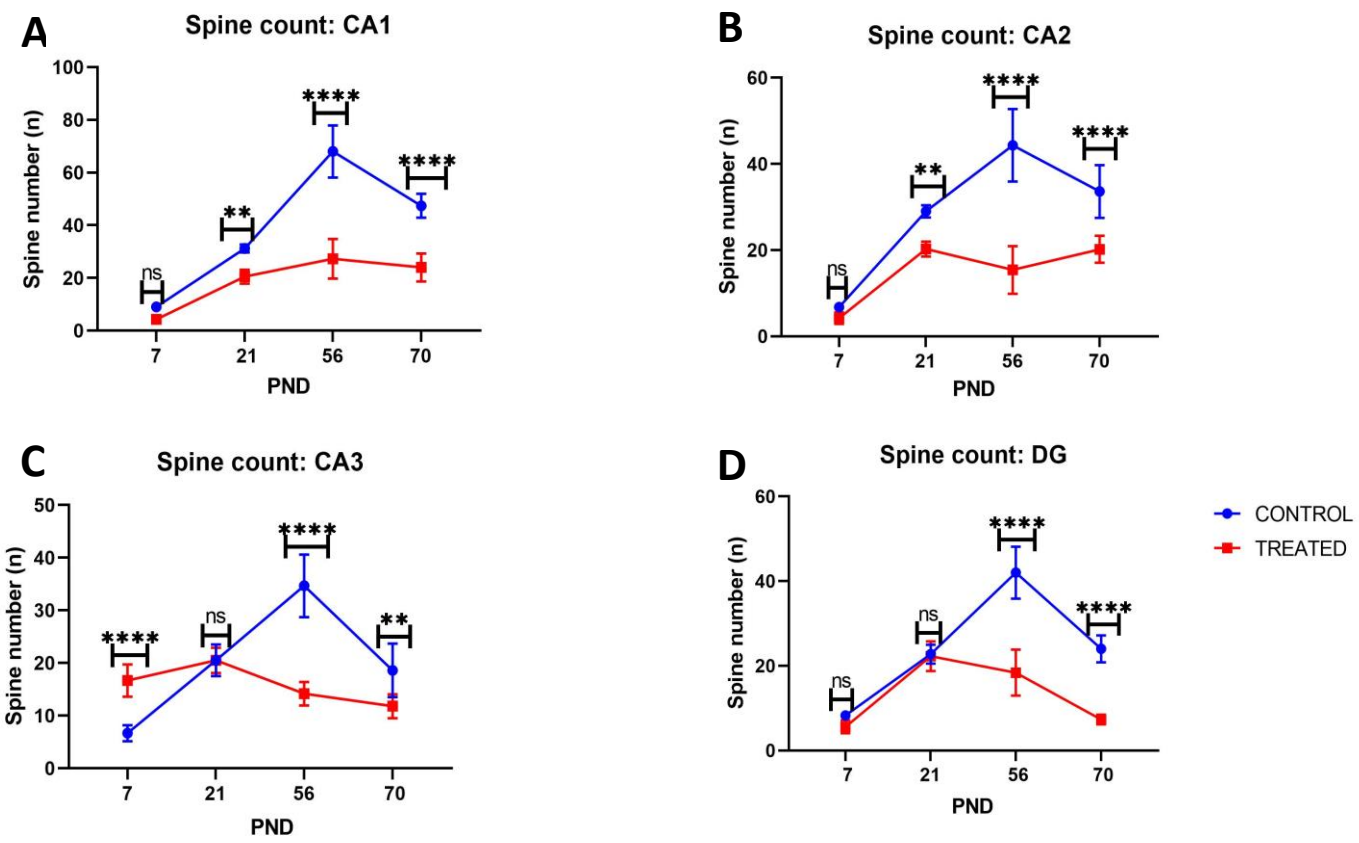



**Figure 4.4.6.1:** Effect of prenatal kolanut consumption on mean hippocampal dendritic spine density of CA1 (**A**), CA2 (**B**), CA3 (**C**), DG (**D**) in rat after birth from PND 7-70. Data are represented as mean ± S.E.M.; significant differences are shown in P-value, **/**** against control.

### 4.4.6.2 Effect of prenatal kolanut consumption on density of hippocampal dendritic spine branch after birth.

As presented in figure 4.4.6.2, prenatal exposure to kolanut exacerbated increase and decreased number of dendritic spine branches (primary, secondary, tertiary and axonal branches) in the CA1-3 and the DG of the hippocampus of the animals with age-dependent effect in comparison with the control.

#### 4.4.6.2.1 CA1 hippocampal region

The prenatal kolanut treatment significantly increase and decrease the number of dendritic spine branches in the CA1 of the hippocampus when compared to the control as shown by the total number of primary branch [mean effect interaction: ($F(3,56) = 77.89$; $p < 0.0001$), mean effect of treatment: ($F(1,56) = 201.88$; $p < 0.0001$) and Age × treatment ($F(3,56) = 481.67$; $p < 0.0001$)] (figure 4.4.6.2.1 A), secondary branch [mean effect interaction: ($F(3,56) = 213.25$; $p < 0.0001$), mean effect of treatment: ($F(1,56) = 1610.82$; $p < 0.0001$) and Age × treatment ($F(3,56) = 1848.14$; $p < 0.0001$)] (figure 4.4.6.2.1 B), tertiary branch [mean effect interaction: ($F(3,56) = 198.44$; $p < 0.0001$), mean effect of treatment: ($F(1,56) = 148.12$; $p < 0.0001$) and Age × treatment ($F(3,56) = 415.96$; $p < 0.0001$)] (figure 4.4.6.2.1 C), and axonal branch [mean effect interaction: ($F(3,56) = 274.24$; $p < 0.0001$), mean effect of treatment: ($F(1,56) = 155.28$; $p < 0.0001$) and Age × treatment ($F(3,56) = 187.50$; $p < 0.0001$)] (figure 4.4.6.2.1 D). Comparative effect of kolanut treatment within the kolanut-treated brains relative to the ages showed that there was significant difference in the total number of CA1 primary (PND 70), secondary (PND 56), tertiary (PND 56 and 70) and axonal (PND 21, 56 and 70) branch when compared to the PND 7 (figure 4.4.6.2.1 A-D). However, the results showed further that there was significant difference in the total number of CA1 primary (PND 70) and tertiary (PND 56 and 70) branch when compared to the PND 21 (figure 4.4.6.2.1 C) and in the total number CA1 primary (PND 70) and secondary (PND 70) branch when compared to the PND 56 (figure 4.4.6.2.1 A-B).

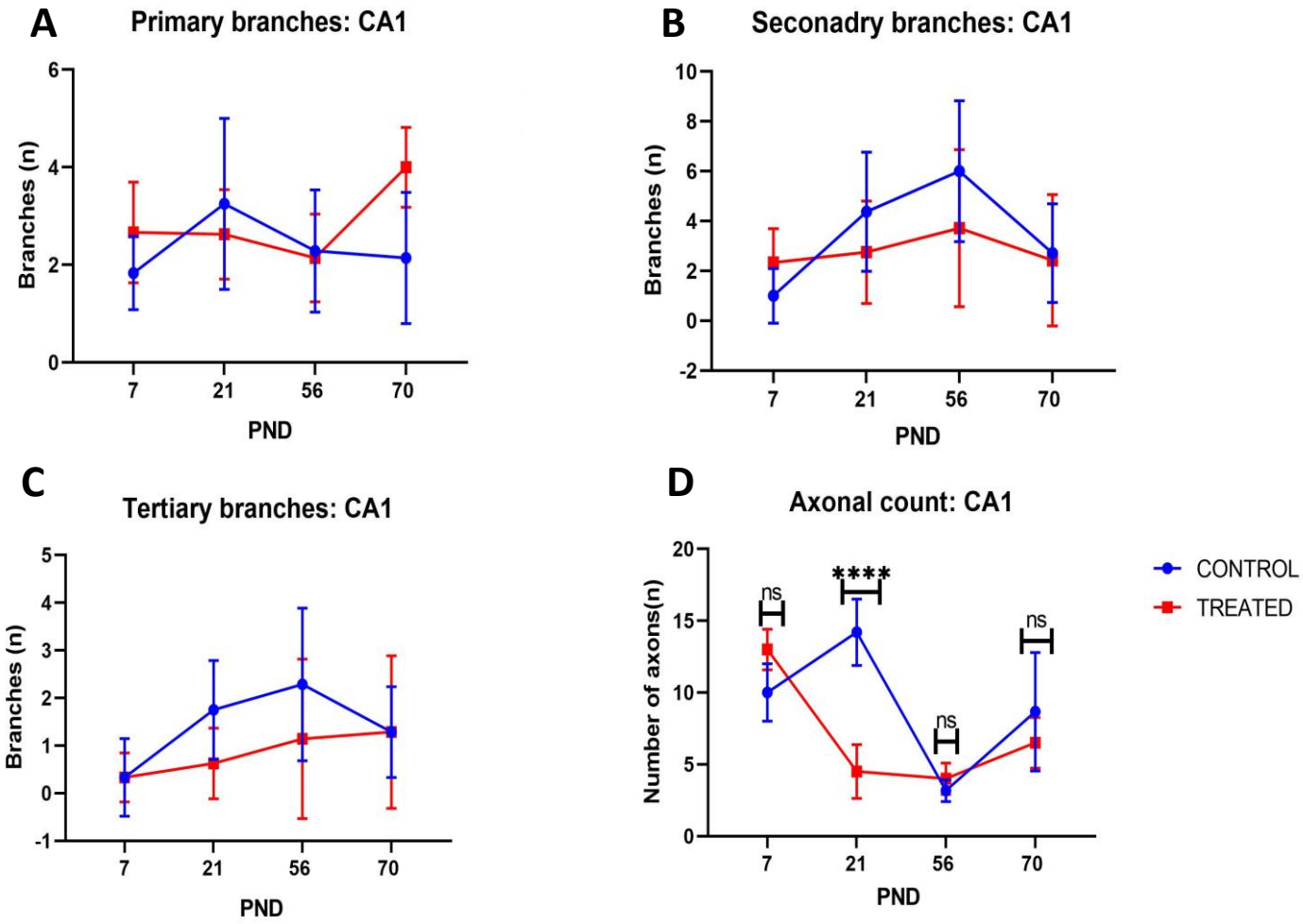



**Figure 4.4.6.2.1:** Effect of prenatal kolanut consumption on density of hippocampal dendritic primary (**A),** secondary (**B**), tertiary (**C**), and axonal (**D**) spine branch of CA1 in rat after birth from PND 7-70. Data are represented as mean ± S.E.M.; significant differences are shown in P-value, **** against control.

#### 4.4.6.2.2 CA2 hippocampal region

As presented in figure 4.4.6.2.2 A-D, the prenatal kolanut exposure moderately altered the number of dendritic spine branches in the CA2 at different segments of the hippocampus when compared to the control. The results showed moderate or no significant difference as indicated by the total number of primary branch [mean effect interaction: ($F(3,56) = 77.89$; $p < 0.0001$), mean effect of treatment: ($F(1,56) = 201.88$; $p < 0.0001$) and Age × treatment ($F(3,56) = 481.67$; $p < 0.0001$)] (figure 4.4.6.2.2 A), secondary branch [mean effect interaction: ($F(3,56) = 213.25$; $p < 0.0001$), mean effect of treatment: ($F(1,56) = 1610.82$; $p < 0.0001$) and Age × treatment ($F(3,56) = 1848.14$; $p < 0.0001$)] (figure 4.4.6.2.2 B), tertiary branch [mean effect interaction: ($F(3,56) = 198.44$; $p < 0.0001$), mean effect of treatment: ($F(1,56) = 148.12$; $p < 0.0001$) and Age × treatment ($F(3,56) = 415.96$; $p < 0.0001$)] and Age × treatment ($F(2,42) = 133.25$; $p < 0.0001$)] (figure 4.4.6.2.2 C) and axonal branch [mean effect interaction: ($F(3,56) = 274.24$; $p < 0.0001$), mean effect of treatment: ($F(1,56) = 155.28$; $p < 0.0001$) and Age × treatment ($F(3,56) = 187.50$; $p < 0.0001$)] (figure 4.4.6.2.2 D). The comparative effect of kolanut treatment within the kolanut-treated brains relative to the ages showed significant difference in the total number of CA2 secondary (PND 56), tertiary (PND 21 and 70) and axonal (PND 21, 56 and 70) branches when compared to the PND 7 (figure 4.4.6.2.2 B and D). However, the results showed further a significant difference in the total number of CA2 secondary (PND 56) and tertiary (PND 56 and 70) branches when compared to the PND 21 (figure 4.4.6.2.2 B-C) and in the total number CA2 tertiary branch (PND 70) when compared to the PND 56 (figure 4.4.6.2.2 C).

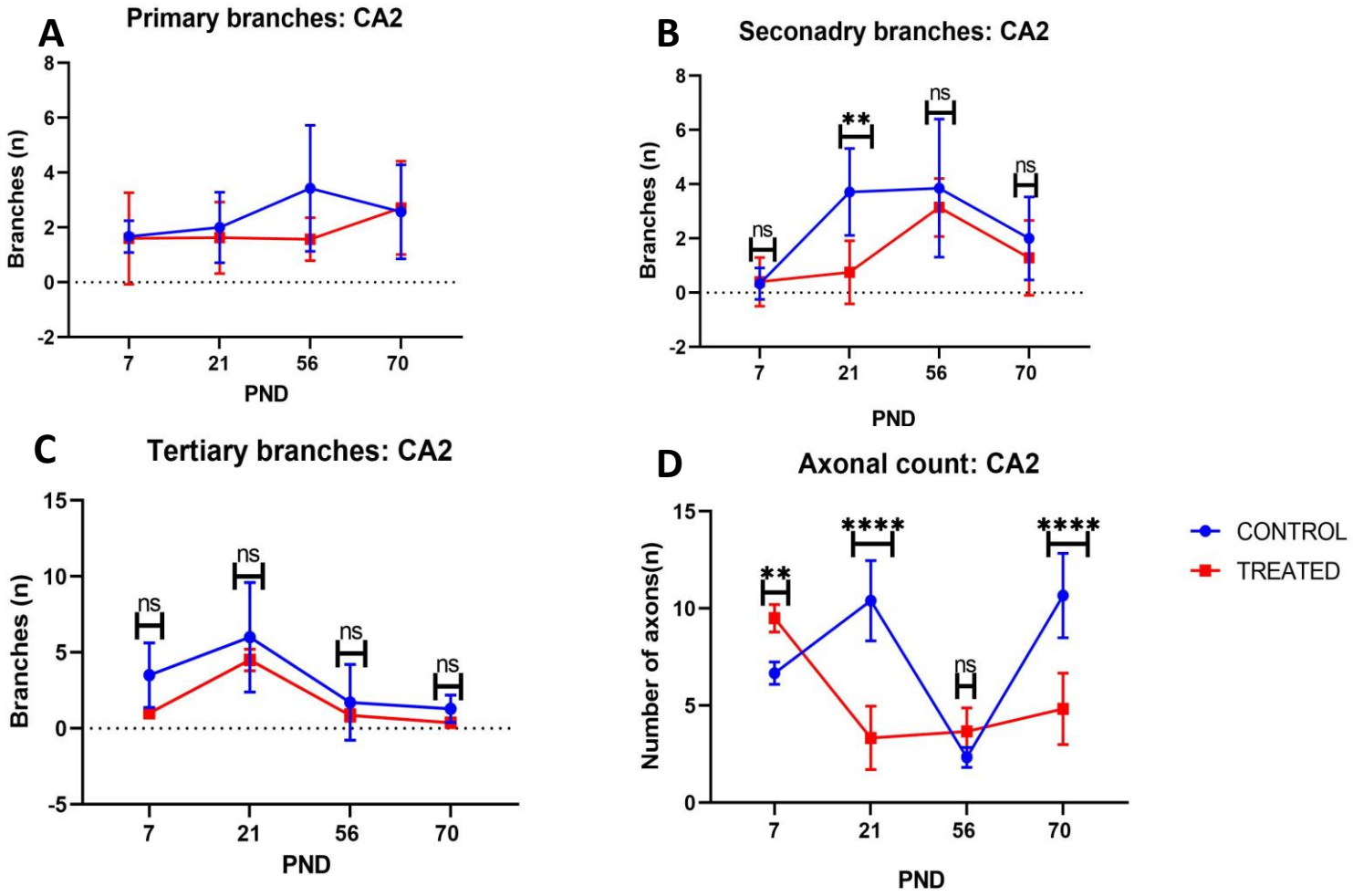



**Figure 4.4.6.2.2:** Effect of prenatal kolanut consumption on density of hippocampal dendritic primary (**A),** secondary (**B**), tertiary (**C**), and axonal (**D**) spine branch of CA2 in rat after birth from PND 7-70. Data are represented as mean ± S.E.M.; significant differences are shown in P-value, **/ **** against control.

#### 4.4.6.2.3 CA3 hippocampal region

Further, the spine density estimation at the CA3 hippocampal region showed that the prenatal kolanut treatment moderately affected the number of dendritic spine branches in the CA3 of the hippocampus compared to the control as shown by the total number of primary branches [mean effect interaction: ($F(3,56) = 77.89$; $p < 0.0001$), mean effect of treatment: ($F(1,56) = 201.88$; $p < 0.0001$) and Age × treatment ($F(3,56) = 481.67$; $p < 0.0001$)] (figure 4.4.6.2.3 A), secondary branch [mean effect interaction: ($F(3,56) = 213.25$; $p < 0.0001$), mean effect of treatment: ($F(1,56) = 1610.82$; $p < 0.0001$) and Age × treatment ($F(3,56) = 1848.14$; $p < 0.0001$)] (figure 4.4.6.2.3 B), tertiary branch [mean effect interaction: ($F(3,56) = 198.44$; $p < 0.0001$), mean effect of treatment: ($F(1,56) = 148.12$; $p < 0.0001$) and Age × treatment ($F(3,56) = 415.96$; $p < 0.0001$)] and Age × treatment ($F(2,42) = 133.25$; $p < 0.0001$)] (figure 4.4.6.2.3 C), and axonal branch [mean effect interaction: ($F(3,56) = 274.24$; $p < 0.0001$), mean effect of treatment: ($F(1,56) = 155.28$; $p < 0.0001$) and Age × treatment ($F(3,56) = 187.50$; $p < 0.0001$)] (figure 4.4.6.2.3 D). However, the kolanut treatment effect was compared within the kolanut-treated brains relative to the ages and there was moderately significant difference in the total number of CA3 primary (PND 21), secondary (PND 21, 56 and 70), tertiary (PND 21, 56 and 70) and axonal (PND 21, 56 and 70) branch when compared to the PND 7 (figure 4.4.6.2.3 A-D). In addition, there was further significant difference in the total number of CA3 primary (PND 70), secondary (PND 70), tertiary (PND 56 and 70) and axonal (PND 70) branch when compared to the PND 21 (figure 4.4.6.2.3 A-D) and in the total number CA3 secondary and axonal (PND 70) branch when compared to the PND 56 (figure 4.4.6.2.3 B and D).

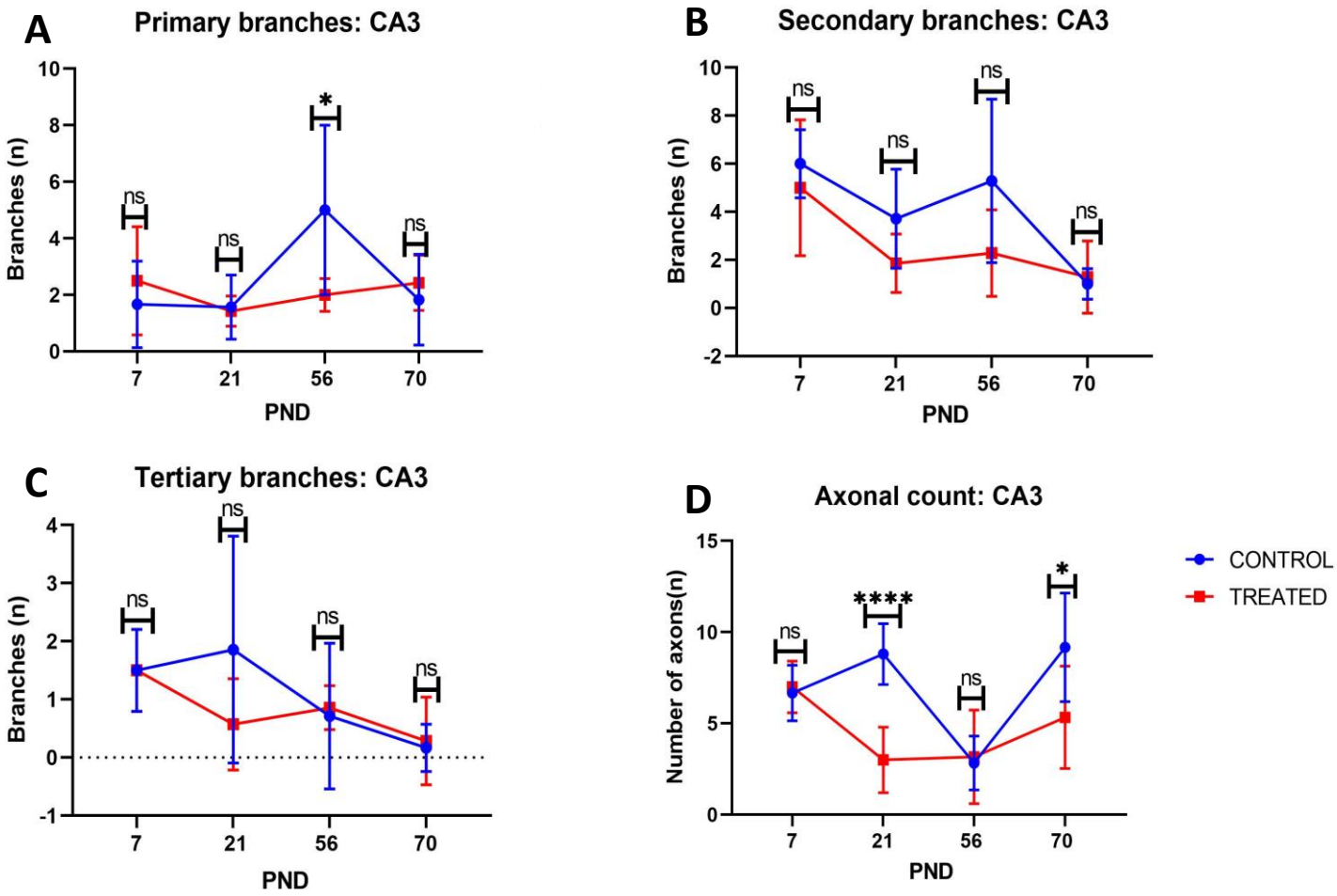



**Figure 4.4.6.2.3:** Effect of prenatal kolanut consumption on density of hippocampal dendritic primary (**A),** secondary (**B**), tertiary (**C**), and axonal (**D**) spine branch of CA3 in rat after birth from PND 7-70. Data are represented as mean ± S.E.M.; significant differences are shown in P-value, */**** against control.

#### 4.4.6.2.4 Dentate Gyrus (DG)

To complete the estimation of spine branches in the hippocampus, the total number of spine branches was also estimated in the dentate gyrus of the exposed brains. Figure 4.4.6.2.4 A-D showed that prenatal kolanut treatment significantly affect the number of dendritic spine branches in the DG of the hippocampus when compared to the control as shown by the total number of primary branch [mean effect interaction: ($F(3,56) = 77.89$; $p < 0.0001$), mean effect of treatment: ($F(1,56) = 201.88$; $p < 0.0001$) and Age × treatment ($F(3,56) = 481.67$; $p < 0.0001$)] (figure 4.4.6.2.4.A), secondary branch [mean effect interaction: ($F(3,56) = 213.25$; $p < 0.0001$), mean effect of treatment: ($F(1,56) = 1610.82$; $p < 0.0001$) and Age × treatment ($F(3,56) = 1848.14$; $p < 0.0001$)] (figure 4.4.6.2.4 B), tertiary branch [mean effect interaction: ($F(3,56) = 198.44$; $p < 0.0001$), mean effect of treatment: ($F(1,56) = 148.12$; $p < 0.0001$) and Age × treatment ($F(3,56) = 415.96$; $p < 0.0001$)] (figure 4.4.6.2.4 C), and axonal branch [mean effect interaction: ($F(3,56) = 274.24$; $p < 0.0001$), mean effect of treatment: ($F(1,56) = 155.28$; $p < 0.0001$) and Age × treatment ($F(3,56) = 187.50$; $p < 0.0001$)] (figure 4.4.6.2.4 D). Comparative effect of kolanut treatment within the kolanut-treated brains relative to the ages showed that there was significant difference in the total number of DG primary (PND 21 and 70), secondary (PND 21, 56 and 70), tertiary (PND 21, 56 and 70) and axonal (PND 21 and 70) branch when compared to the PND 7 (figure 4.4.6.2.4 A-D). However, the results showed further that there was significant difference in the total number of DG secondary (PND 56 and 70), tertiary (PND 56 and 70) and axonal (PND 56 and 70) branch when compared to the PND 21 (figure 4.4.6.2.4 B-D) and in the total number DG secondary (PND 70), tertiary (PND 70) and axonal (PND 70) branch when compared to the PND 56 (figure 4.4.6.2.4 B-D).

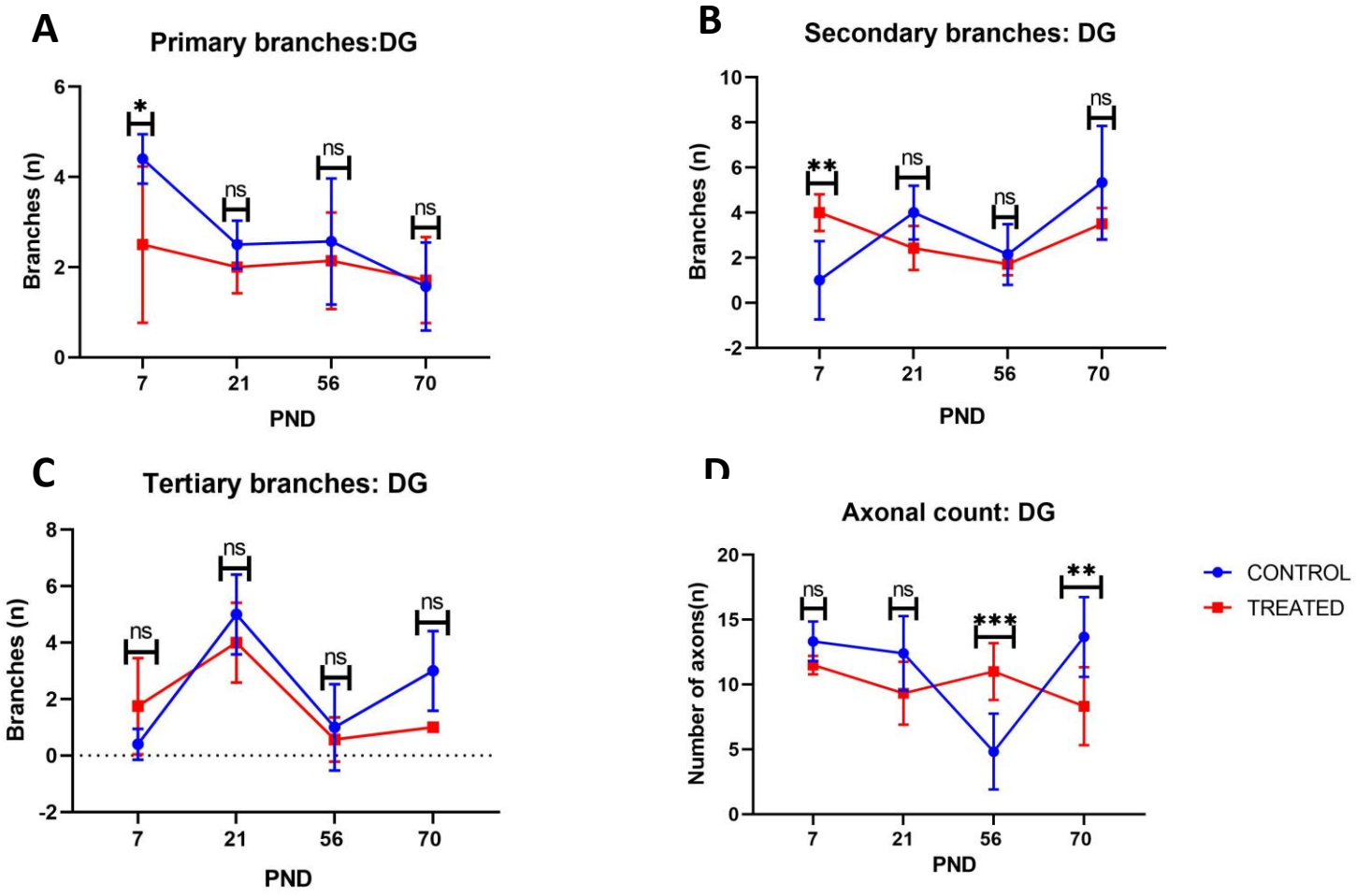



**Figure 4.4.6.2.4:** Effect of prenatal kolanut consumption on density of hippocampal dendritic primary (**A),** secondary (**B**), tertiary (**C**), and axonal (**D**) spine branch of DG in rat after birth from PND 7-70. Data are represented as mean ± S.E.M.; significant differences are shown in P-value, */ **/ *** against control.

### 4.4.7 Effect of prenatal kolanut consumption on spine morphology in the different regions of the hippocampus after birth

The effects of prenatal kolanut exposure on the spine morphology (filopodia, long-thin, thin, stubby, mushroom and branched) within the CA1-3 and DG region in the hippocampus of the experimental animals is captured in table 4.5. There was significant increase in filopodia spine density in the CA1 (PND 21), CA2 (PND 21 and 56), CA3 (PND 21, 56 and 70) and DG (PND 56) relative to their respective controls. Further comparative significant difference was evident in the filopodia spine density as a function of kolanut treatment exposure between the groups. Conversely, when examining the spine morphology, a significant increase in long-thin spines in the CA1 (PND 21 and 70), CA2 (PND 21, 56 and 70), CA3 (PND 21 and 56) and DG (PND 21 and 56) with a concomitant decrease in stubby spines [CA1 (PND 21, 56 and 70), CA2 (PND 21, 56 and 70), CA3 (PND 21 and 56) and DG (PND 21, 56 and 70)], mushroom [CA1 (PND 21), CA2 (PND 21, 56 and 70), CA3 (PND 21, 56 and 70) and DG (PND 21 and 56)], branched spines [CA1-3 and DG (PND 21, 56 and 70)] and an inconsistent increase/decrease spine morphology observed in the thin spines [CA1 (PND 21 and 56), CA2 (PND 21), CA3 (PND 70) and DG (PND 56 and 70)] relative to the controls. Although, a decrease in long-thin spine in the CA3 and DG at PND 70 and an increase in stubby spines [CA1 (PND 21 and 56) and CA3 (PND 70)] and mushroom [CA1 (PND 56 and 70) and DG (PND 70)] was also observed. A comparative significant difference was also seen in long-thin, stubby, thin, mushroom and branched spine density as a function of kolanut treatment exposure between the groups (Table 4.5).

**Table 4.5: Effect of kolanut extract on spine morphology in the different regions of the hippocampus in rat after birth.**

| | | PND 21 | PND 56 | PND 70 |
|---|---|---|---|---|
| **FILOPODIA** | | | | |
| **CA1** | **Control** | 0 | 0 | 0 |
| | **Treated** | 4* | 0 | 0 |
| **CA2** | **Control** | 0 | 0 | 0 |
| | **Treated** | 5.56* | 30*, a | 0 |
| **CA3** | **Control** | 0 | 0 | 0 |
| | **Treated** | 5.26* | 16.67*,a | 54.55*,a,b |
| **DG** | **Control** | 0 | 2.70 | 0 |
| | **Treated** | 0 | 4.76*,a | 0 |
| **LONGTHIN** | | | | |
| **CA1** | **Control** | 3.03 | 0 | 8.57 |
| | **Treated** | 16* | 0 | 18.75*,a,b |
| **CA2** | **Control** | 8.33 | 6.67 | 9.38 |
| | **Treated** | 11.11* | 30*,a | 63.33*,a,b |
| **CA3** | **Control** | 0 | 13.33 | 27.78 |
| | **Treated** | 31.58* | 16.67*,a | 9.09*,a,b |
| **DG** | **Control** | 8 | 2.70 | 29.41 |
| | **Treated** | 30.43* | 47.62*,a | 0*,a,b |
| **THIN** | | | | |
| **CA1** | **Control** | 33.33 | 63.64 | 31.43 |
| | **Treated** | 44* | 43.67* | 37.5 a,b |
| **CA2** | **Control** | 30.56 | 26.67 | 28.13 |
| | **Treated** | 55.56* | 30 a | 26.67 a |
| **CA3** | **Control** | 41.18 | 36.67 | 27.78 |
| | **Treated** | 47.37 | 41.67 | 18.18* a,b |
| **DG** | **Control** | 48 | 43.24 | 41.18 |
| | **Treated** | 47.83 | 28.57* | 75* |
| **STUBBY** | | | | |
| **CA1** | **Control** | 9.09 | 6.06 | 14.29 |
| | **Treated** | 16* | 20*,a | 0*,a,b |
| **CA2** | **Control** | 5.46 | 10 | 28.13 |
| | **Treated** | 0* | 0* | 0* |
| **CA3** | **Control** | 29.41 | 13.33 | 5.56 |
| | **Treated** | 0* | 8.33*,a | 18.18*,a,b |
| **DG** | **Control** | 16 | 18.92 | 5.88 |
| | **Treated** | 4.35* | 4.76* | 0*,a,b |
| **MUSHROOM** | | | | |
| **CA1** | **Control** | 42.42 | 6.06 | 17.14 |
| | **Treated** | 16* | 20* | 37.5*,a,b |

| | | | | |
|---|---|---|---|---|
| **CA2** | **Control** | 27.78 | 16.67 | 15.63 |
| | **Treated** | 16.67* | 0*,a | 6.67*,a,b |
| **CA3** | **Control** | 17.65 | 20 | 22.22 |
| | **Treated** | 10.52* | 16.67*,a | 0*,a,b |
| **DG** | **Control** | 20 | 16.22 | 17.65 |
| | **Treated** | 13.04* | 9.52* | 25* |
| **BRANCHED** | | | | |
| **CA1** | **Control** | 12.12 | 24.24 | 28.57 |
| | **Treated** | 4* | 13.33*,a | 6.25*,b |
| **CA2** | **Control** | 27.78 | 40 | 18.75 |
| | **Treated** | 11.11* | 10*,a | 3.33*,b |
| **CA3** | **Control** | 11.77 | 16.67 | 16.67 |
| | **Treated** | 5.26* | 0*,a | 0*,a |
| **DG** | **Control** | 8 | 16.22 | 5.88 |
| | **Treated** | 4.35* | 4.76* | 0*,b |

**Table 4.5** shows results of effect of kolanut on the spine morphology in the different regions of the hippocampus in the treated and control animals from PND 21, 56 and 70. Data is represented by mean $\pm$ SEM with 10 animals in each group. * indicates significant differences as compared to control.

### 4.4.8 Effect of prenatal kolanut consumption on the total synaptic density in the different regions of the hippocampus after birth

The total synaptic count was estimated from the different regions of the hippocampal brain sections after prenatal exposure to kolanut. From the results obtained and as represented in figure 4.4.8. A-D, there was significant difference in the mean synaptic count between the different treatments groups as indicated by CA1 [mean effect interaction: ($F(3,56) = 58.25$; $p < 0.0001$), mean effect of treatment: ($F(1,56) = 64.23$; $p < 0.0001$) and Age × treatment ($F(3,56) = 454.33$; $p < 0.0001$)] (figure 4.4.8 A), CA2 [mean effect interaction: ($F(3,56) = 273.82$; $p < 0.0001$), mean effect of treatment: ($F(1,56) = 882.00$; $p < 0.0001$) and Age × treatment ($F(3,56) = 284.32$; $p < 0.0001$)] (figure 4.4.8 B) and CA3 [mean effect interaction: ($F(3,56) = 419.94$; $p < 0.0001$), mean effect of treatment: ($F(1,56) = 1456.72$; $p < 0.0001$) and Age × treatment ($F(3,56) = 2159.50$; $p < 0.0001$)] (figure 4.4.8 C) when compared to the control groups. Meanwhile, there was no significant difference in the DG synaptic count [mean effect interaction: ($F(3,56) = 45.70$; $p < 0.0001$, mean effect of treatment: ($F(1,56) = 3.12$; $p = 0.0830$) and Age × treatment ($F(3,56) = 506.18$; $p < 0.0001$)] comparative to the control (figure 4.4.8 D). Further comparative effect of prenatal kolanut exposure was also observed in the kolanut-treated brains with significant different treatment effect in the number of hippocampal synaptic density at PND 56 (CA1 and CA3) and at PND 70 (CA1 and DG) when compared to the kolanut-treated brains at PND 7 (figure 4.4.8 A, C-D). However, a similar comparative significant different treatment effect in the number of hippocampal synaptic density at PND 56 (CA3) and at PND 70 (CA1 and CA3) relative to the Kolanut-treated brains at PND 21 (figure 4.4.8 A and D). In addition, the number of hippocampal synaptic density in PND 70 showed significant decreased treatment effect (CA3) when compared to the Kolanut-treated brains at PND 56 (figure 4.4.8 C).

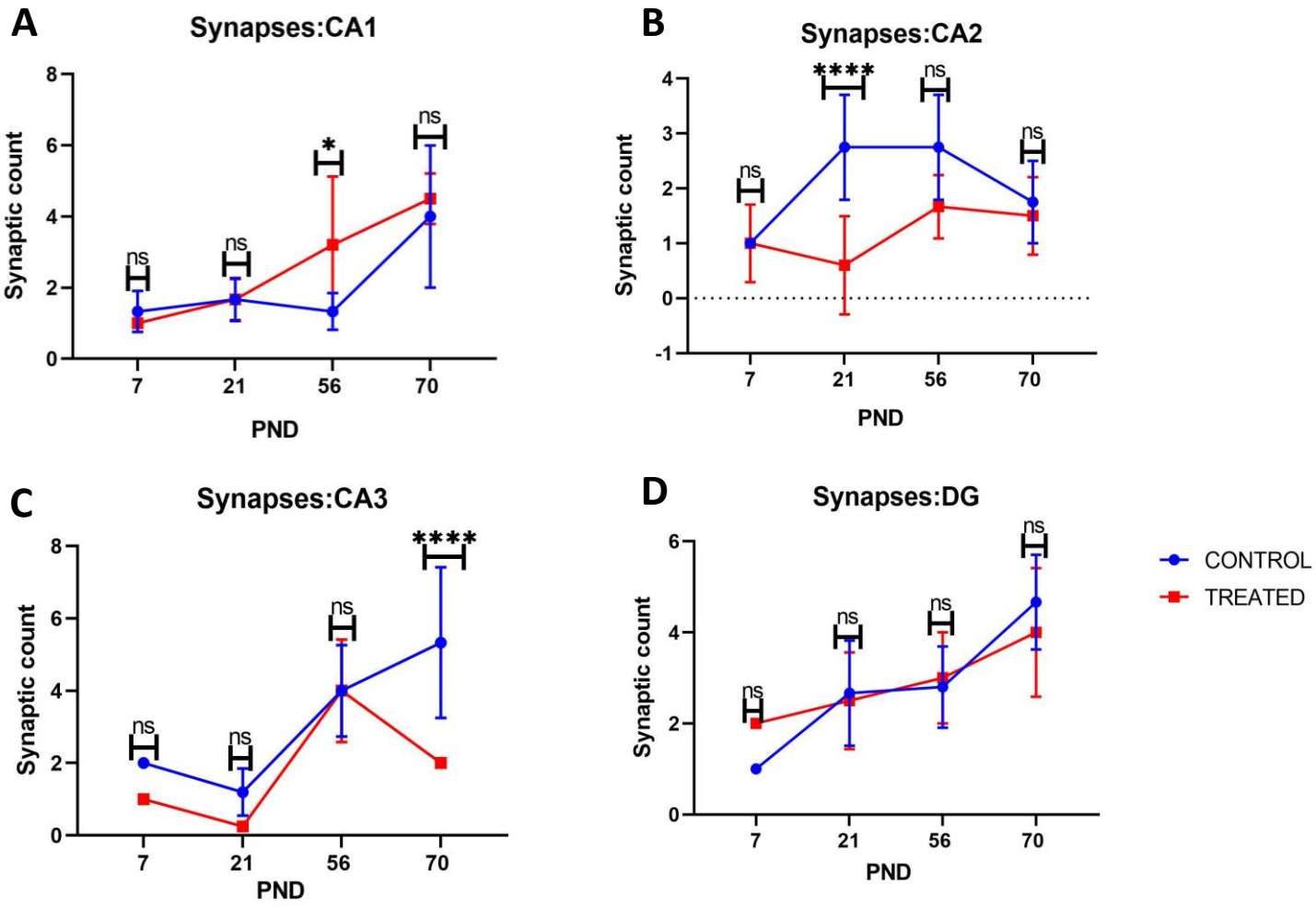



**Figure 4.4.8:** Effect of prenatal kolanut consumption the total synaptic density in the CA1 (**A),** CA2 (**B**), CA3 (**C**), and DG (**D**) in rat hippocampus after birth from PND 7-70. Data are represented as mean ± S.E.M.; significant differences are shown in P-value, */**** against control.

## 4.5 Histomorphometry evaluation of the hippocampus and prefrontal cortex in pups following exposure to prenatal kolanut consumption.

### 4.5.1 Effect of prenatal kolanut consumption on the color intensity of Nissl, Ki-67 and DCX stains in the hippocampus of rat after birth

Figure 4.5.1 A-C (I-IV) represents the photomicrographs and quantifications of the staining intensity of the subgranular zone (SGZ) in the DG. The results showed significant difference in the Nissl mean expression of staining intensity [mean effect of treatment: ($F_{(2,12)} = 18.72$; $p = 0.0002$), mean effect of age: ($F_{(1,12)} = 2.408$; $p = 0.1467$) and mean interaction effect of treatment × age ($F_{(2,12)} = 1.042$; $p = 0.3826$)] in the SGZ of the DG at the different PNDs (21, 56 and 70) relative to their respective controls (figure 4.5.1 A-C (I-II)). To elucidate the anti-neurogenic effect of intrauterine kolanut consumption on the neuronal differentiation in the pup hippocampi, the data showed significant increase $DCX^+$ mean expression intensity in SGZ in the DG [mean effect of treatment: ($F_{(2,12)} = 125.7$; $p < 0.0001$), mean effect of age: ($F_{(1,12)} = 191.1$; $p < 0.0001$) and mean interaction effect of treatment × age ($F_{(2,12)} = 4.18$; $p = 0.0419$)] across the PNDs at 21, 56 and 70 relative to their respective control after prenatal administration of kolanut (figure 4.5.1 A-C (III-IV)). In addition, the endogenous proliferating (cell cycle) marker, Ki-67 at PND 21, 56 and 70 and as represented in figure 4.5.1 A-C (V-VI) indicated no significant difference on the intensity of the Ki-67+cells [mean effect of treatment: ($F_{(2,12)} = 4.70$; $p = 0.0311$), mean effect of age: ($F_{(1,12)} = 0.32$; $p = 0.5817$) and mean interaction effect of treatment × age ($F_{(2,12)} = 0.80$; $p = 0.4732$)] in contrast to their respective controls (figure 4.5.1 A-C (V-VI)).

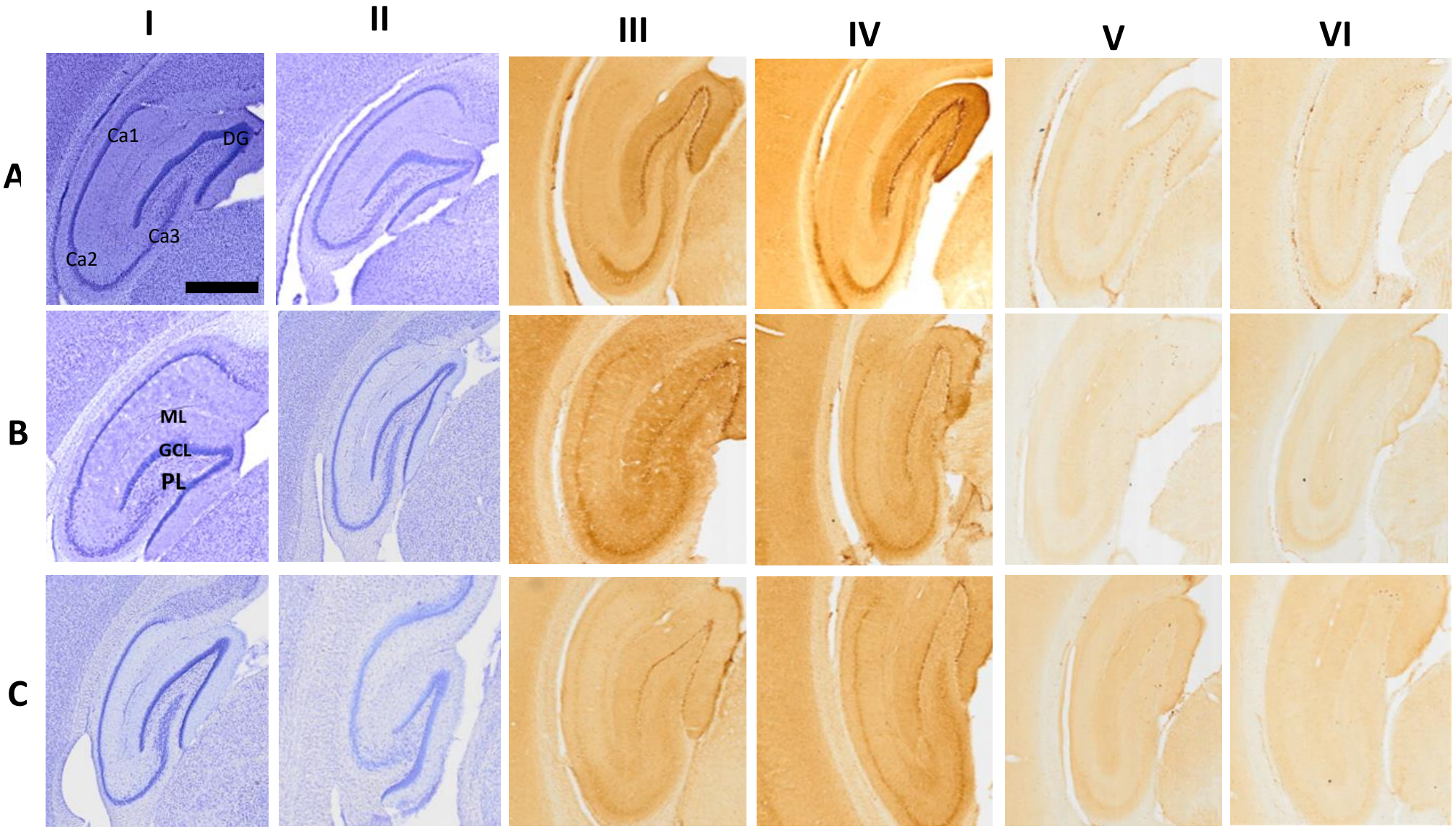



**Figure 4.5.1a**

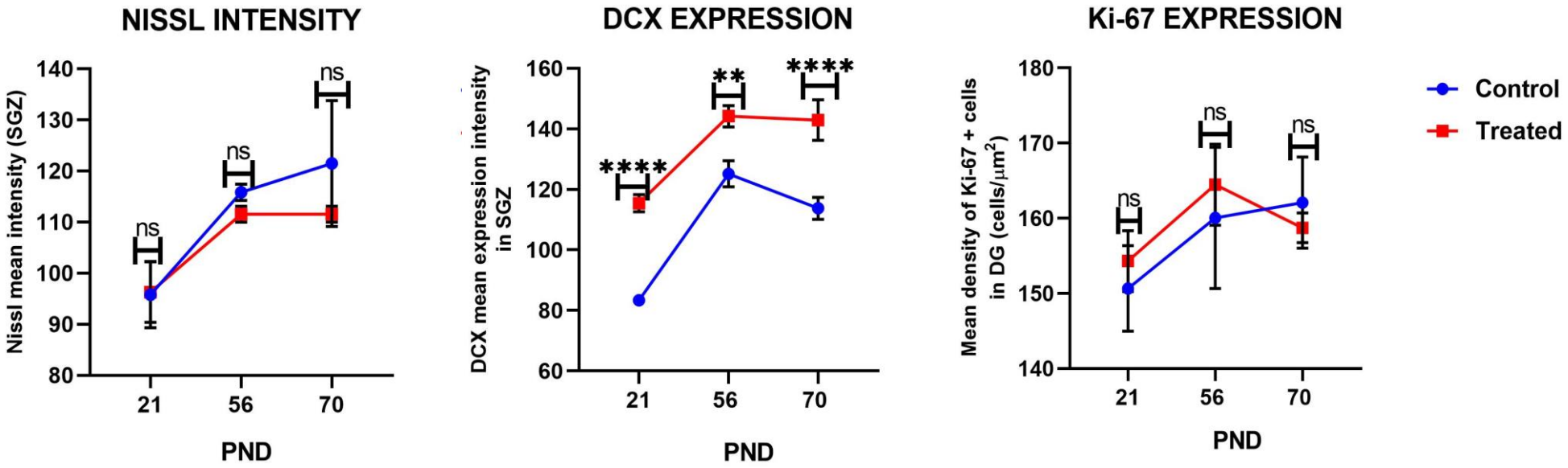
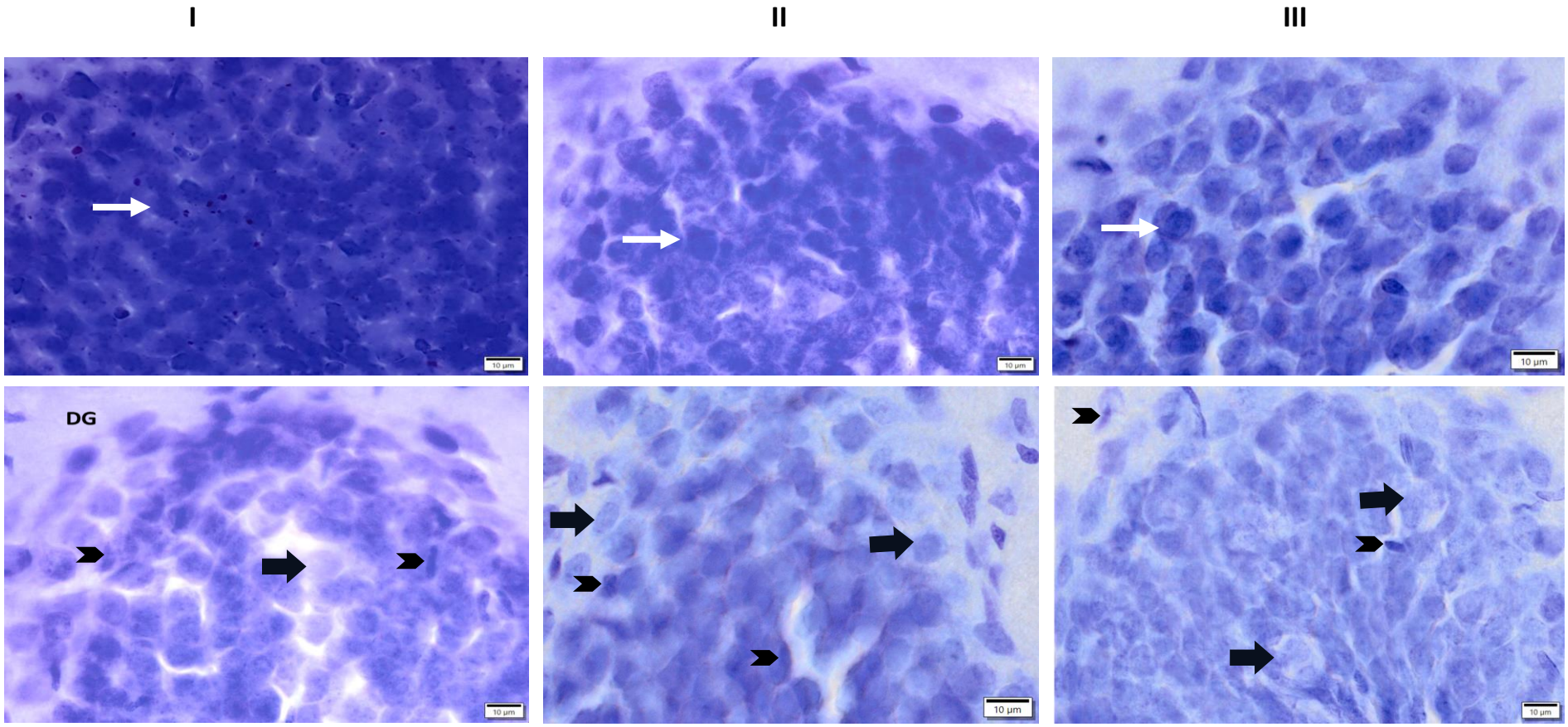



**Figure 4.5.1:** Effect of prenatal exposure to kolanut on colour intensity of Nissl, Ki-67 and DCX stains. A cross-section of stains; Nissl (column I, II), DCX (column III, IV), Ki-67 (column V, VI) of the hippocampus of PNDs 21, 56 and 70 (n = 8) (figure 4.5.1 a). while figure 4.5.1 b shows the colour intensity of the stains in the hippocampus. CA 1 (Cornu ammonis 1), CA2 (Cornu ammonis 2), and CA3 (Cornu ammonis 3). A, PND 21, B, PND 56, and C, PND 70 (Mg. X4). Data are represented as mean ± S.E.M.; significant differences are shown in P-value, **/ **** against control.

### 4.5.2 Effect of prenatal kolanut consumption on the neuronal morphology on the dentate gyrus after birth

As represented in figure 4.5. 2 A, B (I-III) and C, prenatal exposure to kolanut treatment predisposes the pups to neuronal degeneration and histo-architectural changes as evidenced by; decrease in mean density of neuronal cells at PND 21 and 56 [mean effect of treatment: ($F_{(2,24)} = 51.05$; $p < 0.0001$), mean effect of age: ($F_{(1,24)} = 92.51$; $p < 0.0001$), and mean interaction effect of treatment × age ($F_{(2,24)} = 5.96$; $p = 0.0079$)], decrease in mean area of neuronal cells at PND 21 and 56 [mean effect of treatment: ($F_{(2,23)} = 1.82$; $p = 0.1853$), mean effect of age: ($F_{(1,23)} = 45.97$; $p < 0.0001$) and mean interaction effect of treatment × age ($F_{(2,23)} = 1.21$; $p = 0.3153$)], decrease in mean diameter of neuronal cells at PND 21 and 56 [mean effect of treatment: ($F_{(2,22)} = 1.65$; $p = 0.2155$), mean effect of age: ($F_{(1,22)} = 33.14$; $p < 0.0001$) and mean interaction effect of treatment × age ($F_{(2,22)} = 2.37$; $p = 0.1173$)], and increase number of pyknotic and chromatolytic cells at PND 21, 56 and 70 [mean effect of treatment: ($F_{(2,21)} = 2.24$; $p = 0.1311$), mean effect of age: ($F_{(1,21)} = 85.21$; $p < 0.0001$) and mean interaction effect of treatment × age ($F_{(2,21)} = 1.20$; $p = 0.3234$)] when compared to their respective controls. In addition, the photomicrograph showed neuronal atrophy and clumping as well as gliosis of the hippocampal DG cells (figure 4.5. 3 BI-III and C).


I
II
III
A
B
DG
10 µm

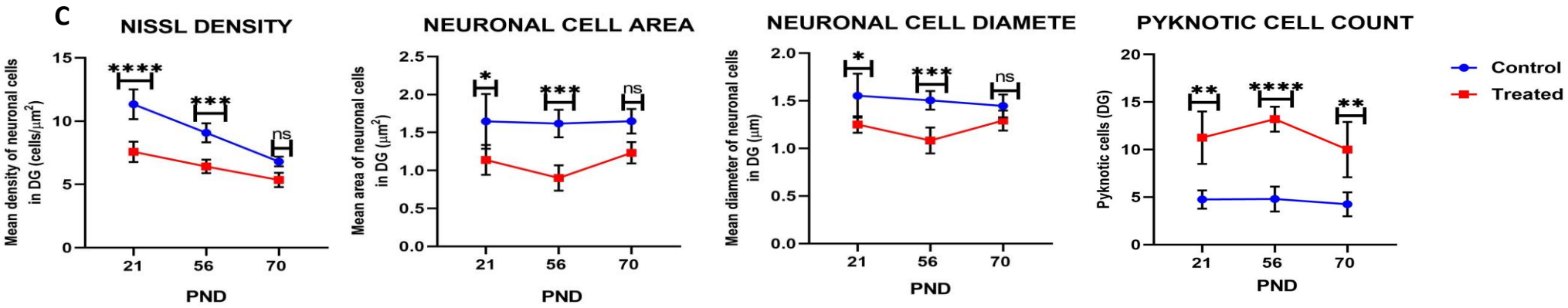



Figure 4.5. 2: Effect of prenatal kolanut administration on neuronal morphology in rat hippocampal dentate gyrus. Nissl stain of dentate gyrus (DG) showed morphological changes in the treated group (B I, II, III) as compared to the control group (A I, II, III). The white thin arrows show darkly stained Nissl neurons, the black arrowheads indicate necrotic neurons, and the black thick arrows show nuclei chromatolysis in the subgranular zone. There is reduction and disruption of layering of the cells and changes in the cell shapes of the DG in the B plate. C shows quantitative analysis of Nissl-stained dentate gyrus (DG) at PND 21, 56, and 70 (n = 8). Data are represented as mean ± S.E.M.; significant differences are shown in P-value, */**/***/**** against control. Scalebar=20μm.

### 4.5.3 Effect of prenatal kolanut consumption on $DCX^+$ expression in the subgranular zone of the hippocampal dentate gyrus after birth

The immature neurons via $DCX^+$ expression in the DG was estimated further as presented in figure 4.5.3. In contrast to the respective control animals at the different PNDs (21, 56 and 70), the prenatal kolanut administration induced a significant decrease in mean density count [mean effect of treatment: ($F_{(2,42) = 100.7}$; $p < 0.0001$), mean effect of age: ($F_{(1,42) = 120.6}$; $p < 0.0001$) and mean interaction effect of treatment × age ($F_{(2,42) = 9.53}$; $p = 0.0004$)], decrease in mean area count [mean effect of treatment: ($F_{(2,39) = 9.41}$; $p = 0.0005$), mean effect of age: ($F_{(1,39) = 184.8}$; $p < 0.0001$) and mean interaction effect of treatment × age ($F_{(2,39) = 6.46}$; $p = 0.0038$)], and decrease in mean diameter count [mean effect of treatment: ($F_{(2,42) = 2.87}$; $p = 0.0678$), mean effect of age: ($F_{(1,42) = 152.9}$; $p < 0.0001$) and mean interaction effect of treatment × age ($F_{(2,42) = 3.80}$; $p = 0.0304$)] of $DCX^+$ in the SGZ of the DG. These results showed that prenatal kolanut administration inhibited the ability of the neural stem cells proliferations (figure 4.5.3 A-C).

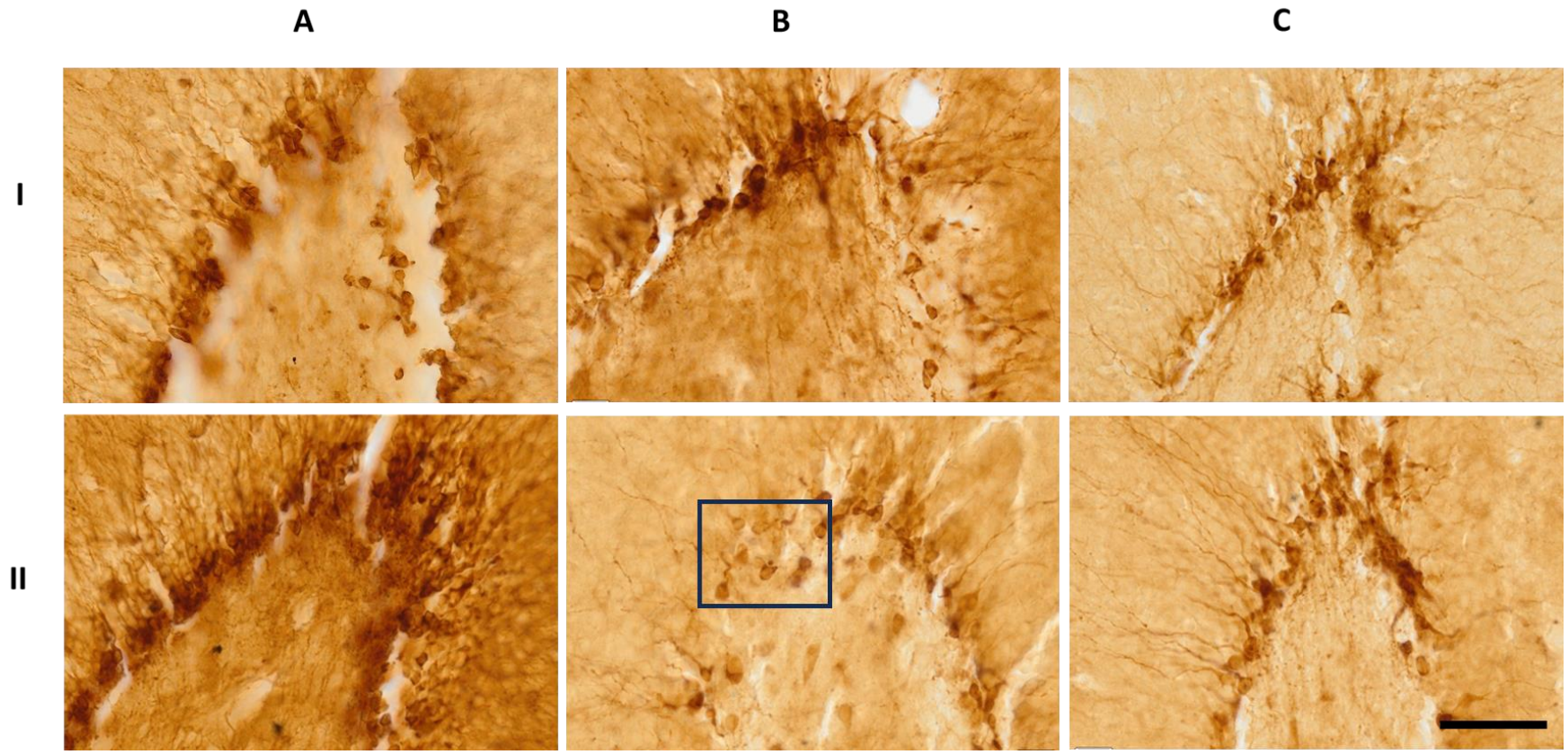



**Figure 5.5.3a.**

III

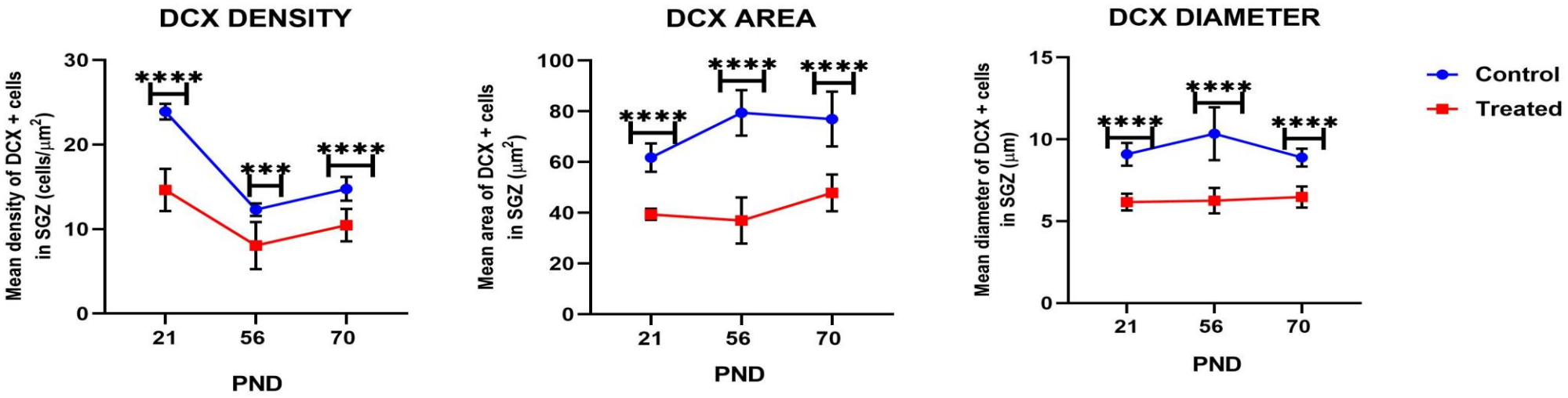
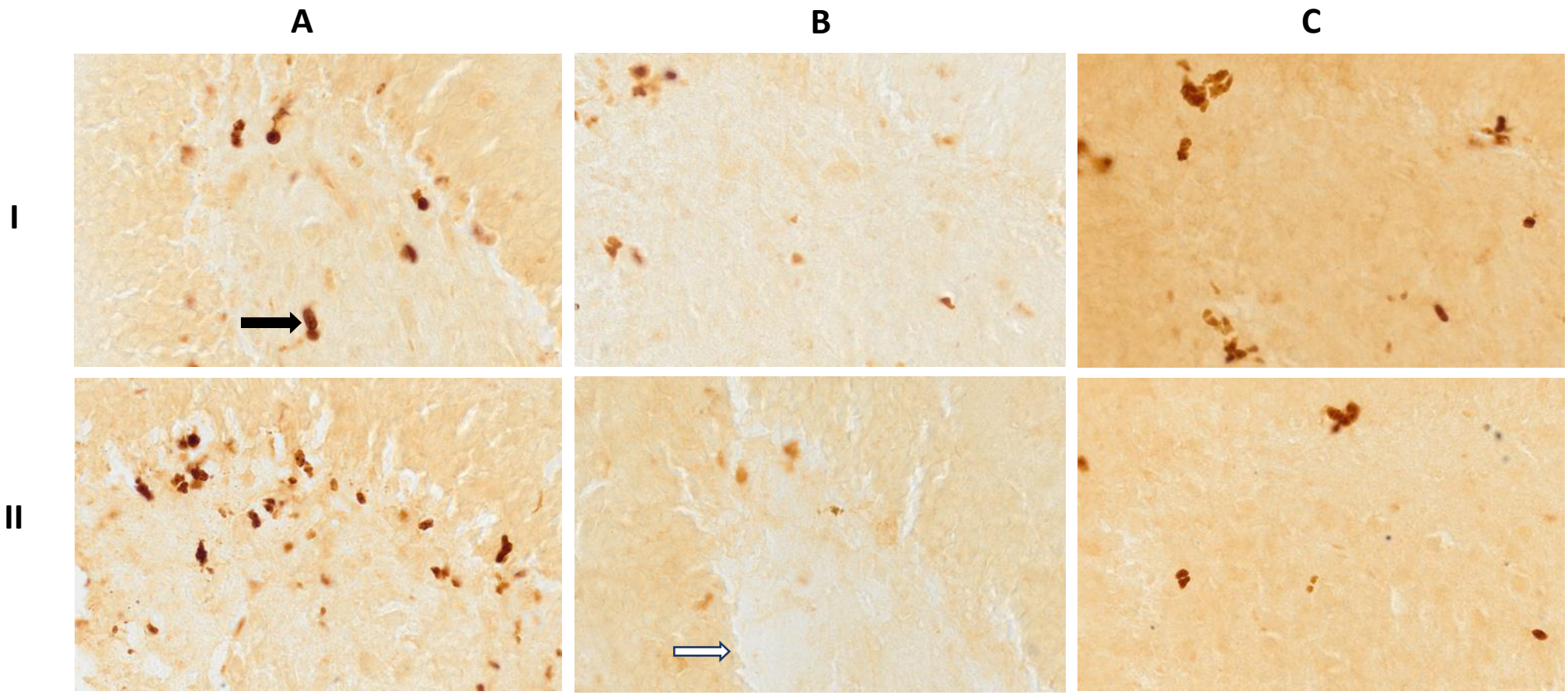



**Figure 4.5.3b**

**Figure 4.5.3:** Effect of prenatal kolanut effect on DCX+ expression in the subgranular zone in the dentate gyrus. Photomicrograph of doublecortin (DCX) immunopositive cells. There is loss of DCX immunopositive neurons in the subgranular zone of the dentate gyrus (DG) after intrauterine exposure of kolanut to the PND 21, 56, 70 treated groups (II) as compared to the control group (I). BII shows multi layering of the neuronal cells (black box) (figure 4.5.3a). Quantitative analysis (III) shows significant reduction of DCX cells in kolanut exposed rats (n = 8) (figure 4.5.3b). Data are represented as mean ± S.E.M.; significant differences are shown in P-value, ***/**** against control. Scale bar = 20 μm.

### 4.5.4 Effect of prenatal kolanut consumption on Ki-67 expression in the sub-granular zone of the hippocampal dentate gyrus after birth

The photomicrographs showed that kolanut exposure accentuates loss of proliferating cells in SGZ and hilus of the DG (figure 4.5.4 A-C). Furthermore, the stereological estimate of the mean density, mean area, and mean diameter of the proliferating cells in the DG of the dorsal hippocampus was used to confirm the prenatal effect of kolanut administration on neuronal proliferation in the pups. As represented in figure 4.5.4 (III), the number of Ki-67+ cells in the DG was significantly suppressed as indicated by decrease in mean density of Ki-67+ cells (PND 21) [mean effect of treatment: ($F_{(2,31)} = 23.72$; $p < 0.0001$), mean effect of age: ($F_{(1,31)} = 17.75$; $p = 0.0002$) and mean interaction effect of treatment × age ($F_{(2,31)} = 1.97$; $p = 0.1570$)], decrease in mean area of Ki-67+ cells (PND 21 and 70) [mean effect of treatment: ($F_{(2,40)} = 4.21$; $p = 0.0219$), mean effect of age: ($F_{(1,40)} = 30.24$; $p < 0.0001$) and mean interaction effect of treatment × age ($F_{(2,40)} = 1.00$; $p = 0.3777$)], and decrease in mean diameter of of Ki-67+ cells (PND 21 and 70) [mean effect of treatment: ($F_{(2,42)} = 8.99$; $p = 0.0006$), mean effect of age: ($F_{(1,42)} = 52.57$; $p < 0.0001$) and mean interaction effect of treatment × age ($F_{(2,42)} = 2.57$; $p = 0.0886$)] in the DG when compared to their respective control.


A
B
C
I
II

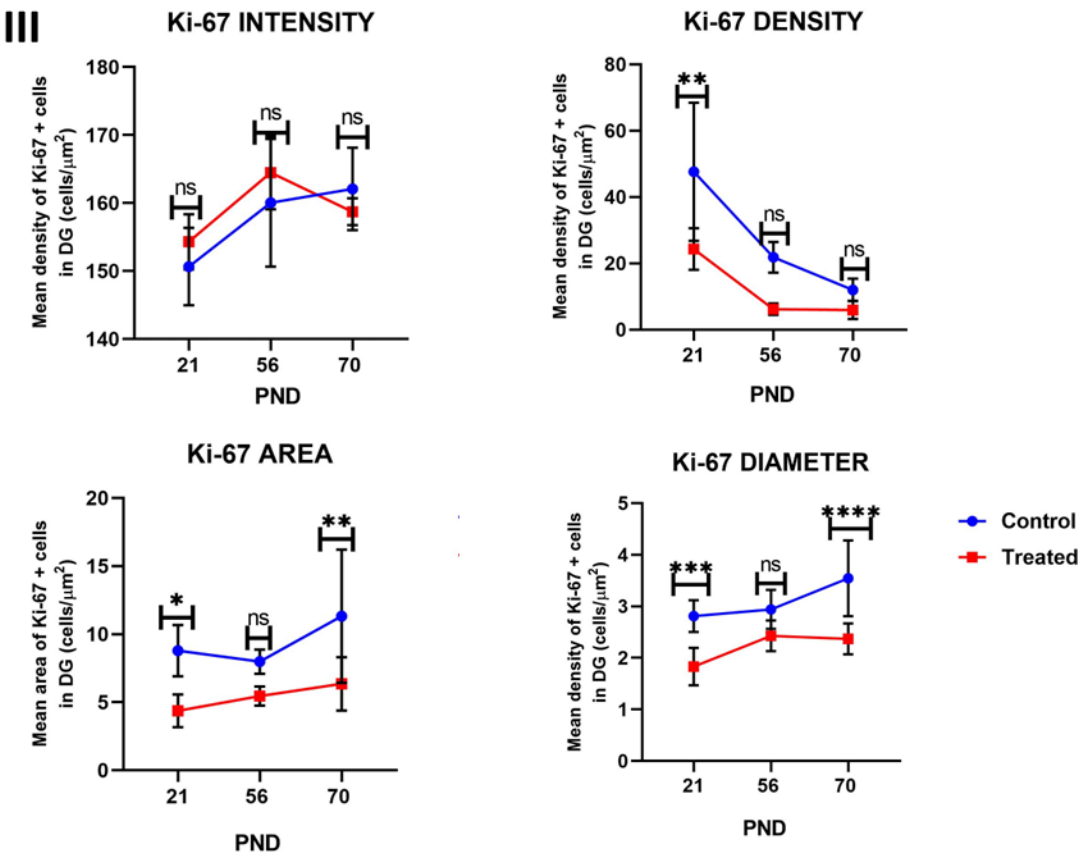



**Figure 4.5.4:** Effect of prenatal kolanut administration alters the cumulative neurogenesis via decrease Ki-67 expression in the dentate gyrus. Ki67 immunohistochemistry shows that kolanut exposure accentuates loss of proliferating cells in subgranular zone and hilus of the dentate gyrus (DG) (AII, BII, CII) of PND 21, 56, 70. III Quantitative analysis shows that kolanut exposure reduces the Ki-67-labelled proliferating cells in the dentate gyrus (DG) at PND 21, 56, and 70 (n = 8). The black arrow shows a positive stained neuron while the white arrow showed area devoid of neuron. Data are represented as mean ± S.E.M.; significant differences are shown in P-value, */**/***/**** against control. Scale bar = 20 µm.

### 4.5.5 Effect of prenatal kolanut consumption on the histomorphometry of Nissl stain in the prefrontal cortex after birth.

The photomicrographs showed that kolanut exposure significantly accentuates pyknotic activity and reduction in neuronal cell density. Different degrees of cell death were observed as stated in figure 4.5.5 a & b. Also, the stereological estimate of the mean density, mean area, and mean diameter of the neuronal cells in the prefrontal cortex showed that prenatal administration of kolanut could significantly reduce the neuronal mean cell density across age groups [mean effect of treatment: ($F_{(2,24)} = 51.05$; $p < 0.0001$), mean effect of age: ($F_{(1,24)} = 92.51$; $p < 0.0001$), and mean interaction effect of treatment × age ($F_{(2,24)} = 5.96$; $p = 0.0079$)] and mean expression of staining intensity of the prefrontal neuronal cells [mean effect of treatment: ($F_{(2,12)} = 18.72$; $p = 0.0002$), mean effect of age: ($F_{(1,12)} = 2.408$; $p = 0.1467$) and mean interaction effect of treatment × age ($F_{(2,12)} = 1.042$; $p = 0.3826$)] with increase in the pyknotic cells [mean effect of treatment: ($F_{(2,21)} = 2.24$; $p = 0.1311$), mean effect of age: ($F_{(1,21)} = 85.21$; $p < 0.0001$) and mean interaction effect of treatment × age ($F_{(2,21)} = 1.20$; $p = 0.3234$)] in PNDs 21, 56 and 70 when compared to their respective control.

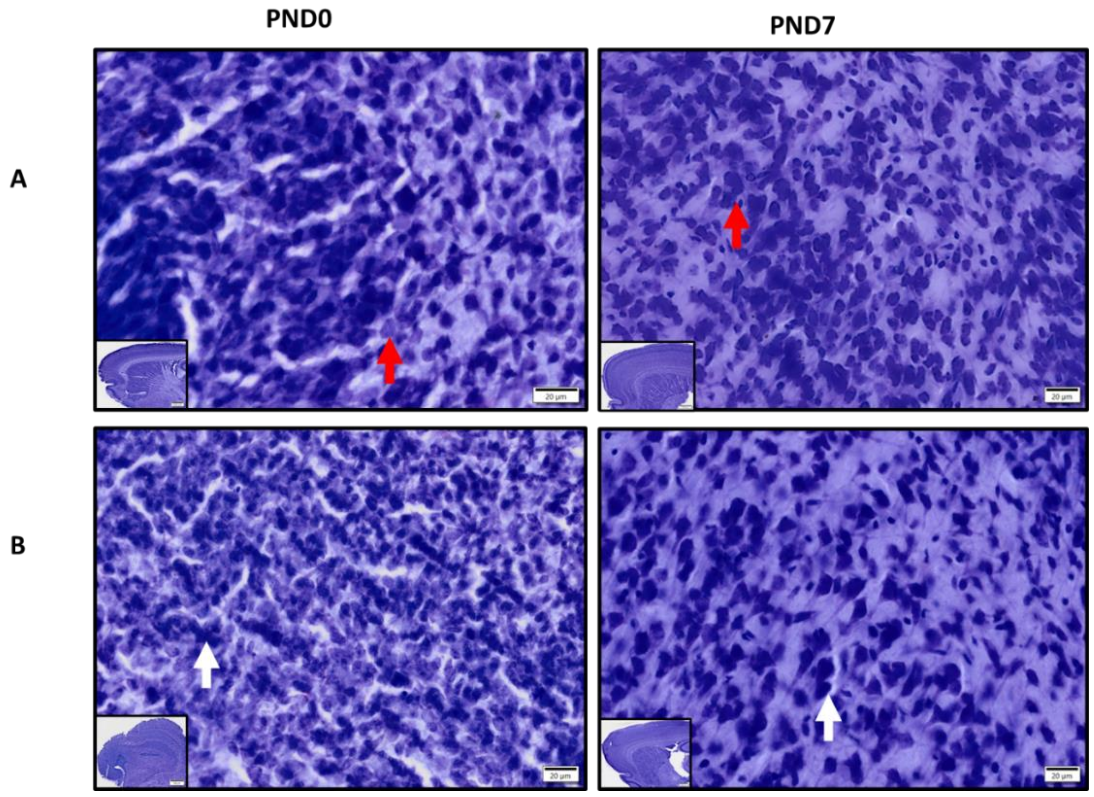



**Figure 4.5.5a:** Effect of prenatal kolanut administration causes neuronal necrosis in the prefrontal cortex (white arrows) in the kolanut treated rats (PND 0 and 7) relatively normal neurons (red arrows).

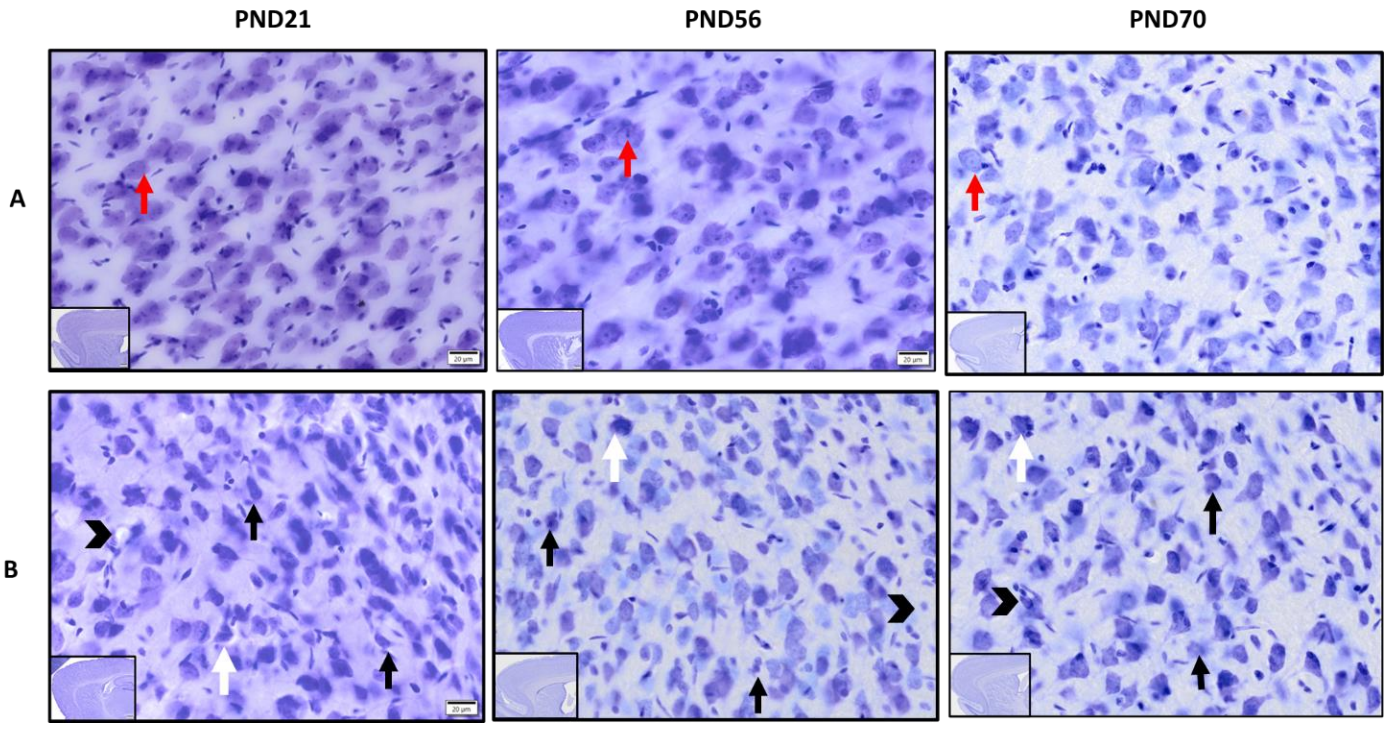



**Figure 4.5.5b:** Effect of prenatal kolanut administration causes neuronal necrosis in prefrontal cortex. Black arrows show acute necrosis. Red arrows show relatively normal neurons, and the arrow heads show a pyknotic nucleus with vacuolation. White arrows show ferrugination of neurons.

### 4.5.6 Effect of prenatal kolanut consumption on the intensity of Nissl stain in the prefrontal cortex (PFC) after birth

Figure 4.5.6 showed the quantifications of the level of Nissl staining intensity of the prefrontal cortex SGZ in the DG. The results showed that there was a significant difference in the Nissl mean expression of staining intensity [mean effect of treatment: ($F_{(2,12)} = 18.72$; $p = 0.0002$), mean effect of age: ($F_{(1,12)} = 2.408$; $p = 0.1467$) and mean interaction effect of treatment × age ($F_{(2,12)} = 1.042$; $p = 0.3826$)] in the SGZ of the DG at the PND 21 and 70 when compared to the respective controls (figure 4.5.6).

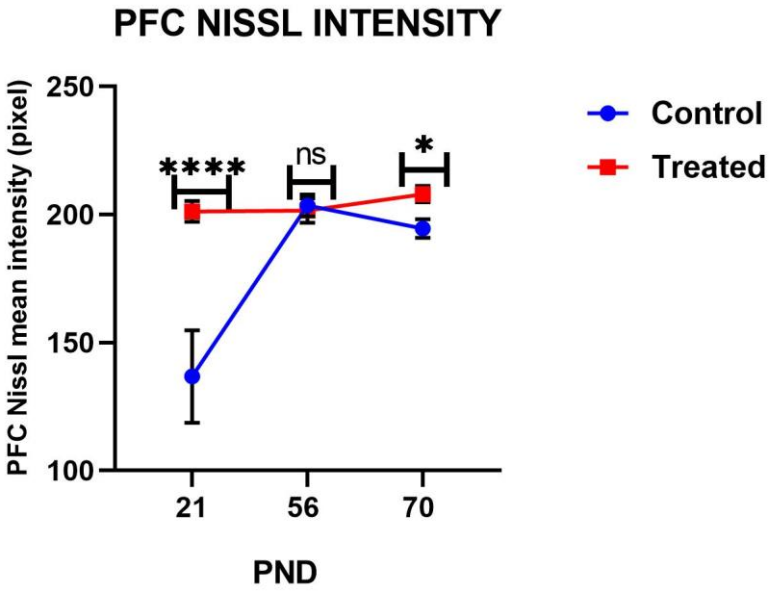



**Figure 4.5.6:** Effect of pre-natal kolanut consumption on the intensity of Nissl stain in the prefrontal cortex after birth. A cross-section of stains; Nissl of the prefrontal cortex of PNDs 21, 56 and 70 (n = 8) showing the intensity of the stains in the prefrontal cortex. A, PND 21, B, PND 56, and C, PND 70. Data are represented as mean ± S.E.M.; significant differences are shown in P-value, *, **** against control.

### 4.5.7 Effect of prenatal kolanut consumption on the mean Nissl density of neuronal cells in rat prefrontal cortex after birth

As represented in figure 4.5.7, there was a decrease in mean Nissl density of neuronal cells at PND 21, 56 and 70 [mean effect of treatment: ($F_{(2,24)} = 51.05$; $p < 0.0001$), mean effect of age: ($F_{(1,24)} = 92.51$; $p < 0.0001$), and mean interaction effect of treatment × age ($F_{(2,24)} = 5.96$; $p = 0.0079$)] when compared to the controls.

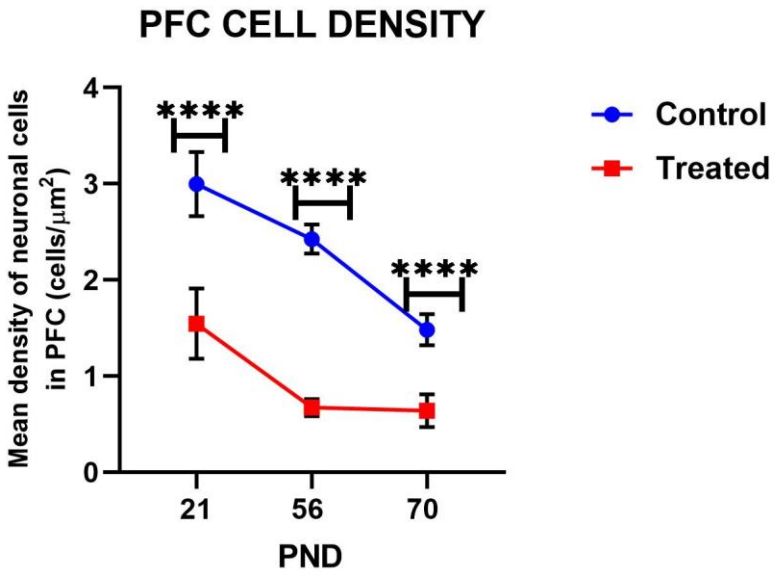



**Figure 4.5.7:** Effect of pre-natal kolanut consumption on the mean Nissl density of neuronal cells in prefrontal cortex after birth. Data are represented as mean ± S.E.M.; (n = 8), significant differences are shown in P-value, **** against control.

### 4.5.8 Effect of prenatal kolanut consumption on the neuronal cell area of the prefrontal cortex after birth

As shown in figure 4.5.8, there was no significant differences in the mean area of the neuronal cells in the prefrontal cortex at PND 21, 56 and 70 [mean effect of treatment: ($F_{(2,23)} = 1.82$; $p = 0.1853$), mean effect of age: ($F_{(1,23)} = 45.97$; $p < 0.0001$) and mean interaction effect of treatment × age ($F_{(2,23)} = 1.21$; $p = 0.3153$)] when compared to the controls.

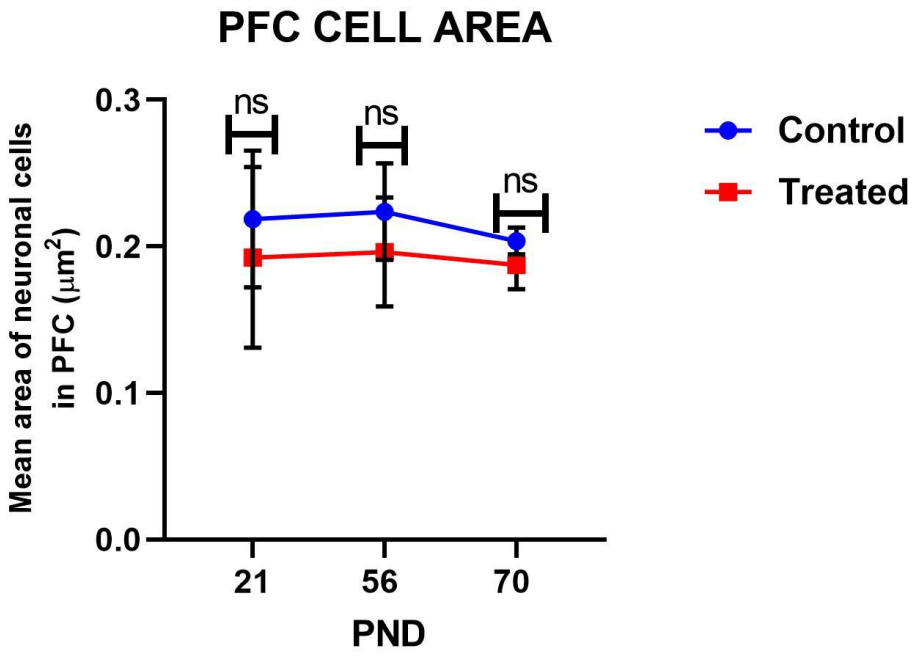



**Figure 4.5.8:** Effect of pre-natal kolanut consumption on the neuronal cell area of rat prefrontal cortex after birth. Data are represented as mean ± S.E.M.; (n = 8).

### 4.5.9 Effect of prenatal kolanut consumption on the neuronal cell diameter of prefrontal cortex after birth

Figure 4.5.9 showed no significant differences in the mean diameter of the neuronal cells in the rat prefrontal cortex at PND 21, 56 and 70 [mean effect of treatment: ($F_{(2,23)} = 1.82$; $p = 0.1853$), mean effect of age: ($F_{(1,23)} = 45.97$; $p < 0.0001$) and mean interaction effect of treatment × age ($F_{(2,23)} = 1.21$; $p = 0.3153$)] when compared to the controls.

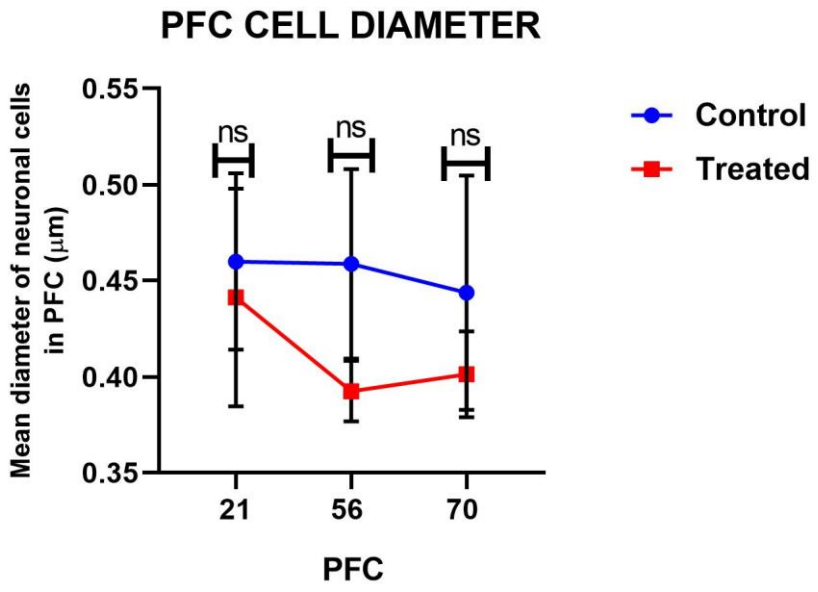



**Figure 4.5.9:** Effect of prenatal kolanut consumption on the neuronal cell diameter of prefrontal cortex after birth. Data are represented as mean ± S.E.M.; (n = 8).

### 4.5.10 Effect of prenatal kolanut consumption on the neuronal cell viability of prefrontal cortex after birth

As shown in figure 4.5.10, there was a significant increase in the number of pyknotic and chromatolytic cells at PND 21, 56 and 70 [mean effect of treatment: ($F_{(2,21)} = 2.24$; $p = 0.1311$), mean effect of age: ($F_{(1,21)} = 85.21$; $p < 0.0001$) and mean interaction effect of treatment × age ($F_{(2,21)} = 1.20$; $p = 0.3234$)] when compared to the controls.

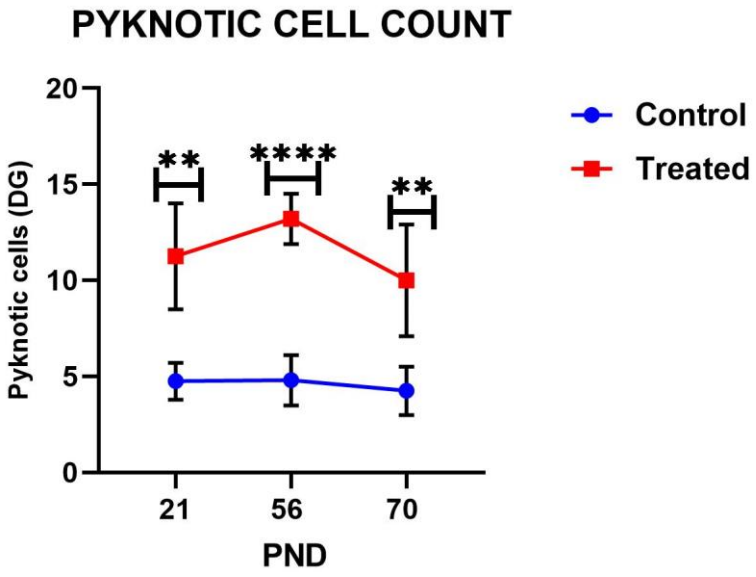



**Figure 4.5.10:** Effect of prenatal kolanut consumption on the neuronal cell viability of prefrontal cortex after birth. Data are represented as mean ± S.E.M.; (n = 8), significant differences are shown in P-value, **/**** against control.

## 4.6 Biochemical estimation in the hippocampus pups following exposure to pre-natal kolanut consumption.

### 4.6.1. Effect of prenatal kolanut consumption on the malondialdehyde (MDA) level

The level of malondialdehyde (MDA) µm/l, a thiobabituric acid reactive substance was assessed in the hippocampal tissues of the experimental animals. There was a significant increase in MDA levels in the brain of the animals exposed to kolanut across all age groups (mean effect of age ($F_{(1,30)} = 12.53$; p= 0.0013) and mean effect of treatment ($F_{(4,30)} = 4.366$; p= 0.0067) compared to the controls as shown in table 4.6. And no significant difference in the interaction between treatment and age ($F_{(4,30)} = 2.406$; p= 0.0715).

**Table 4.6.1: Effect of kolanut extract on malondialdehyde level.**

| PND | | 0 | 7 | 21 | 56 | 70 |
|---|---|---|---|---|---|---|
| **MDA** | **Control** | 1.36 ± 0.04 | 1.20 ± 0.28 | 1.65 ± 0.15 | 1.38 ± 0.27 | 1.27 ± 0.22 |
| (µm/l) | **Treated** | 1.26 ± 0.01 | 1.60 ± 0.36* | 1.74 ± 0.08 | 1.78 ± 0.09* | 1.63 ± 0.20* |

**Table 4.6.1** shows results of effect of kolanut on the state of oxidation in the treated and control animals from PND 0, 7, 21, 56 and 70. Data is represented by mean ± SEM with 10 animals in each group. * $p<0.01$ indicates significant differences as compared to control.

### 4.6.2. Effect of prenatal kolanut consumption on the superoxide dismutase level

The level of superoxide dismutase (SOD) (U $mg^{-1}$) was assessed in the hippocampal tissues of the experimental animals. The result showed no significant differences in SOD levels in the brain of the animals exposed to kolanut across all age groups when compared to the controls as shown in table 4.6.2.

**Table 4.6.2: Effect of kolanut extract on superoxide dismutase level.**

| PND | | 0 | 7 | 21 | 56 | 70 |
|---|---|---|---|---|---|---|
| **SOD** | **Control** | 5.23 ± 0.42 | 5.13 ± 0.51 | 4.69 ± 0.47 | 4.73 ± 0.30 | 4.95 ± 0.12 |
| **(Umg$^{-1}$)** | **Treated** | 5.79 ± 0.16 | 5.45 ± 0.32 | 4.96 ± 0.14 | 5.11 ± 0.30 | 4.84 ± 0.15 |

**Table 4.6.2** shows results of effect of kolanut on the state of antioxidation in the treated and control animals from PND 0, 7, 21, 56 and 70. Data is represented by mean ± SEM with 10 animals in each group.

### 4.6.3. Effect of prenatal kolanut consumption on the glutathione peroxidase level after birth

The level of glutathione peroxidase (GPx) (µmole) was assessed in the hippocampal tissues of the experimental animals. The result showed that there was a significant difference and a steady increase in GPx levels in the brain of the animals exposed to kolanut in the PND 7, 21, 56, and 70 groups when compared to the controls as shown in table 4.6.3.

**Table 4.6.3: Effect of kolanut extract on glutathione peroxidase level.**

| PND | | 0 | 7 | 21 | 56 | 70 |
|---|---|---|---|---|---|---|
| **GPX** | **Control** | 1.11 ± 0.31 | 1.06 ± 0.04 | 1.58 ± 0.07 | 1.27 ± 0.21 | 1.67 ± 0.11 |
| **(µmole)** | **Treated** | 1.29 ± 0.13 | 1.60 ± 0.36* | 1.74 ± 0.08* | 1.94 ± 0.10* | 1.77 ± 0.10 |

Table 4.6.3 shows results of effect of kolanut on the state of antioxidation in the treated and control animals from PND 0, 7, 21, 56 and 70. Data is represented by mean ± SEM with 10 animals in each group. * $p<0.01$ indicates significant differences as compared to control.

### 4.6.4. Effect of prenatal kolanut consumption on the brain derived neurotropic factor levels after birth.

As shown in table 4.6.4, a significant difference in the hippocampal BDNF level (ng/ml) was observed among the treated animals when compared with the controls across all age groups [(mean effect of age ($F_{(1,30)} = 73.55$; $p = 0.0001$)), mean effect of treatment ($F_{(4,30)} = 11.70$; $p = 0.0001$), and interaction between treatment and age ($F_{(4,30)} = 5.229$; $p = 0.0026$)].

**Table 4.6.4: Effect of kolanut extract on brain derived neurotropic factor levels in rats.**

| PND | 0 | 7 | 21 | 56 | 70 |
|---|---|---|---|---|---|
| **BDNF Control** | 4.11 ± 0.08 | 4.19 ± 0.21 | 4.98 ± 0.04 | 5.31 ± 0.17 | 4.48 ± 0.32 |
| **(ng/ml) Treated** | 5.33 ± 0.34* | 5.34 ± 0.79* | 4.87 ± 0.42 | 6.14 ± 0.11* | 5.85 ± 0.26* |

**Table 4.6.4** shows results of effect of kolanut on the state of neurogenesis in the treated and control animals from PND 0, 7, 21, 56 and 70. Data is represented by mean ± SEM with 10 animals in each group. * $p<0.01$ indicates significant differences as compared to control.

### 4.6.5. Effect of prenatal kolanut consumption on acetylcholine (ACh) levels

The level of ACh (nmol/L) estimated in the hippocampal tissues of the experimental animals showed that treatment with kolanut had a significant effect on the level of acetylcholine in the hippocampus of the treated animals across age group ($F_{(1,30)} = 8.438$; $p = 0.0068$) and treatment ($F_{(4,30)} = 15.61$; $p = 0.0001$), but not on interaction between treatment and age ($F_{(4,30)} = 1.196$; $p = 0.3327$) when compared to the controls (Table 4.6.5).

**Table 4.6.5: Effect of kolanut extract on acetylcholine levels.**

| PND | | 0 | 7 | 21 | 56 | 70 |
|---|---|---|---|---|---|---|
| ACH (Nmol/L) | Control | 7.19 ± 0.81 | 5.05 ± 0.33 | 7.37 ± 0.29 | 8.42 ± 2.01 | 5.78 ± 0.78 |
| | Treated | 6.51 ± 1.87 | 4.03 ± 0.02 | 6.49 ± 1.62* | 5.71 ± 0.63* | 4.23 ± 0.13 |

**Table 4.6.5** shows results of effect of kolanut on the state of cholinergic activity in the treated and control animals from PND 0, 7, 21, 56 and 70. Data is represented by mean $\pm$ SEM with 10 animals in each group. * $p<0.01$ indicates significant differences as compared to control.

## 4.7 Association between the behavioral tests, biochemical levels and neurogenesis markers following exposure to pre-natal kolanut consumption.

### 4.7.1 Association between the behavioral tests, malondialdehyde, brain derived neurotropic factor and acetylcholine level after pre-natal kolanut consumption in rats.

The association between the behavioral tests and the biochemical analyses were investigated using logistic regression analysis. Behavioral correlates of BDNF, ACh and MDA were obtained. Even though our study did not show a positive association between the reduced memory indicators of the behavioral tests and the level of ACh, it is still of note that the level of ACh was reduced in kolanut-exposed animals (Table 4.7.1).

**Table 4.7.1a. Correlation between pairs of traits in Sprague Dawley rats fed kolanut extract**

| Pairs of traits[a] | Correlation (r) | Probability |
|---|---|---|
| SRT vs. MDA in PND 7 | 0.99 | 0.0057 |
| Grooming vs. BNDF in PND 21 | 0.76 | 0.0297 |
| Grooming vs MDA in PND 56 | 0.77 | 0.0252 |
| Rearing vs MDA in PND 56 | 0.62 | 0.0032 |
| Faecal and MDA in PND 21 | 0.76 | 0.0284 |
| Rearing vs. Ach in PND 56 | -0.83 | 0.0115 |
| NOL vs. MDA in PND 56 | -0.69 | 0.0164 |
| NOR vs. MDA in PND 21 | 0.63 | 0.0502 |
| RAM vs. BDNF in PND 56 | 0.95 | 0.0491 |

[a]SRT = Surface righting test; MDA = Malondialdehyde; BNDF = Brian derived neurotropic; Ach = Acetylcholine; NOL = Novel object location; NOR = Novel object recognition; RAM = Radial arm maize; PND 7 = 7 days after birth; PND 21 = 21 days after birth; PND 56 = 56 days after birth.

**Table 4.7.1b: Effect of kolanut extract on malondialdehyde, brain derived neurotropic factor and acetylcholine levels.**

| | | 0 | 7 | 21 | 56 | 70 |
|---|---|---|---|---|---|---|
| **MDA** | **Control** | 1.36 ± 0.04 | 1.20 ± 0.28 | 1.65 ± 0.15 | 1.38 ± 0.27 | 1.27 ± 0.22 |
| **(µm/l)** | **Treated** | 1.26 ± 0.01 | 1.60 ± 0.36* | 1.74 ± 0.08 | 1.78 ± 0.09* | 1.63 ± 0.20* |
| **BDNF** | **Control** | 4.11 ± 0.08 | 4.19 ± 0.21 | 4.98 ± 0.04 | 5.31 ± 0.17 | 4.48 ± 0.32 |
| **(ng/ml)** | **Treated** | 5.33 ± 0.34* | 5.34 ± 0.79* | 4.87 ± 0.42 | 6.14 ± 0.11* | 5.85 ± 0.26* |
| **ACh** | **Control** | 7.19 ± 0.81 | 5.05 ± 0.33 | 7.37 ± 0.29 | 8.42 ± 2.01 | 5.78 ± 0.78 |
| **(nmol/l)** | **Treated** | 6.51 ± 1.87 | 4.03 ± 0.02 | 6.49 ± 1.62* | 5.71 ± 0.63* | 4.23 ± 0.13 |

**Table 4.7.1b** shows results of effect of kolanut on the state of oxidation, neurogenesis and cholinergic activity in the treated and control animals from PND 0, 7, 21, 56 and 70. Data is represented by mean ± SEM with 10 animals in each group. * $p<0.01$ indicates significant differences as compared to control.

### 4.7.2. Relationship between mean density of Ki-67 cells, MDA and BDNF concentration in the hippocampus

At PND 70, the mean density of Ki-67 cells was negatively correlated to MDA concentration (Pearson's correlation, $r$ = - 0.9787, $p$ = 0.0213, R = 0.9578) and BDNF concentration (Pearson's correlation, $r$ = - 0.9362, $p$ = 0.0638, R = 0.8764). However, there was significant negative correlation between the mean density of Ki-67 cells and hippocampal MDA activity. In addition, there was insignificant negative correlation between the mean density of Ki-67 cells and hippocampal BDNF activity.

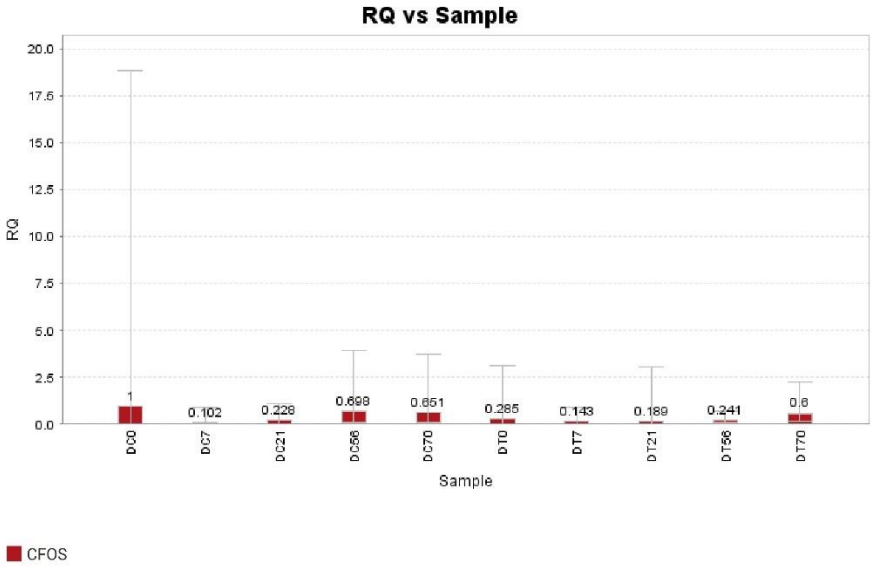
### 4.7.3. Relationship between mean area of DCX cells, MDA and BDNF expression in the hippocampus

At PND 70, we found that mean area of DCX cells was negatively correlated to MDA concentration (Pearson's correlation, $r$ = - 0.04475, $p$ = 0.9553, R = 0.002002) and BDNF expression (Pearson's correlation, $r$ = - 0.9983, $p$ = 0.0017, R = 0.9766). However, there was insignificant negative correlation between the mean area of $DCX^+$ cells and hippocampal MDA activity. In addition, there was a significant negative correlation between the mean area of DCX cells and hippocampal BDNF expression.

## 4.8. Molecular Result

### 4.8.1 Effect of kolanut on gene *c-fos* mRNA for neuronal proliferation and differentiation

Kolanut affected the expression of *c-fos* mRNA levels in the hippocampal tissues of the treated animals, thereby downregulated the level of *c-fos* in the treated animals. However, this was not significant across age group ($F_{(4, 20)} = 2.832$; $p = 0.0519$), treatment ($F_{(1, 20)} = 2.012$; $p = 0.1714$), and interaction ($F_{(4, 20)} = 2.627$; $p= 0.0652$) when compared to the controls (figure 4.8.1).



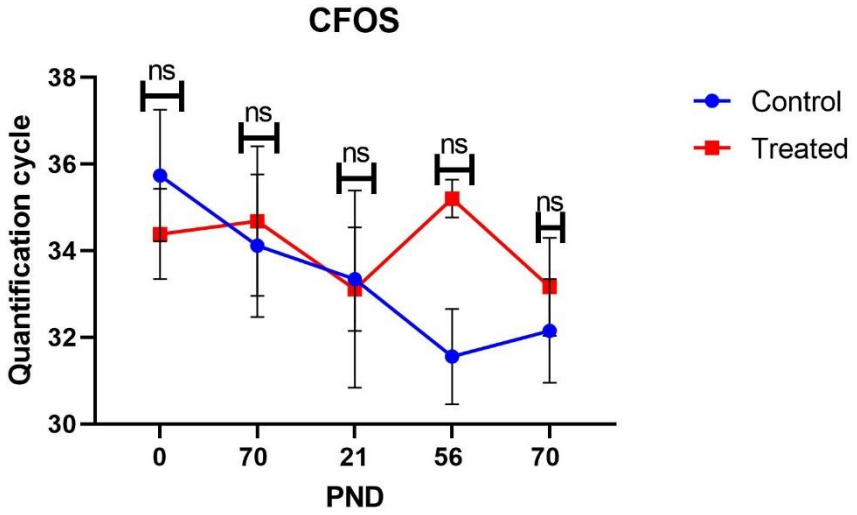
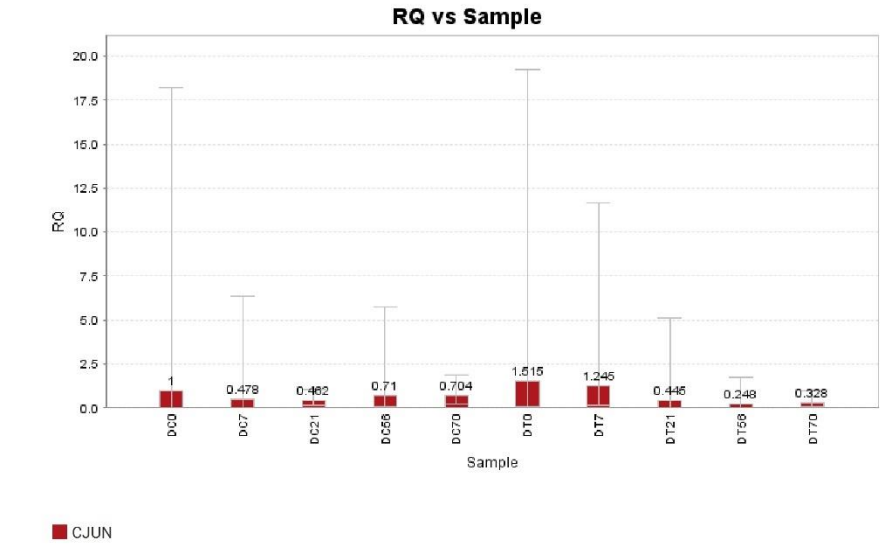




**Figure 4.8.1:** Effect of prenatal kolanut consumption on the level of *c-fos* mRNA in the hippocampal tissue from PND 0, 7, 21, 56 and 70. Data is represented by mean ± SEM with 3 animals in each group.

### 4.8.2. Effect of kolanut on *c-jun* mRNA gene for neuronal proliferation, plasticity and programmed cell death.

Kolanut caused a decrease the level of *c-jun* mRNA levels of the experimental tissues thereby causing a significant effect on the neuronal proliferation, plasticity and programmed cell death in the hippocampus of the treated animals across age group [ ($F_{(4, 20) = 3.090}$; $p = 0.0392$), treatment ($F_{(1, 20) = 1.345}$; $p = 0.2599$), interaction ($F_{(4, 20) = 4.894}$; $p = 0.0065$)] when compared to the controls (figure 4.8.2).



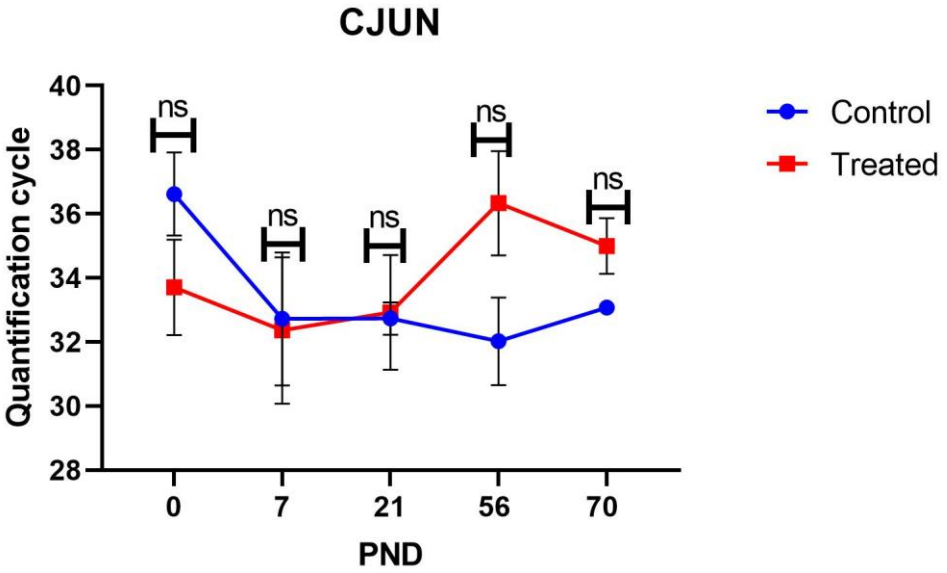
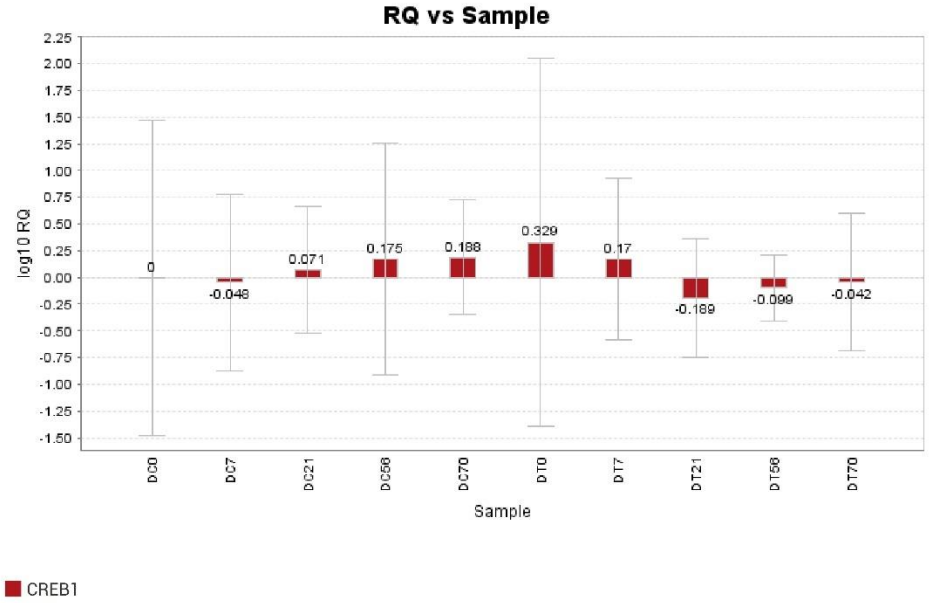




**Figure 4.8.2:** Effect of prenatal kolanut consumption on the level of *c-jun* mRNA in the hippocampal tissue from PND 0, 7, 21, 56 and 70. Data is represented by mean ± SEM with 3 animals in each group. $P < 0.01$ indicates significant differences as compared to control.

### 4.8.3. Effect of kolanut on memory-related synaptic plasticity

The level of *CREB1* estimated in the hippocampal tissues of the experimental animals showed that treatment with kolanut had a significant effect on the level of *CREB1* in the hippocampus of the treated animals across age group [($F_{(4, 20) = 3.110}$; $p = 0.0384$), treatment ($F_{(1, 20) = 0.1826}$; $p = 0.6738$), and interaction ($F_{(4, 20) = 4.622}$; $p = 0.0083$)] when compared to the controls (figure 4.8.3). this was observed in the reduction of synapses in the brain sections (figure 4.4.1.1).



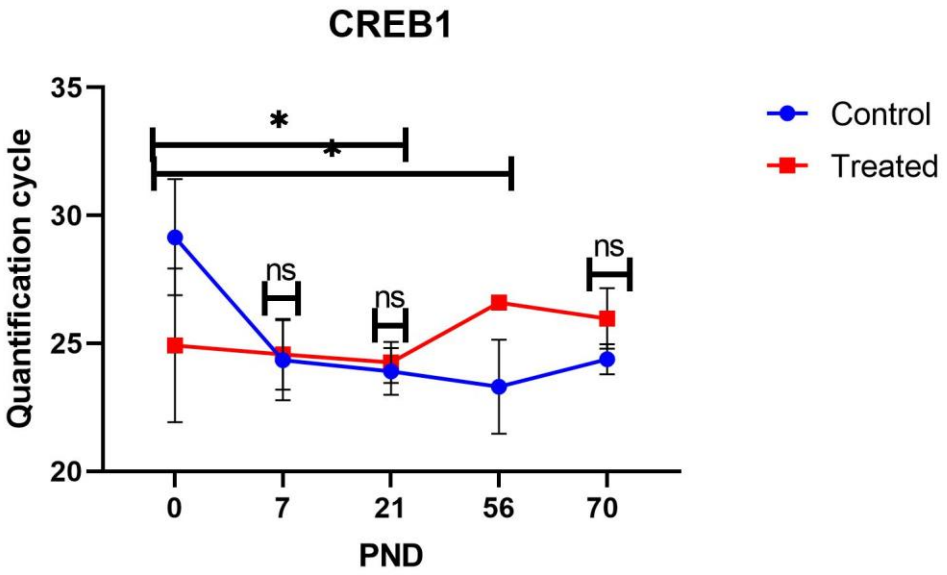




**Figure 4.8.3:** Effect of prenatal kolanut consumption on the level of *CREB1* mRNA in the hippocampal tissue from PND 0, 7, 21, 56 and 70. Data is represented by mean ± SEM with 3 animals in each group. P <0.01 indicates significant differences as compared to control.

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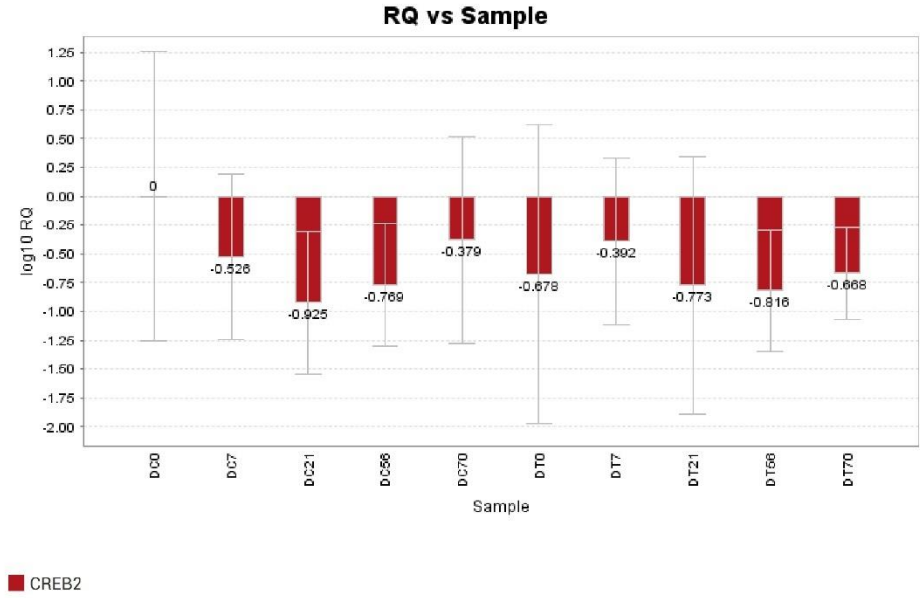
### 4.8.4. Effect of kolanut on induction of long-term synaptic plasticity

Kolanut caused an increase in the level of *CREB2* mRNA estimated in the hippocampal tissues of the experimental animals. *CREB2* mRNA level was supposed to be less than the *CREB1* mRNA in a normal process, thus the integrity of the synapses was further compromised. Kolanut also had a significant effect on the expression of *CREB2* mRNA levels in the hippocampus of the treated animals across age group [($F_{(4, 20) = 3.594}$; $p = 0.0230$), treatment ($F_{(1, 20) = 0.1811}$; $p = 0.6750$), and interaction ($F_{(4, 20) = 3.078}$; $p = 0.0397$)] when compared to the controls (figure 4.8.4).



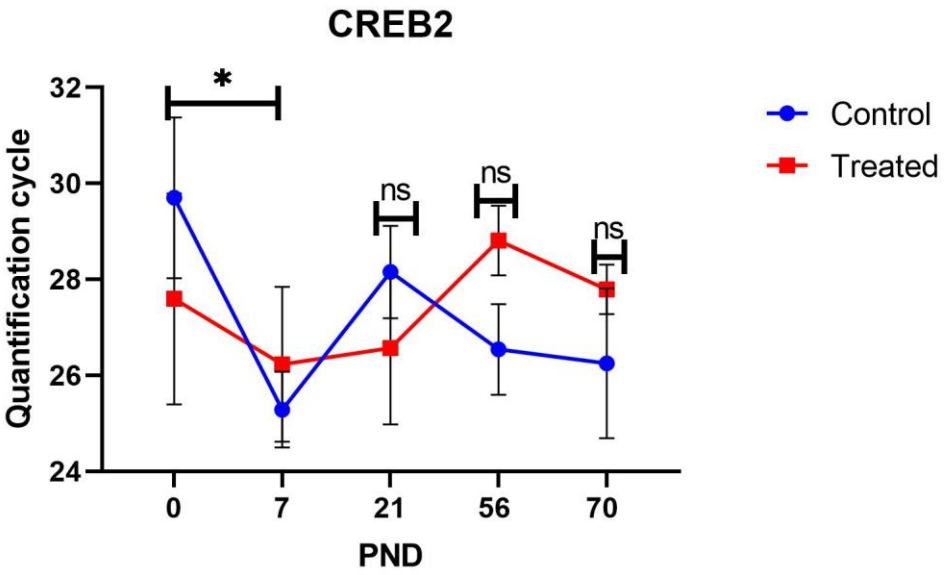
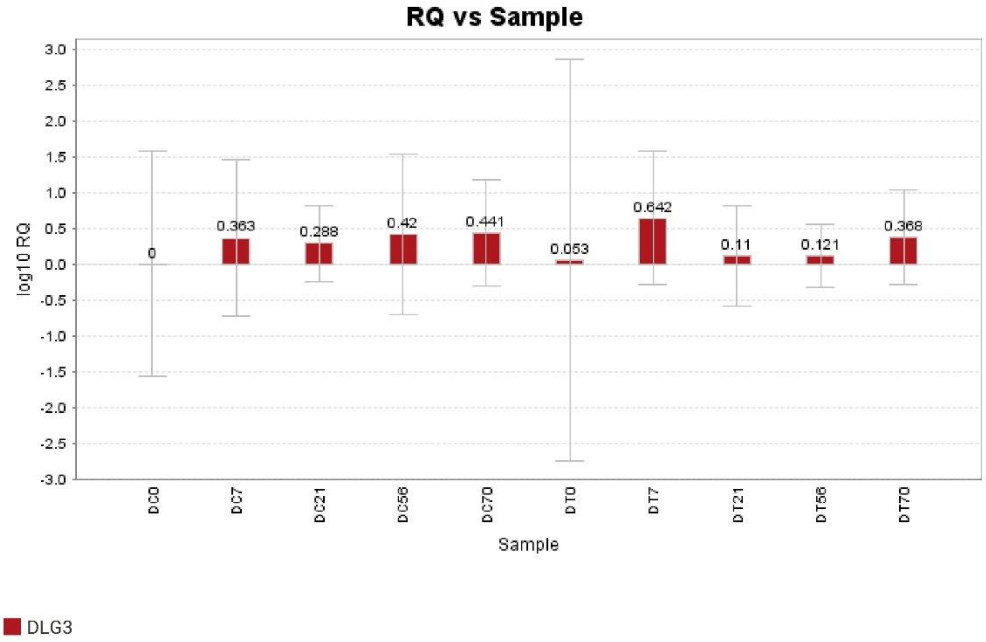




**Figure 4.8.4:** Effect of prenatal kolanut consumption on the level of *CREB2* mRNA gene in the hippocampal tissue from PND 0, 7, 21, 56 and 70. Data is represented by mean ± SEM with 3 animals in each group. $P < 0.01$ indicates significant differences as compared to control.

### 4.8.5. Effect of kolanut on synaptic and cognitive functions

The level of *DLG3* mRNA estimated in the hippocampal tissues of the experimental animals was reduced by kolanut. Thereby affecting the synaptic and cognitive functions of kolanut treated animals. A significant effect on the level of *DLG3* mRNA in the hippocampus of the treated animals was observed across age group ($F_{(4, 20) = 10.80}$; $p = < 0.0001$) and treatment ($F_{(1, 20) = 8.382}$; $p = 0.0090$), but not on interaction between treatment and age ($F_{(4, 20) = 0.5278}$; $p = 0.7166$) when compared to the controls (figure 4.4.1.1, 4.4.1.2 and 4.4.8).



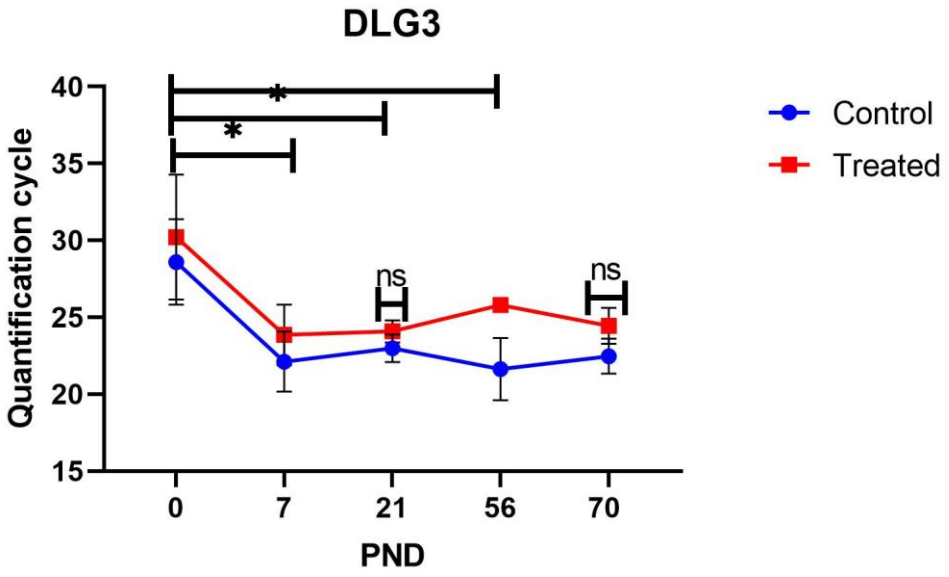
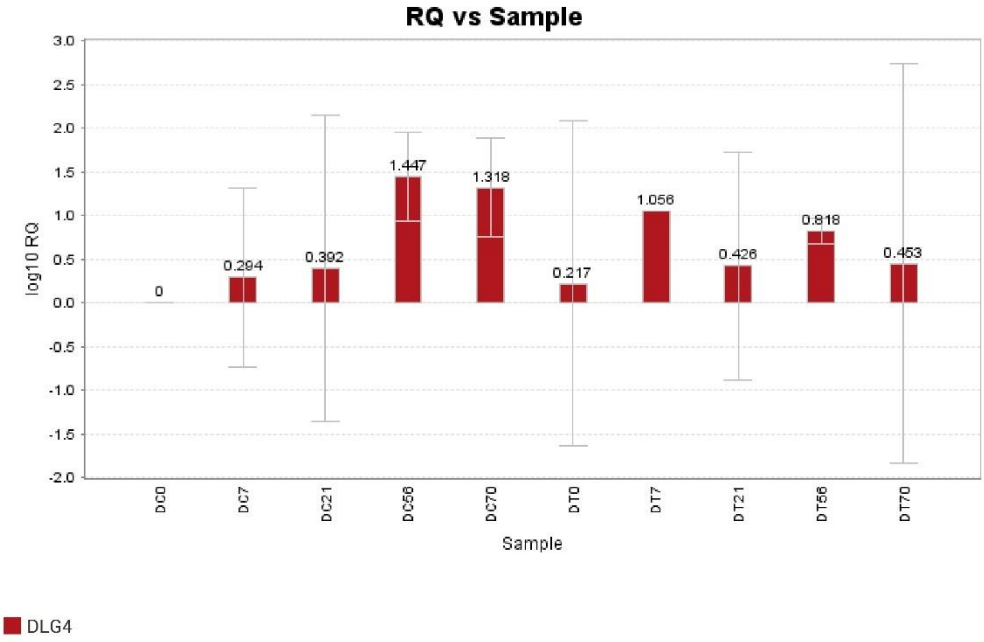




**Figure 4.8.5:** Effect of prenatal kolanut consumption on the level of DGL3 mRNA in the hippocampal tissue from PND 0, 7, 21, 56 and 70. Data is represented by mean ± SEM with 3 animals in each group. $P < 0.0001$ indicates significant differences as compared to control.

### 4.8.6. Effect of kolanut on dendrites and spine morphology

Kolanut caused a reduction in the level of *DGL4* mRNA estimated in the hippocampal tissues of the experimental animals as compared to the control group. Kolanut treatment had a significant effect on the level of DGL4 mRNA in the hippocampus of the treated animals across age group ($F_{(4, 20) = 6.429}$; $p = 0.0017$), treatment ($F_{(1, 20) = 0.5223}$; $p = 0.4782$) with a significant interaction between treatment and age ($F_{(4, 20) = 3.518}$; $p = 0.0249$) when compared to the controls (figure 4.8.6). This could be the mechanism in which the dendrites and spine morphogenesis of the kolanut-treated animals were affected and causing reduced densities (figure 4.4.1.3, figure 4.4.6.1, table 4.5)



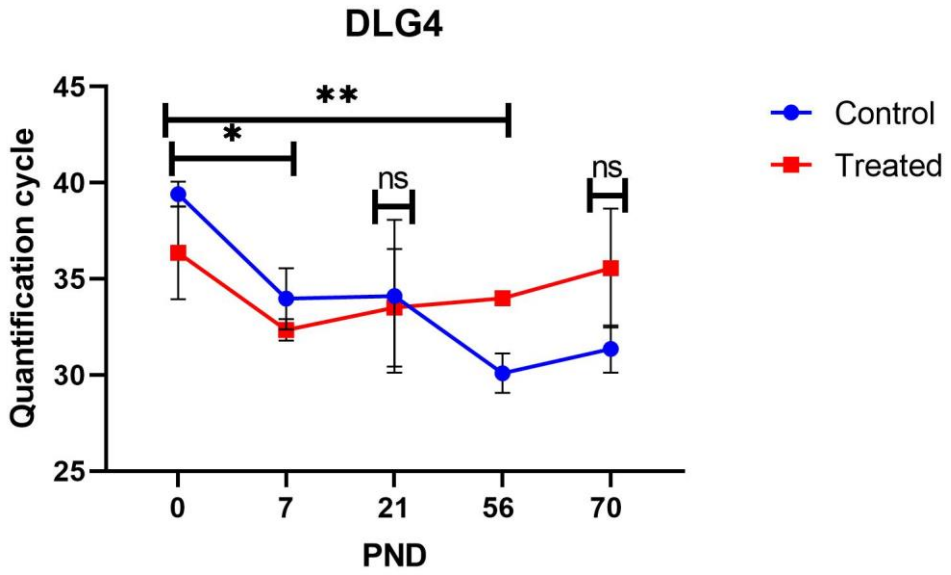




**Figure 4.8.6:** Effect of prenatal kolanut consumption on the level of DGL4 mRNA in the hippocampal tissue from PND 0, 7, 21, 56 and 70. Data is represented by mean ± SEM with 3 animals in each group. $P < 0.01$ indicates significant differences as compared to control.

# CHAPTER FIVE

# DISCUSSION

Ethnomedicine is currently gaining significant traction and attention worldwide because of continuing investigations and establishment of its efficacy, potency, availability, and low toxicity rate when taken moderately and above all very low comparative price to contemporary medicines (Onyiapat, 2014; Forray, 2016; Atiba et al., 2023). Meanwhile, few studies have reported the harmful impact of plant-based treatment (plant natural products) extracts on the different organs of the body. Several reports focused only on the phytochemical constituents of these plant-based natural products and overlook the critical likely harmful responses that might be elicited and become visible after a period of time. Kolanut has been identified as one of these plant-based products that can illicit such responses. The brain, a complex organ involved in numerous tasking activities, is vulnerable to effects of free radicals and consequent decreased functionality. Also, the hippocampus and prefrontal cortex, a co-regulator, along with the other brain regions responsible for processing memory and learning and cognitive functions are not exempted from this (Guitart-Masip et al., 2010; Lisman et al., 2017)

## 5.1. General observations and gross morphometry of pups and dams

The effect of prenatal consumption of kolanut that was observed in the behavior of the kolanut-treated dams and pups was similar to the results of a previous study (Atiba et al., 2016). However, the specific impact of prenatal kolanut consumption as observed and reported in this study under these conditions remains to be determined. Meanwhile, from previous studies (Atiba et al., 2017, 2021), some animals treated with *Moringa oleifera* and kolanut during gestation reported similar cases of amelia, meromelia, still birth and inability to carry their pregnancy to term. Merr and colleagues (2010) also discovered the teratogenicity of crude extracts of *Goniothalamus amuyon* and *Alstonia macrophylla* causing morphological malformations. Likewise, Green et al. (2013) also reported developmental defects when the developing fetuses were exposed to plant products and alkaloids. There was an increase in congenital malformed infants that their mothers chewed *Khat* (a plant product) in pregnancy as compared to women without Khat (Mekonnen et al., 2020). Therefore, the use of herbs especially of plant origin can potentiate miscarriages, still birth with or without the presence of congenital malformation in developing fetuses.

A significant body weight loss ($p < 0.0001$) was observed in kolanut treated dams as compared to the control that had a steady increase in body weight. This could imply that the decreased or inconsistent body weight seen in the treated dams is attributed to inadequate food consumption throughout the gestational period which can be due to kolanut intake in the treated dams. From previous study, decrease in body weight was observed following gestational consumption of kolanut (Atiba et al., 2021) which apparently reduced the body weights of the pups from the treated dams. Other studies have reported that some plants products reduce body weight of animals and humans exposed to them. One of such cases is the Chinese herbal extract number ten (NT) (York et al., 2007) which caused reduced body weight in animals treated with the extract. Also, some plants products reduced body fats thus reducing the body weights of the treated animals (Astell et al., 2013; Kasali et al., 2022). In addition, higher brain weights observed in the treated dams and pups could be due to inflammatory process probably caused by the presence of glial cells. Kolanut could have acted as a neurotoxicant thus generating inflammation and caused oxidative stress (Atiba et al., 2021; James, 2023).

This finding indicates that prenatal kolanut consumption affects the level of food and water intake in dams after birth. Specifically, there was a significant decrease in the amount of food consumed by the kolanut treated dams compared to the control group, starting from day 15. The difference in food consumption between the two groups increased with age, reaching a maximum difference on the 24th day after the start of the experiment. On the other hand, there was a significant steady increase ($p = 0.0005$) in the volume of water consumed by the treated dams compared to the control dams throughout the duration of the experiment, except on the 24th day. These findings align with some studies on the effects of kolanut consumption. Kolanut, which contains caffeine, theobromine, catechins, and tannins, has been found to affect food and water intake in animals (Atiba et al., 2021). Report from a study on the comparative effects of chronic consumption of kolanut (*Cola nitida*) and caffeine diets on locomotor behavior and body weights in mice indicated that, chronic consumption of kolanut resulted in a decrease in food intake and body weight (Umoren et al., 2009). This is consistent with the observation of a significant decrease in food consumption by the kolanut-treated dams. The decrease in food intake could be due to the appetite-suppressing effects of caffeine, a major component of kolanut (Umoren et al., 2009). On the other hand, the same study found that caffeine-diet significantly decreased water intake (Umoren et al., 2009). In this study, result indicates a steady increase in water consumption by the

treated dams. This discrepancy might be due to differences in the experimental setup, the concentration of kolanut consumed, or the physiological responses of the dams. It is important to note that kolanut consumption during pregnancy has been associated with various adverse effects. For instance, it has been found to induce histological alterations in pup's cerebellum, by causing retention of external granular layer beyond PND 21, destruction of Bergmann glial cells, multiple layering of Purkinje cells layer and cell chromatolysis (Atiba et al., 2021). Therefore, the decreased food intake and increased water consumption observed in this study could be part of a broader range of physiological changes induced by prenatal kolanut exposure.

**5.2. Behavioral outcome in the pups**

The effect of kolanut on spatial and non-spatial memory behavior were evaluated through spontaneous locomotor reflex behavior, anxiety and explorative behavior in rats, using cliff avoidance (CA), surface righting test (SR), spontaneous movement, open field, object recognition and location, and RAM test models. Previous studies have reported that either neurochemical and structural deregulation or metabolic disturbances are implicated in the disruption of neurocellular activities in the juvenile pups post-birth (Khazen et al., 2019). These disturbances could be attributed to the exposure to environmental toxins, drugs, or any other form of stressors (Khazen et al., 2019).

Ahmadalipour and Rashidy-Pour (2015) reported that prenatal exposure to morphine or throughout pregnancy results in prolonged or permanent neurochemical and behavioral impairment, including a reduction in learning and memory behavior in children of addicted mothers (Ahmadalipour and Rashidy-Pour, 2015). Studies have also uncovered that exposure to stressful environmental or traumatic events during the gestation period exacerbates the vulnerability to psychopathology-induced neuroendocrine alterations. These are associated with a decrease in emotion, learning, and memory behaviors that appear in the early life stages (Chagas et al., 2021).

In a previous study on kolanut extract obtained from *Cola nitida* (Vent.) Schott & Endl., it was reported that the administration of the extract to pregnant rats induced significant impairment of motor and behavioral activity (Atiba et al., 2021). However, in the present study, evaluation of the postnatal motor reflex behavior in pups following intrauterine exposure to kolanut (*Cola nitida*)

extract was done. The immediate developmental reflex and memories in the pups at PNDs 4, 5, 6, and 7 were investigated. The results showed that the developmental reflex behavior decreases with increased latency of memory retention when subjected to CA (PND 5, 6, and 7) and SR (PND 5). Moreover, on PND 7, the pups showed no further signs of positive behavior, suggesting impaired developmental reflex behaviors and symptoms of hypo-locomotion (sluggish movement).

These data are further affirmed by the delayed/decreased number of head raising and pivoting. In addition, the time taken to pivot reduces as observed in the kolanut-treated pups, which could also be attributed to the impaired developmental reflex behavior. Head raising and pivoting have already been associated with early maturity; therefore, it is noteworthy that this defect could have been caused by kolanut. Thus, delaying the maturity of the treated pups, since the developmental reflexes and memory function were expected to improve in the pups with age, and that was not observed in the kolanut-treated pups. To affirm it further, the delay of developmental reflex activity and memory retention ability were also observed in the kolanut-treated animals at an advanced age (PND 21 and 56). The data showed no signs of increased locomotion, as depicted by no change in mean velocity and in the total distance covered.

The kolanut-treated animals exhibited no marked freezing episode, however, there were elevated grooming behavior, defecation, and urination—indicators of explorative activity reduction and anxiety-like behavior. Moreover, rearing activity increased in the kolanut-treated animals. This activity is regarded as maladaptive behavior due to the failure of the pups to retain previous memory or consolidate the fact that they were previously present in that environment. These responses could mean that prenatal kolanut consumption provokes a decline in cognitive function and memory in the pups, possibly at an advanced age. This is in line with the findings of Kalueff et al.(2005) that grooming frequency and exploratory activities could be increased in the presence of stress, anxiety, fear (Smolinsky et al., 2009) and increase in response to novelty (Pisula et al., 2006).

The burden of negative cognitive phenotypes, such as amnesia, was also observed in this study. Specifically, kolanut caused an irregular retention of previous memory. This could have been worsened by unsustainable cognitive functions due to the reduced amount of residual knowledge

in NOR and NOL. In addition to this, the radial arm maze assessment followed a similar paradigm with decreased spatial memory function in the pups. Perhaps in this study, prenatal consumption of kolanut negatively affected the development and behavioral index for cognitive and spatial memory and tilled more to the deleterious effects of kolanut when consumed in pregnancy. A previous observation was reported by Atiba et al. (2021), indicating that the consumption of kolanut in pregnant animals is detrimental to the developing cerebellar neurons, which might be implicated in spatial memory and motor/cognitive impairment.

**5.3. Effects of kolanut on the morphology of hippocampal neurons**

The neurotoxic effect of prenatal exposure to kolanut on the hippocampal neuronal morphology were investigated in the experimental animals. The findings from this study revealed that prenatal exposure to kolanut downregulated hippocampal neuron development by reducing the mean neuronal cell body (soma count), soma diameter and area, axon count and length, number and length of dendritic branch, spine morphology, neuronal synapses count as well as the dendritic arborization in the hippocampus.

Preclinical, clinical, and post-mortem investigations posits that diverse hippocampal-signalling molecules are involved in neuronal remodelling (Duric et al., 2013). These include neurotransmitter receptors and neurotrophic expressions, which play important roles in the aetiology of neuronal and behavioural development (Leggio et al., 2013). As a result, gestational kolanut consumption could tamper with the hippocampal-signalling molecules as evident by downregulation of hippocampal neuron development in this study.

Moreover, data collected from previous studies have also shown strong evidence that stress-related effect on neuro-circuitry and the body's defense system formed an integrated part that gave protection during developmental exposure to environmental stressors (Juster et al., 2010). These stressors could trigger several inflammatory cascades that activate inflammatory and/or oxidant molecules which then downregulate key proteins involved in neuronal development (Sharma and Mehdi, 2023).

Thus, investigating the mechanism through which prenatal exposure to kolanut downregulated hippocampal neuronal morphology through expression of distinct spine types was observed in this study. The results obtained from this study show that prenatal exposure to kolanut reduces the

density and size (area and diameter) of soma in the CA1, CA2, CA3 and DG region of the hippocampus with age-dependent effects. Furthermore, a decrease in density and size (area and diameter) of soma in the CA1, CA2, CA3 and DG regions of the hippocampus with increase in age was observed after exposure. Although this observation was not consistent in some regions of the hippocampus, the significant reduction in soma density and size (area and diameter) was noticed in comparison to the respective non-exposed animals. Therefore, it could be inferred that kolanut when consumed prenatally might impose a notable post-birth threat throughout the hippocampal neurons' development.

Arborization is the branching of dendrites in a tree-like form and this helps in the number, rate and pattern of branches and synapses received by each neuron (Mcallister, 2000; Schmuck et al., 2020). Dendritic arbors develop in a systematic order whereby the apical dendrites are formed by the processes of migration and the basal dendrites project right from the bodies of the neurons. Moreover, proper growth and arborization of the dendrites are important in adequate functioning of the nervous system (Schmuck et al., 2020). In this study, it showed that kolanut disrupted the migration of the neurons (Atiba et al., 2021), thereby disrupted arborization of the dendrites, thus limiting the number of synapses and plasticity formed by the dendrites (Arikkath, 2012). It made more sense why the behavioral indices for cognitive memory and recall were reduced in kolanut-treated animals.

In addition, prenatal exposure to kolanut altered the spine morphology as well as the dendritic spines density in the pups up to adolescence. This report corroborates previous studies in adult mice and rat models, where significant decrease in the overall spine densities within the regions of the hippocampus after stress exposure was identified (Spano et al., 2019; Jiang et al., 2020). Specifically, the CA1-3 and DG dendrites of kolanut-treated pups had decrease spines. Their characteristics range from no stubby (neck), more spines having a long neck (long-thin) and an inconsistent number of spines having a short neck (thin) with age-dependent effect (PND 21-70).

Therefore, it could be speculated that the changes in the specific spine-type, is as a result of prenatal kolanut exposure with significant age-dependent effect. It is expected that the pups hippocampi should be going through a substantial overproduction and pruning of synapses (Andersen and Teicher, 2008; Iñiguez et al., 2016), but was not the case in this study.

Coincidentally, studies have revealed that other stressor types via different route or forms of exposure can deregulate the signalling molecules. For example, brain derived neurotrophic factor, myelin basic protein and synaptic neurotransmitter affect the developmental pattern of hippocampal dendritic spines (Kellner et al., 2014). Therefore, marked increase in long-thin spines with concomitant decrease in stubby spines and irregular thin spines pattern corresponds to a notable alteration in the spine stability as an implication of prenatal kolanut exposure.

In addition to this, an increase in filopodia (long neck effect) and a decrease in mushroom spine density in the CA1-3 and DG of the hippocampus further depict that prenatal exposure of kolanut delays the maturation of the spines. Similarly, the age-dependent decrease spine length and branch density observed at different regions of the hippocampus could also be as a result of prenatal consumption of kolanut.

Previous studies on CA1 pyramidal neurons reported that the length of the spine neck could restricts calcium entry into the dendritic shaft which then impede the spread of calcium within the dendrite after stimulation, required for excitation (action potential) (Takasaki and Sabatini, 2014). Calcium regulates the dendritic formation and modulates the amplitude, propagation and integration of synaptic input (Adasme et al., 2011). Also, reduction in calcium level leads to reduction in BDNF level (Segal and Korkotian, 2014) in the dendritic spines, thus reducing the cognition and memory function of the hippocampus.

Therefore, this study proposes that prenatal kolanut exposure might be altering excitation of synapse in the rat hippocampus by altering the spine morphology and decreasing the densities and length/branches of the spines (i.e., decrease stubby/mushroom spines and increase long thin/filopodia spines).

**Kolanut effects on neurogenesis and neuroplasticity of the pups' brains**

Studies have revealed that prenatal changes or alterations due to immunotoxins or neurotoxin contributes to or suppresses neuronal plasticity and function including spatio-cognitive and memory functions (Atiba et al., 2021). The search for the toxicological effect of intrauterine kolanut consumption during pregnancy on the hippocampal and cortical neurogenesis is an attempt to provide a new insight into the toxic component of kolanut. The findings of this study

do indicate that kolanut administration during pregnancy can alter the postnatal (adolescent and young adult) proliferation, generation, and development of new neurons in the hippocampus and cortex, specifically in the subgranular zone (SGZ) of the dentate gyrus (DG) of the hippocampus.

In the dentate gyrus, generation of new granule cells occur from neural progenitor cells. Following the activation of these newly produced neural progenitor cells, they further give rise to transit enlarging progenitor cells and/or intermediate progenitor cells. These then generate neuroblasts which differentiate into immature neurons and lengthen its dendrites to the granular cells and molecular layer and finally project their axons down to the CA3 region (Zhao et al., 2008; Zhang et al., 2016b). After several weeks, these new born neurons mature into functional dentate granule neurons that form the synaptic connections and subsequently integrate into the hippocampal circuitry (Laplagne et al., 2006; Zhao et al., 2008). This study elucidated the effect of prenatal kolanut consumption on the color intensity of Nissl, Ki-$67^{+}$ cell, an endogenous cell cycle or cell proliferating marker for mature neurons, and $DCX^{+}$ neural progenitor cells in the DG of the hippocampus.

The color intensity of Nissl in the prefrontal cortex was evaluated. From the findings, there was no changes in the level of Nissl and Ki-$67^{+}$ staining intensity of the neurons in the DG at the different timelines, but an increase in the level of Nissl staining intensity of the neurons in the prefrontal cortex occurred. Whitney et al. (2008) reported that specific brain cell such as the Purkinje cells in human cases expresses decreased or unexpected Nissl staining intensity. Although, the reasons were not clear but was speculated to be due to pre-mortem circumstances since it was not considered as a post-mortem delay effect or other processing variables (Whitney et al., 2008). However, observation from experimental rats relative to their controls expressed no significant changes in the hippocampus but a significant change in the cortex was observed when exposed to the prenatal kolanut consumption.

The level of Nissl staining intensity ($p$ = 0.0002) was reduced compared to the hippocampus of the control animals while an increased intensity was seen in the cortex of the kolanut-treated brain tissues. Reduction in the level of staining intensity of Nissl substance is usually considered as an indicator of decreased cellular metabolic activity, as it is associated with protein synthesis (Usman et al., 2022). Although, this is mostly observed in exposure-related tissue damage and might

explain further the observed alteration in DCX+ and Ki-67+ cells with associated tissue injury and cell death (chromatolytic and pyknotic cells). The Ki-67+ staining was also noticed to be highly expressed at PND 70. Similar to previous findings, this study reports that the increased Ki-67+ expression indicated decreased level of cell proliferation in the DG (Whitney et al., 2008; Alsaegh et al., 2023). Meanwhile, it was observed that DCX+ expression were more pronounced in the SGZ of the DG at the different PNDs at 21, 56 and 70. In line with previous reports, exposure to toxicants or some treatment drugs affect the development of new born neurons and their morphology in the SGZ of the DG as indicated by reduced DCX+ cells, thus, increase DCX+ expression was observed in this study (Wang et al., 2017, 2019). To substantiate these findings further, the level of neuronal degeneration was estimated. This was done via histomorphological assessment characterized by alterations in neurocellular morphology by measuring the area, diameter and density of neurons. This helped to determine the degree of changes caused by prenatal kolanut consumption in the adolescent and/or young adult hippocampal DG. Interestingly, from the findings, intrauterine kolanut consumption altered the architecture of granular layer of the DG and the cortex evidenced by decrease mean area and diameter and with decrease density in the hippocampus and the prefrontal cortex.

On PND 21 and 56, a significant loss of mean area, diameter and density in the hippocampus was observed. And on PND 21, 56 and 70, a significant decrease in cortical neurons with no changes in mean area and diameter in the kolanut-treated groups coupled with a concomitant increase in pyknotic cells, dampened and fragmented cytoplasm, condensed nuclei and numerous chromatolytic cells were observed in both the hippocampus and the prefrontal cortex. It is important to note that the intrauterine exposure to kolanut treatment predisposed the animals to neuronal degeneration and histo-architectural changes as in this study. It is also suggested that the presence of these methylxanthine constituents (caffeine and theobromine) in the kolanut might be the cause of the high pyknotic cells count reported in this study which still needs further clarification. Moreover, it is consistent with previous study which reported that prenatal kolanut consumption inhibited neuronal function and also act as a development suppressant in the cerebellum of the pup when fed during gestation (Atiba et al., 2021).

In contrast to the exposure model in this study but similar in the results obtained, in the exposure to acute radiofrequency electromagnetic radiation. Study reported by Singh et al, 2023 on the

mitigation of neurogenesis and concomitantly exacerbation of neuronal DNA fragmentation and distorted morphology in young/adolescent rat. This was evidenced by pyknotic or karyorrhectic nuclei in the DG region of the hippocampus.

The involvement of $DCX^+$ cells, a post-mitotic progenitors and early immature neurons used to estimate the neural stem cells growth was further elucidated. From the results, it was observed that intrauterine administration of kolanut affect the nature of the $DCX^+$ expression by decreasing the number of mean areas, mean diameter and mean density of the new neurons thus suppressing the neural stem cells at PNDs 21, 56 and 70.

The intrauterine kolanut consumption was also found to inhibit the activation of progenitor cells proliferation in the SGZ at different PNDs (21, 56 and 70) in the rats. These results illustrated that intrauterine kolanut consumption down-regulates the proliferation of neural stem cells post-birth and continuous until adolescent and young adulthood. From this study, the decrease in the number of $DCX^+$ cells indicated suppressed post-mitotic progenitors and decrease immature neurons (Brown et al., 2003; Náměstková et al., 2005; Mori et al., 2020; Sorrells et al., 2021).

Previous reports have shown that exposure to toxicants or some treatment drugs tamper with the development of new born neurons and their morphology in the SGZ of the DG (Okada et al., 2016; Wang et al., 2017, 2019; Ben-Mimouna et al., 2018). These data suggest that intrauterine kolanut consumption does not promote the neural stem cell differentiation and could be impeding $DCX^+$ cell migration in prenatal and post-natal neurogenesis.

Although, it appeared that the neurotoxicological signs of kolanut consumption are consistent with some of overt neurotoxicity signs previously reported by Ikegwuonu et al. (1981) and Atiba et al. (2021) in rat model. It would, thus, be suggested that the alteration reported in the post-mitotic progenitors and non-maturing early neurons, i.e., $DCX^+$ expression, may be due to the presence of methylxanthine constituents in the kolanut, mainly the caffeine and the theobromine which still needs further clarification. As previously mentioned, intrauterine consumption of kolanut during gestation in the rats resulted to aberrant, low mean count (area, diameter, and density) $DCX^+$ cells (post-mitotic progenitor and early immature neurons), and with high or exaggerated expression leading to defect in neuronal migration in the SGZ of the DG (Atiba et al., 2021).

To explore the impact of intrauterine kolanut consumption on the maturation of neurons, under expression of Ki-67 (an endogenous cell cycle/proliferating marker for mature neurons) proteins in the DG was detected and analysed. Ki-67 protein expression is known to enhance the physiological growth of neurons (Klintsova et al., 2007). From this study, the Ki-$67^+$ cell (mean area, diameter, and density) and in both adolescent and/or young adult hippocampal DG decreases showing that the hippocampal cell proliferation were influenced by the prenatal administration of kolanut. As previously stated, the intensity of the Ki-$67^+$ were not affected as reported in this study, and the reason for this outcome is unknown.

According to the results, the level of this basic proteins in the kolanut-exposed rat might have undergone some significant changes which could suppress the hippocampal cell proliferation and the neurogenesis in the adult brain as revealed by the decreased Ki-$67^+$ and $DCX^+$ cells. Although, it was reported that intrauterine kolanut consumption impeded $DCX^+$ expression and possibly inhibited cellular migration during neurogenesis (Atiba et al., 2021). These results indicate that prenatal kolanut consumption exhibited strong influence on the rate of survival of neurons and does not promote growth and maturation of neurons. To some extent, intrauterine kolanut consumption could provoke the loss of newborn neurons and prevents the rebuilding of neural pathways in the adolescent and/or young adult hippocampal DG. This possibly enhances the loss of spatial cognitive and memory functions as previously mentioned. Herein, it was deduced that prenatal kolanut consumption remarkably reduced neurocellular development, proliferation, differentiation, and maturation in SGZ of the hippocampal DG and prefrontal cortex in the rat brain.

**5.4. Effects of kolanut on pro-oxidant and antioxidant enzymes in the pups**

The brain is a complex organ involved in numerous tasking activities. It utilizes glucose and ketose for proper functioning and extremely vulnerable to free radical damage due to its low usage of oxygen and low concentration of antioxidant enzymes. The hippocampus remains one of the key brain structures responsible for memory and learning, together with the formation of emotional responses (Lisman et al., 2017).

The antioxidant defense system which is made up of the enzymatic antioxidants and the non-enzymatic antioxidants act as the first line of defense against the insult of free radicals and

reactive oxygen species generated in the system or acquired through the toxic substances consumed or exposed to. The antioxidant defense system mitigates the deleterious effect of reactive oxygen species, and thereby averting the cellular oxidative damage implicating the structural and functional alterations (Atiba et al., 2016; Zhang and Tsao, 2016; Zhang et al., 2016a; Farella et al., 2020; Onasanwo et al., 2021; Adebayo et al., 2022).

In this study, induction of oxidative stress in the hippocampal tissue was observed across all kolanut-treated groups through the increase of malondialdehyde concentration. Hinckle et al. (2017) and Erukainure et al. (2017) observed a decrease in malondialdehyde levels in the liver of rats exposed to $H_2O$ and treated with kolanut extract. These changes in malondialdehyde level could be attributed to dose-dependent and tissue-specific differences since the higher dose was used in this study. It is important to note that the brain is highly susceptible to free radical damage resulting from the excessive accumulation of reactive oxygen species which induces and exacerbates mitochondrial damage, likewise lipid peroxidation, apoptosis, and inflammation (Kang and Yang, 2020). An increase in malondialdehyde level in the hippocampal tissue could depict the probable pro-oxidant role of the kolanut in the tissue, thereby subjecting it to lipid peroxidation, and possible tissue damage. The increase in pro-oxidant activity observed in the hippocampal tissues of the kolanut-treated pups could be mediating the decrease developmental reflex activity and impaired memory retention ability as well as altered the histoarchitecture and morphometry recorded in this study.

On the other hand, it was expected that gestational kolanut administration would boost the endogenous antioxidant enzymes and subsequently induces remarkable reduction in the level of lipid peroxidation in the hippocampus. However, the interpretation of the data generated in this study showed otherwise as the prenatal kolanut exposure triggered dysfunction of the reduction-oxidative system/reaction by directly up-regulating or down-regulating the endogenous glutathione peroxidase (GPx) and superoxide dismutase (SOD) at the different timelines. Superoxide dismutase is an antioxidant enzyme that catalyzes the dismutation of oxygen radicals (superoxide anion) to molecular oxygen ($O_2$) and hydrogen peroxide ($H_2O_2$) (Zhang et al., 2016b; Farella et al., 2021). The ability of this nut to induce alterations in target tissues could be attributed to the presence of caffeine, an active component found in kolanut (Erukainure &

Oyebode et al., 2017). However, studies have also shown that its toxicity level could be both dose and time-dependent (Adeosun et al., 2017; Atiba et al., 2021).

Caffeine is a central nervous system stimulant that regulates the CNS by mechanisms involving different pathways, thus enhancing cognitive function in both humans and animals (Schubert et al., 2017). In this study, SOD activity was observed to be initially enhanced in pups in groups PND 0, 7, 21, and 56, although its inhibition was evident in pups in group PND 70. It could be gathered that SOD activity tends to decrease over time in the treated animals.

Additionally, the same trend was observed in Glutathione peroxidase activity (GPx), involved in catalyzing the reduction of peroxidases like hydrogen peroxide to water and oxygen through the oxidation of reduced glutathione (Brigelius-Flohé and Maiorino, 2013), in the hippocampal tissue of the treated animals. Glutathione peroxidase activity increased in groups PND 0, 7, 21, and 56, but decreased in group PND 70. A plausible explanation for these observations could be associated with the unique properties of kolanut which could act as an antioxidant and as a stressor owing to its rich flavonoid and caffeine contents.

Therefore, it could then be deduced that over time, treatment with kolanut extract exhibited a deleterious effect on the hippocampal tissue through mechanisms that involve the decrease in SOD and GPx activities thereby increasing its susceptibility to oxidative damage. Corroborating this result, Erukainure et al. (2021) observed an increased level of SOD activity in hepatic tissues of rats treated with kolanut extract. In previous studies of sleep deprivation, there was no observed changes in SOD and GPx activities in animals exposed to kolanut extract (Oboh et al., 2018; Edo et al., 2023). Although, existing data reported decrease level of endogenous antioxidant enzymes when exposed to neurotoxicants at gestation, our data corroborate these reports even though the neurotoxicant exposed to are different.

**5.5. Effects of kolanut on neurotropic and cholinergic system in the pups**

Neuroplasticity, also known as synaptic plasticity, plays a crucial role in brain functionality and adaptive capacity. It is the ability of the extraordinary structure of the brain, to alter functionally and physically in response to external stimuli such as behavioral, cognitive, or environmental demands (Gulyaeva, 2017). The mechanism of neuroplasticity is mediated by a class of proteins known as neurotrophins which play crucial roles in synapses. These include but are not limited to

the nerve growth factor (NGF) which plays an essential role in the cholinergic system, and the Brain-Derived Neurotrophic Factor (BDNF), which functions in the adult brain as a regulator of synaptic plasticity (Isaev et al., 2017).

Brain-derived neurotrophic factor is broadly distributed throughout the brain and its expression is most prominent in the hippocampus (Linz et al., 2019; Colucci-D'amato et al., 2020). The production and release of BDNF elicits neuroprotection through the activation of its high-affinity receptor, tropomyosin-related kinase B (TrKB) (Numakawa et al., 2018). This involves three essential signaling pathways namely, the phospholipase-c-gamma (PLC-y), phosphatidylinositol-3-kinase (PI3K), and the mitogen-activated phosphokinase (MAPK) resulting to the activation and release of downstream proteins involved in neuronal survival, plasticity, cellular energy balance, and mitochondria biogenesis (Gulyaeva, 2017; Raefsky and Mattson, 2018).

Investigations and reports have shown that BDNF upregulates antioxidant enzymes and repairs damaged DNA in neurons (Schmitz et al., 2017), prevents neuronal apoptosis through the downregulation of pro-apoptotic protein (Bax and Bad), and the upregulation of anti-apoptotic protein (BCl2), amongst others (Chen et al., 2018). Studies have shown that BDNF regulates the release of many neurotransmitters and plays a significant role in neurogenesis through the enhancement of protective pathways and inhibition of deleterious pathways (Palasz et al., 2020). The relationship between BDNF concentration and stress in the brain has been highly explored (Tognoli et al., 2010; Linz et al., 2019).

However, this present study reported increased concentration level of the hippocampus derived neurotrophic factor of the prenatal kolanut-treated pups. The increased hippocampus derived neurotrophic factor concentration may indicate an adaptive response of the neurotrophin in the neuronal circuits even in the presence of oxidative burden, implicating the decreased developmental reflex behaviour and impaired memory and cognitive function in the pups (Zhang and Tsao, 2016). In addition, the hippocampal brain derived neurotrophic factor expression could be attributed to the caffeine present in kolanut which functions in part as a neuronal function and development suppressant as reported in a previous study (Atiba et al., 2021).

Corroborating this effect, the activity of the cholinergic system decreased with decrease in acetylcholine release in the pups' hippocampi. The cholinergic system in the brain plays a vital

role in memory and cognitive functions (Bekdash, 2021). Cholinergic neurons which are responsible for the production of acetylcholine are found throughout the brain (Frahm et al., 2015). They are involved in cholinergic signaling, an anti-inflammatory mechanism that confers protection against neuroinflammation in the brain (Maurer and Williams, 2017). Disruption in this system could lead to cognitive impairment and memory deficits as a result of neuro-inflammation which is evident in some neurodegenerative diseases mostly observed in the aged (Haam and Yakel, 2017).

Acetylcholine is a neuromodulator and a neurotransmitter that plays a major role in cognition (Haam and Yakel, 2017; Madrid-López et al., 2017). It is synthesized from choline and Acetyl-CoA through the action of choline acetyltransferase (ChAT) (Maurer and Williams, 2017). Acetylcholine receptors are widely expressed throughout the brain, the hippocampus inclusive, where they are thought to be involved in regulating excitability and cognitive functions. Activation of these cholinergic receptors impacts neuronal network activity in the hippocampus (Haam and Yakel, 2017).

In this study, a steady decrease in acetylcholine concentration was observed in the kolanut-treated pups inciting a decreased developmental reflex behaviour and impaired memory and cognitive function reported in this study. A study by Maurer and Williams, (2017) had shown that decrease in acetylcholine release affect cognitive memory indices. Therefore, it can be concluded that the adaptive production of the brain derived neurotrophic factor protein, decreased activity of the cholinergic system, and increased redox status observed in the kolanut-treated pups' hippocampi may be accountable for the reduced developmental reflex behaviour and impaired memory and cognitive function observed in this study.

**5.6. Relationship between behavioral tests, biochemical levels and neurogenesis markers**

The relationship between the behavioral test, biochemical levels and the markers of neurogenesis were further investigated using logistic regression analysis. Behavioral correlates of BDNF, ACh and MDA were obtained, and a significant association and positive correlation was observed between the SRT and MDA at PND 7 and rearing and MDA at PND 56. Also, a significant negative correlation was found between NOL and MDA at PND 56, NOR and MDA at PND 21, and RAM and BDNF at PND 56. This may be due to the increased in the level of

malondialdehyde and reduced acetylcholine level in the hippocampi of kolanut treated pups. Even though the study did not show a positive association between the reduced memory indicators of the behavioral tests and the level of ACh, it is still of note that the level of ACh was reduced in kolanut-exposed animals.

The behavioral tests showed mixed results, with some tests showing a positive correlation with MDA levels, and others showing a negative correlation. It is of note that MDA is a marker of oxidative stress, and these results suggest that kolanut consumption might affect brain function in ways related to oxidative stress.

Previous research has shown that kolanut consumption can affect behavior. For instance, a study found that co-administration of alcohol and kolanut decreased brain total protein, DNA, RNA levels, and inhibited gene expression (Obochi et al., 2007c). This aligns with findings of this present study of altered behavior in pups exposed to kolanut. As found at the biochemical level in this study, ACh which is a neurotransmitter involved in many functions, including memory and muscle activation, suggests that these functions might be affected by kolanut consumption. In addition, the observation of reduced ACh levels in kolanut-exposed animals is consistent with the known effects of caffeine contained in kolanut, which can influence neurotransmitter levels as reported in a study on the benefits and effects of kolanut consumption (Adelusi et al., 2020).

Furthermore, the findings showed a significant correlation between the mean area of DCX+ cells and the expression of BDNF protein, although, negatively correlated. In contrast, there was no significant relationship observed between the mean area of DCX+ cells and the MDA activity. The result further indicated no significant relationship between the mean density of Ki-67+ cells and the expression of BDNF protein. Moreover, a significant relationship was observed between the Ki-67+ cells and the MDA activity. Both BDNF protein and MDA activity were negatively correlated to the mean density of Ki-67+ cells. These results suggest that DCX+ cells, Ki-67+ cells and BDNF protein play crucial roles in neurogenesis during the development of the hippocampus from fetal to adult stage (Obochi et al., 2007c).

DCX+ and Ki-67+ cells are markers for new neuron formation, while BDNF is a protein that supports the survival of existing neurons and encourages the formation of new neurons and synapses. In this study, the results showed a significant but negative correlation between these

markers and BDNF protein or MDA activity. While some previous studies reported antioxidant properties of kolanut use (Fabunmi and Arotupin, 2015; Joshua et al., 2017), this present study found a negative correlation between these markers and BDNF protein and MDA activity. Also, this study indicates a complex relationship between kolanut exposure and neurogenesis markers, thus suggesting kolanut consumption might negatively affect neurogenesis, or the formation of new neurons from fetal to adult stage.

### 5.7. Effects of kolanut on molecular basis of proliferation, differentiation, synapses, plasticity, and programmed cell death

Recent studies have suggested that kolanut may have an effect on neuronal proliferation and differentiation, specifically through the modulation of *c-fos* mRNA levels in hippocampal tissues (Hansson et al., 2003; Obochi et al., 2007b; Calabrese et al., 2014; Zhang et al., 2016b). *C-fos* is an immediate early gene, and its protein product, *fos*, is often used as a marker of neuronal activity (Krukoff, 1999; Cruz-Mendoza et al., 2022; Aparicio et al., 2022).

In a previous study, the expression of *c-fos* has been shown to be rapidly activated after a stimulus, with the length of time that it remains active depending on the type of stimulus and the structure studied (Aparicio et al., 2022). This aligns with the observation from this study that following treatment with kolanut extract, there was a downregulation of *c-fos* mRNA levels in the hippocampal tissues of treated animals, suggesting a modulatory effect on neuronal activity. It was also observed that the downregulation of *c-fos* was not significant across age groups and treatments. This may suggest that the effect of kolanut on *c-fos* mRNA levels may not be directly influenced by the age of the animal nor the specific treatment regimen. While there is limited literature specifically addressing the influence of age and treatment regimen on *c-fos* mRNA levels, it is known that the expression of *c-fos* can be influenced by various factors such as the type of stimulus and the structure studied (Calabrese et al., 2014; Zhang et al., 2016b; Aparicio et al., 2022).

Research on the effects of kolanut on neuronal activity is still emerging. Knowing that *c-fos* is important in neurogenesis, cognitive and memory functions, one could imply that kolanut caused the downregulation of the *c-fos*, thus resulting in deleterious effects on neurogenesis and

neuroplasticity. These effects also extend to cognitive and memory impairment observed in this study.

C-*jun* is a transcription factor involved in various cellular events, including gene expression, neuronal plasticity, regeneration, and cell death (Bonetto et al., 2020; Shao et al., 2021). It is part of the c-*jun* N-terminal kinases (JNKs) family, which plays a central role in stress signaling pathways (Yarza et al., 2016). C-*jun* has been implicated in processes such as neuronal plasticity (Biggi et al., 2017) and programmed cell death in neuronal health (Roffler-Tarlov et al., 1996; Kase et al., 2020). The observed increase in *c-jun* mRNA levels in the hippocampus of kolanut-treated pups in this study could suggest that kolanut may induce stress responses in neuronal cells, leading to changes in gene expression and potentially affecting neuronal plasticity and survival. This aligns with previous studies indicating that substances like caffeine found in kolanut can stimulate the central nervous system (Akinpelu, 2020; Egoro et al., 2023). However, it is important to note that the specific effects of kolanut on neuronal health are complex. This may depend on various factors, including the dosage and duration of kolanut consumption, the specific neuronal cell types involved, and the overall physiological context. For instance, Egoro et al. (2023) found that chronic consumption of kolanut may influence hepato-renal disorders; acute kidney failure, liver failure, suggesting that long-term kolanut use could have systemic effects that indirectly impact neuronal health. It is evident that the role of *c-jun* in neuronal health is multifaceted. For example, *c-jun* has been found to play a role in neuronal plasticity, with evidence of presynaptic localization and function of the *c-jun* N-terminal Kinase (JNK) in synaptic plasticity (Biggi et al., 2017). *C-jun* has been implicated in programmed cell death, with studies showing that phosphorylation of *c-jun* is one of the earliest biochemical changes detected in dying neurons (Roffler-Tarlov et al., 1996).

The impact of kolanut in this study was also observed on memory-related synaptic plasticity in changes in the level of CREB1, a gene that encodes the cAMP responsive element binding protein 1, a transcription factor implicated in numerous biological processes including memory (Kandel, 2012; Ortega-Martínez, 2015). The findings from the study indicate that treatment with kolanut had a significant effect on the level of CREB1 on the hippocampus of the treated animals across different age groups. This is evident among the treated pups which showed a higher quantification cycle compared to the control animals across different postnatal days. Whereas Ortega-Martínez

(2015) found that a higher quantification cycle suggests lower expression of the CREB1 gene. This suggests that kolanut may have a detrimental effect on memory-related synaptic plasticity which is as a result of the crucial role CREB1 plays in the formation and consolidation of memory (Kandel, 2012; Rosenberg et al., 2014). Studies have shown that its involvement in the regulation of gene expression is a key process in long-term memory formation and synaptic plasticity (Kandel, 2012; Ortega-Martínez, 2015). Therefore, a decrease in the level of CREB1 could potentially impair these processes, leading to negative effects on memory. Kolanut is known to naturally stimulate the central nervous system, which may increase alertness and boost energy levels (Adelusi et al., 2020). However, the specific impact of kolanut on memory-related synaptic plasticity and CREB1 levels has not been extensively studied. Therefore, this experiment provides valuable insights into the potential effects of kolanut on memory.

CREB2, also known as ATF4, is a transcription factor that plays a crucial role in long-term memory and synaptic plasticity. It is also involved in various cellular processes, including cell survival and differentiation (Ortega-Martínez, 2015; Steven et al., 2020). The expression of CREB2 is regulated by various signaling pathways, and its dysregulation can lead to various neurological disorders (Steven et al., 2020). The findings of this study indicate that the administration of kolanut had a significant interaction between treatment and age on CREB2 mRNA. This expression suggests that the effect of kolanut on CREB2 expression varies with age. This could potentially imply that the impact of kolanut on synaptic plasticity and memory functions might be age dependent. Previous studies have shown that the expression of CREB2 can be influenced by various factors, including environmental stimuli and pharmacological agents (Ortega-Martínez, 2015; Steven et al., 2020). However, a study on the effect of alcohol and kolanut interaction on biochemical indices of neuronal gene expression in Wistar albino rats showed that alcohol-kolanut co-administration decreased brain total protein, DNA, RNA levels and protein/RNA ratios, and inhibited gene expression (Obochi et al., 2007b). The study further explains that the effects which inhibited DNA transcription, mRNA splicing and protein synthesis, and polypeptide expression, are necessary for the growth, development, differentiation, and cell survival. Hence, it could be hypothesized that the kolanut intervention might have a similar inhibitory effect on gene expression, leading to a decrease in the relative quantification values observed in the samples. This could potentially affect the induction of long-term synaptic

plasticity, as changes in gene expression can influence synaptic strength and, consequently, the ability of neurons to form long-lasting connections.

It also appears that kolanut treatment significantly affects the level of DLG3 mRNA in the hippocampus of the treated pups across different age groups as observed. Moreover, it is of great importance to note that DLG3 is a gene that encodes a member of the membrane-associated guanylate kinase (MAGUK) family, which is involved in synaptic function (Liu et al., 2019; Park and Farris, 2021). Studies have also shown that DLG3 plays a crucial role in both the synaptic and cognitive functions (De Los Reyes et al., 2023). This could imply that the effect of kolanut on DLG3 mRNA levels is consistent across different age groups and is not dependent on the age of the pups at the time of treatment. This aligns with Obochi et al. (2007b) report that showed alcohol and kolanut co-administration decreased brain total protein, DNA, RNA levels, and inhibited gene expression. Although this study focused on the combined effects of alcohol and kolanut, it does provide some context for the potential effects of kolanut on gene expression. Hence, further studies could also help clarify whether these effects are due to the kolanut itself or to other compounds present in the kolanut, such as caffeine.

DLG4, also known as PSD-95, is a major scaffolding protein in the postsynaptic density (PSD) and plays a crucial role in synaptic maturation and plasticity. It is known to affect dendritic spine morphology, which is critical for learning and memory (Qi et al., 2022; Zhang et al., 2016b). Changes in dendritic spine morphology and density are associated with various neurological conditions (Pchitskaya and Bezprozvanny, 2020). The significant interaction between treatment and age as observed in this study implies that the effect of kolanut on DLG4 expression may vary depending on the age of the pups. This could potentially be due to age-related changes in the brain's response to kolanut or age-related changes in DLG4 expression. Tian et al's study on dendritic morphology reported possible effect of dendritic morphology on the properties of back-propagating action potentials, which are crucial for neuronal signal integration and synaptic plasticity (Tian et al., 2022). Dendritic spines are the primary sites of excitatory synaptic input, and their shape and size are correlated with the strength of the synaptic connection (Pchitskaya and Bezprozvanny, 2020). Also, a study by Ortiz-Sanz et al., (2020) proved that changes in dendritic spine morphology and density are associated with various neurological conditions. For instance, a significant alteration in dendritic spine density and maturity were observed in

hippocampal neurons treated with Aβ oligomers in their study. Similar patterns of DLG4 expression were observed in this present study, although no significant impact of kolanut treatment was observed on DLG4 expression.

# CHAPTER SIX

# CONCLUSION AND RECOMMENDATION

Kolanut, an economically rich nut found in Nigeria and in some sub-Sahara countries was studied to document its potential effects on the developing hippocampus. The results provided substantial evidence on the effects of kolanut consumption on behavior, biochemical levels, neurogenesis markers, neuronal activity, and plasticity. This research has also contributed to the growing body of literature on the neurobiological effects of kolanut consumption on the developing hippocampus. The study highlights the potential deleterious effects of kolanut on appetite and water intake, body weight and brain weight. It also caused changes in behavioral activities, thereby reducing the cognitive function and ability to recall events or activities.

It was also established that kolanut possess the potential to affect the histomorphology, size and population of the neurons in the hippocampus. Thereby, reducing neuronal activities, proliferation and connectivity as seen in its suppression of proliferation of new cells (neurogenesis) and synapses (neuroplasticity) of the neuronal cells.

Kolanut probably caused these effects by increasing the level of reactive oxygen species. This led to inflammation and oxidative damage of the neuronal cells by increasing the levels of malonaldehyde, superoxide dismutase and glutathione peroxidase in the hippocampus. These increase in levels might be a way to mitigate and reduce the oxidative stress caused by kolanut.

Kolanut also affected the early genes, *c-fos* and *c-jun*, which mediates neurodegeneration, cell death, repair and plasticity, by causing a downregulation of *c-fos* and upregulation of *c-jun*. Likewise, the levels of transcriptional activators, CREB1 and CREB2 were affected, DLG3 and DLG4 were not spared of the effects of kolanut either. Thus, accounting for the cognitive and memory impairment, reduced dendritic aborization, plasticity and synapses as observed in this study.

**Recommendations**

Given the potential implications of these findings, it is rather advisable that more studies on Nonhuman primates (NHP) models as well as clinical trials are encouraged based on the results found in this study. This can be further developed into translational research.

In addition, further investigation of specific component or fraction (s) of the kolanut that contributed to the observed effects and exploration of other underlying mechanisms would be enlightening. This could include examining potential interventions like antioxidants to mitigate these observed effects.

Finally, even though this study was carried out in animal model, a public health initiatives and stakeholders can incorporate these findings as a sound of warning in developing guidelines and recommendations around kolanut consumption, especially, in the reproductive and pregnant women.

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## APPENDICES

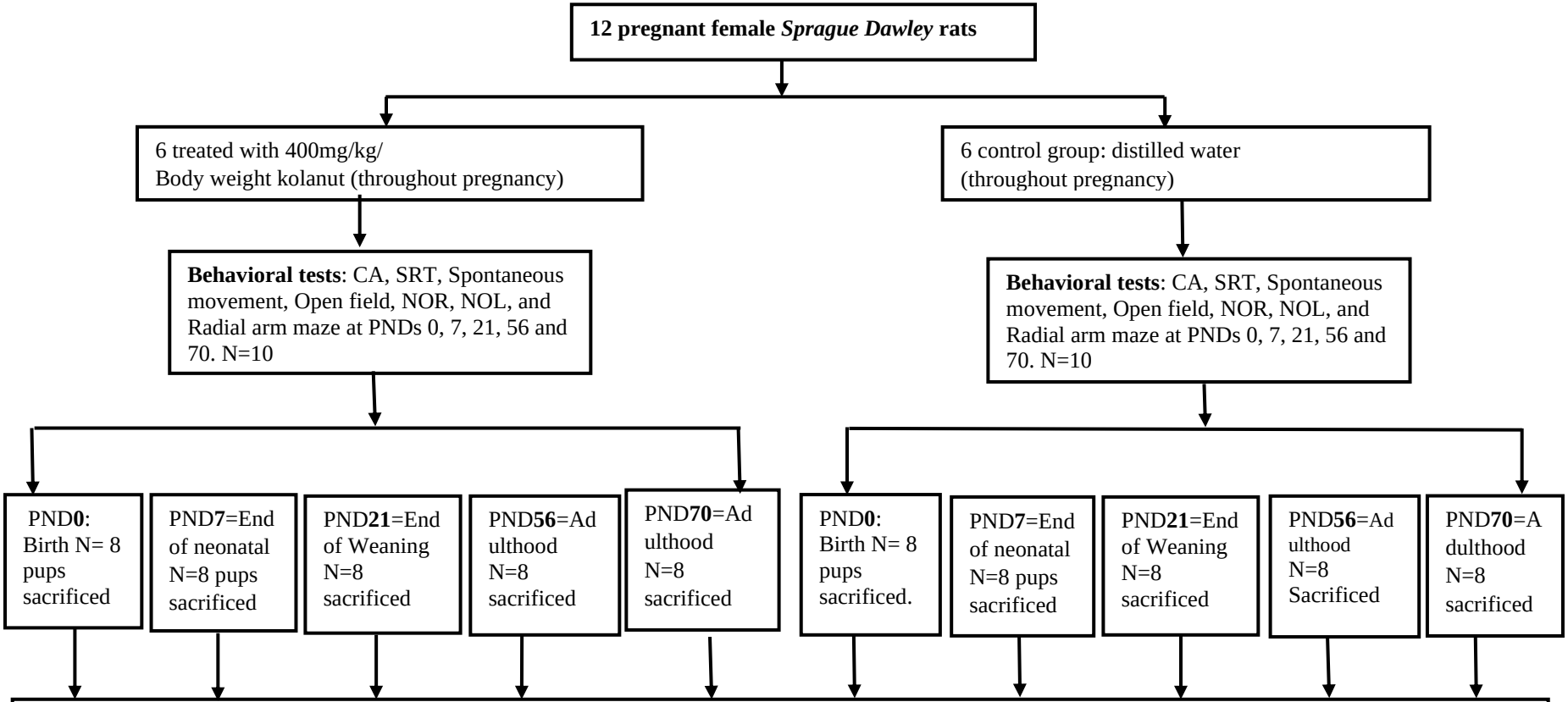
I. Experimental flow chart

II. Ethics certificate

III. Study timeline.

IV. Budget

## EXPERIMENTAL FLOWCHART




Sacrificed by deep anesthesia with an overdose of euthanasia followed by intracardiac perfusion with phosphate buffered saline for molecular, biochemical, and phosphate buffered formalin for histological studies.

Specimen collected.
-Brain tissue
**Offspring**- 2 out of the 8 brains were used for Golgi-Cox. The remaining 6 brains from each group were divided into left hemisphere for molecular and biochemical tests; and right hemisphere for Cresyl violet and immunohistochemistry for Ki67 & DCX.
**Dams**- Daily measurement of food and water intake throughout the experiment were measured. Body and brain weights were recorded.
-All other organs were collected from the dams and their offspring to determine the effects of aqueous extract of kolanut on them and effects on relative organ-body ratio and for further toxicology study.

## ANIMALS RESEARCH ETHICS COMMITTEE (AREC)

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

## STRICTLY CONFIDENTIAL

**CLEARANCE CERTIFICATE NUMBER: 2020/06/02/C**

**APPLICANT: Dr F Atiba**
**School: School of Anatomical Sciences; Department: N/A; Location: CAS**

**PROJECT TITLE: Effect of intrauterine exposure to an aqueous extract of kolanut (Cola Nitida) on the hippocampus formation in the brain of Sprague Dawley Rat offspring;**
**Category: C; Species and Numbers involved:** *12 dams (for breeding) and 72X male & female Sprague Dawley Rats*

Approval is hereby given for the use of animals for the research project named above and described in the application reviewed by a quorate meeting of the AREC held on 31 Mar 2020. This approval remains valid until 12 Oct 2022 and is conditional to the following (if blank there are no special conditions):

| Condition 1 | Condition 2 | Condition 3 | Condition 4 |
|---|---|---|---|
| | | | |

All material changes to the approved research must be reported to the AREC before they are implemented. Failure to do so will invalidate this clearance certificate.

An annual progress report must be provided to the AREC.

The use of these animals is subject to AREC guidelines on the use and care of laboratory animals, is limited to the procedures described in the application and is subject to additional conditions listed below:

I, the Chair of the AREC (or my designated representative) am satisfied that the proposed research is ethical as judged by local law, international standards and University policy.

Signed: _____ _____ Date: ____14/10/2020_____

(Chairperson of the AREC)

I am satisfied that the persons listed in this application are competent to perform the procedures described in the application, in the context of Section 23 (1) (c) of the veterinary and Para-veterinary Professions Act (19 of 1982).

CC: Student supervisor: «Title1» «Initials1» «Supervisor_surname»
Director Central Animals Service: Dr Kim Jardine

Signed: ____ _____Date 15/ 10 2020___

(Registered Veterinarian)

CC: Student supervisor: «Title1» «Initials1» «Supervisor_surname»
Director Central Animals Service: Dr Kim Jardine

## STUDY TIMELINE

| **Year 2019** | **Jan** | **Feb** | **Mar** | **Apr** | **May** | **Jun** | **Jul** | **Aug** | **Sep** | **Oct** | **Nov** | **Dec** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Proposal preparation | | | | | | | | | | | | |
| Harvesting of plant material and extraction | | | | | | | | | | | | |
| | | | | | | | | | | | | |
| **Year 2020** | **Jan** | **Feb** | **Mar** | **Apr** | **May** | **Jun** | **Jul** | **Aug** | **Sep** | **Oct** | **Nov** | **Dec** |
| Trialect training (UK) | | | | | | | | | | | | |
| Proposal submission to ethics | | | | | | | | | | | | |
| Proposal submission to faculty | | | | | | | | | | | | |
| Covid-19 Lockdown | | | | | | | | | | | | |
| Proposal assessment & approval | | | | | | | | | | | | |
| Purchasing of animals and consumables | | | | | | | | | | | | |
| Animal treatment | | | | | | | | | | | | |
| Phases 1 & 2 with termination PND 0 and 7 | | | | | | | | | | | | |
| Phase 3 & termination of PND 21 | | | | | | | | | | | | |
| Results analysis | | | | | | | | | | | | |
| | | | | | | | | | | | | |
| **Year 2021** | **Jan** | **Feb** | **Mar** | **Apr** | **May** | **Jun** | **Jul** | **Aug** | **Sep** | **Oct** | **Nov** | **Dec** |
| Phase 4 & Termination PND 56 & PND 70 | | | | | | | | | | | | |
| Immunohistochemistry | | | | | | | | | | | | |
| Biochemical analysis & genetic determination | | | | | | | | | | | | |
| Joint Advanced seminar 4 (CARTA) | | | | | | | | | | | | |
| **Year 2022** | **Jan** | **Feb** | **Mar** | **Apr** | **May** | **Jun** | **Jul** | **Aug** | **Sep** | **Oct** | **Nov** | **Dec** |
| Microscopy and analysis of results | | | | | | | | | | | | |
| **Year 2023** | **Jan** | **Feb** | **Mar** | **Apr** | **May** | **Jun** | **Jul** | **Aug** | **Sep** | **Oct** | **Nov** | **Dec** |
| Results analysis, thesis and manuscripts writing | | | | | | | | | | | | |
| **Year 2024** | **Jan** | **Feb** | **Mar** | **Apr** | **May** | **Jun** | **Jul** | **Aug** | **Sep** | **Oct** | **Nov** | **Dec** |
| Submission of Thesis and manuscripts. Graduation | | | | | | | | | | | | |

| **BUDGET** | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | |
| **Items** | **Quantity / No of persons** | **Frequency / No of times/ Days** | **No of months** | **Unit cost in Rand** | **Unit cost Equivalent in USD (1R=16.84)** | **Total cost in Rand** | **Unit Cost in Naira** | **Unit Cost Equivalent in USD (1$=~~₦~~360)** | **Total Cost in Naira** | **Total Coat in USD (1$=~~₦~~360) (1R=16.84)** |
| **Section 1: Kolanut** | | | | | | | | | | |
| Purchase of Kolanut | 1 | 3 | 2 | | | | 10,000.00 | 27.78 | 60000.00 | 166.67 |
| Transportation to and fro the plantation | 2 | 3 | 2 | | | | 5,000.00 | 13.89 | 60000.00 | 166.67 |
| Processing kolanut into extract | 1 | 1 | 1 | | | | 220,000 | 611.11 | 220000.00 | 611.11 |
| Agricultural clearance certificate for kolanut | 1 | 1 | 1 | | | | 5,000.00 | 13.89 | 5000.00 | 13.89 |
| **Sub-total** | | | | | | **16138.28** | | | **345000.00** | **958.33** |
| Section 2: Research Animals | | | | | | | | | | |
| Purchase, housing and feeding of animals | 84 | 1 | 6 | 40 | 2.38 | 20160.00 | | | | 1197.15 |
| **Sub-total** | | | | | | **20160.00** | | | | **1197.15** |
| Section 3: Behavioural studies | | | | | | | | | | |
| Construction of Radial arm maze apparatus | 1 | 1 | 1 | 7800 | 463.18 | 7800.00 | | | | 463.18 |
| **Sub-total** | | | | | | **7800.00** | | | | **463.18** |
| Section 4: Histology (Nissil & Golgi Stains) | | | | | | | | | | |
| Cresyl violet acetate | 1 | 1 | 1 | 4631.00 | 275.00 | 4631.00 | | | | 275.00 |
| Gelatine (1000 | 1 | 1 | 1 | 282.00 | 16.75 | 282.00 | | | | 16.75 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 15% Vat 64-65 | | | | 736.95 | 43.76 | 736.95 | | | | 43.76 |
| 2.5l Acetic acid | 1 | 1 | 1 | 153.00 | 9.09 | 153.00 | | | | 9.09 |
| 2.5l Chloroform | 1 | 1 | 1 | 552.00 | 32.78 | 552.00 | | | | 32.78 |
| 2.5l Ethanediol | 1 | 1 | 1 | 255.00 | 15.14 | 255.00 | | | | 15.14 |
| 500ml Hydrogen peroxide | 1 | 1 | 1 | 60.00 | 3.56 | 60.00 | | | | 3.56 |
| 2.5l Methanol | 1 | 1 | 1 | 81.00 | 4.81 | 81.00 | | | | 4.81 |
| 500g Trisodium Citrate | 1 | 1 | 1 | 66.00 | 3.92 | 66.00 | | | | 3.92 |
| 2.5l Propan-2-ol | 1 | 1 | 1 | 289.00 | 17.16 | 289.00 | | | | 17.16 |
| 15% Vat 67-73 | | | | 978.25 | 58.09 | 978.25 | | | | 58.09 |
| **Sub-total** | | | | | | **8084.20** | | | | **480.06** |
| Section 5: Immunohistochemistry | | | | | | | | | | |
| Anti-ki67 (100ug) | 2 | 1 | 1 | 10168.1 | 603.81 | 20336.2 | | | | 1207.61 |
| Anti-Doublecortin | 2 | 1 | 1 | 9703.29 | 577.58 | 19406.58 | | | | 1152.41 |
| Biotinylated anti-rabbit | 1 | 1 | 1 | 5923.16 | 351.73 | 5923.16 | | | | 351.73 |
| ABC Kits | 2 | 1 | 1 | 9082.8 | 539.36 | 18165.6 | | | | 1078.72 |
| 15% Vat | | | | 9574.73 | 568.57 | 9574.73 | | | | 568.57 |
| 3,3'-Diaminobenzidine tetrahydrochloride | 1 | 1 | 1 | 2000.00 | 118.76 | 2000.00 | | | | 118.76 |
| Tri Reagent | 1 | 1 | 1 | 6347.00 | 376.90 | 6347.00 | | | | 376.90 |
| Triton x | 1 | 1 | 1 | 843.00 | 50.06 | 843.00 | | | | 50.06 |
| Bovine serum Albumin | 1 | 1 | 1 | 3824.00 | 227.08 | 3824.00 | | | | 227.08 |
| Trisodium citrate dihydrate | 1 | 1 | 1 | 1444.00 | 85.75 | 1444.00 | | | | 85.75 |
| 15% Vat 82-86 | | | | 2168.70 | 128.78 | 2168.7 | | | | 128.78 |
| **Sub-total** | | | | | | **90032.97** | | | | **5346.38** |
| Section 6: Molecular Studies | | | | | | | | | | |
| Sybr green | 3 | 1 | 1 | 7242.00 | 430.05 | 21726.00 | | | | 1290.14 |
| RNAlater | 1 | 1 | 1 | 2186.00 | 129.81 | 2186.00 | | | | 129.81 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| (100ml) | | | | | | | | | | |
| Freight for ice | 1 | 1 | 1 | 130.00 | 7.72 | 130.00 | | | | 7.72 |
| 15% Vat 90-92 | | | | 1433.70 | 85.14 | 1433.70 | | | | 85.14 |
| CREB 1 | 1 | 1 | 1 | 4210.00 | 250.00 | 4210.00 | | | | 250.00 |
| CREB2 | 1 | 1 | 1 | 5388.80 | 320.00 | 5388.80 | | | | 320.00 |
| DLG3 | 1 | 1 | 1 | 6483.40 | 385.00 | 6483.40 | | | | 385.00 |
| DGL4 | 1 | 1 | 1 | 7849.46 | 466.12 | 7849.46 | | | | 466.12 |
| BDNF 1 | 1 | 1 | 1 | 5397.22 | 320.50 | 5397.22 | | | | 320.50 |
| BDNF II | 1 | 1 | 1 | 5388.80 | 320.00 | 5388.80 | | | | 320.00 |
| BDNF III | 1 | 1 | 1 | 5479.74 | 325.40 | 5479.74 | | | | 325.40 |
| BDNF IV | 1 | 1 | 1 | 5898.21 | 350.25 | 5898.21 | | | | 350.25 |
| 15% Vat 94-101 | | | | 6914.23 | 410.58 | 6914.23 | | | | 410.58 |
| **Sub-total** | | | | | | **78485.56** | | | | **4660.66** |
| Section 7: Biochemistry | | | | | | | | | | |
| Superoxide dismutase kit | 1 | 1 | 1 | 2006.00 | 119.12 | 2006.00 | | | | 119.12 |
| Catalase | 1 | 1 | 1 | 1681.00 | 99.82 | 1681.00 | | | | 99.82 |
| Glutathione Peroxidase | 1 | 1 | 1 | 13588.00 | 806.89 | 13588.00 | | | | 806.89 |
| Malondialdehyde | 1 | 1 | 1 | 822.00 | 48.81 | 822.00 | | | | 48.81 |
| Nitrotetrazolium blue chloride | 1 | 1 | 1 | 873.00 | 51.84 | 873.00 | | | | 51.84 |
| Hydroxylamine hydrochloride | 1 | 1 | 1 | 727.00 | 43.17 | 727.00 | | | | 43.17 |
| Dimethyl Fumarate | 1 | 1 | 1 | 1029.00 | 61.10 | 1029.00 | | | | 61.10 |
| 5,5-Dithiobis(2-Nitrobenzoic acid) | 1 | 1 | 1 | 1782.00 | 105.82 | 1782.00 | | | | 105.82 |
| 15% Vat | | | | 3376.20 | 200.49 | 3376.20 | | | | 200.49 |
| **Sub-total** | | | | | | **25884.20** | | | | **1537.07** |
| Section 8: Laboratory Consumables | | | | | | | | | | |
| 96 well plate | 1 | 1 | 1 | 2310.00 | 137.17 | 2310.00 | | | | 137.17 |
| Microamo adhesive film (100 pieces) | 1 | 1 | 1 | 2255.00 | 133.91 | 2255.00 | | | | 133.91 |
| FG, Optical cap | 1 | 1 | 1 | 1914.00 | 113.66 | 1914.00 | | | | 113.66 |
| Handling Fee | 1 | 1 | 1 | 263.00 | 15.62 | 263.00 | | | | 15.62 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 15% Vat 84-87 | 1 | 1 | 1 | 1011.30 | 60.05 | 1011.30 | | | | 60.05 |
| 48 well TC plates (pk 100) | 1 | 1 | 1 | 2942.00 | 174.70 | 2942.00 | | | | 174.70 |
| 1.5 Tube clear cap (pk500) | 1 | 1 | 1 | 172.00 | 10.21 | 172.00 | | | | 10.21 |
| 0.2ml PCR tube (pk1000) | 1 | 1 | 1 | 500.00 | 29.69 | 500.00 | | | | 29.69 |
| 48 well plate for PCR (pk 50) | 1 | 1 | 1 | 3013.00 | 178.92 | 3013.00 | | | | 178.92 |
| 15% Vat 89-92 | 1 | 1 | 1 | 996.75 | 59.19 | 996.75 | | | | 59.19 |
| **Sub-total** | | | | | | **15377.05** | | | | **913.13** |
| Section 9: Thesis | | | | | | | | | | |
| Stationery and Binding of thesis | 1 | 1 | 1 | 3000.00 | 178.15 | 3000.00 | | | | 178.15 |
| **Sub-total** | | | | | | **3000.00** | | | | **178.15** |
| TOTAL | | | | | | 264962.26 | | | | 15734.10 |
| | | | | | | **R264, 962.26** | | | | **$15, 734.10** |