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Histological, histochemical and immunohistochemical evaluation of the effects of seed and pulp of *Carica papaya* on the visual relay centres in animal model

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The effects of the pulp and seeds of *Carica papaya* (Honey dew variety) on the visual relay centres in rats were investigated. Thirty rats were randomly grouped into three groups (A, B, and C) of ten rats each. Each of the rats in groups A, B and C were fed with 8.50 g of standard rat pellet, 8.50 g of 1:1 mixture of *C. papaya* pulp and the standard rat chow, and 8.50 g of 1:1 mixture of *C. papaya* seeds and standard rats' chow, twice daily, respectively. Twenty-four hours after the last administration, the rats were sacrificed by cervical dislocation, the brain was removed, superior colliculus (SC) and lateral geniculate body (LGB) were carefully excised. Fraction of the SC and LGB meant for quantitative enzyme histochemistry were homogenized in sucrose solution and processed accordingly for the activities of lactate dehydrogenase (LDH), glucose-6-phosphate dehydrogenase (G6PD) and acetylcholinesterase (AChE) while the fraction of the SC and LGB meant for histological and immunohistochemical analysis were fixed in 10% formol-calcium solution and were processed for paraffin embedding, respectively. The histological, histochemical and immunohistochemical studies revealed that *C. papaya* seeds caused neuronal degeneration and disruption in the visual relay centres of the treated rats. The activity of G6PDH was increased in the SC and LGB of rats in groups B and C, and there was statistically significant ($P<0.05$) inhibition in the activity of AChE in both the SC and LGB of rats in group C.

Key words: Quantitative histochemistry, histology, *Carica papaya*, superior colliculus, lateral geniculate body, 10% formol calcium.

INTRODUCTION

In the long history of the old and new world, plants have been used medicinally (Adekomi et al., 2011). A large and increasing number of individuals use medicinal herbs regarding their use ('O' Hara et al., 1998). According to Chang (1987), it has been estimated roughly, that more than half of the total population of the world use herbal drug. Increasing interest in medicinal herbs and/or plants or seek the advice of their physician increased scientific

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evaluation of their therapeutic properties, potential and safety (‘O’ Hara et al., 1998).

Papaya (*Carica papaya*) is a major fruit crop in many tropical parts of the world. It has been ranked first amongst 38 common fruits based on its accordance to the United States Recommended Daily Allowance for many vitamins, and consumption of papaya has been recommended for preventing vitamin A deficiency (Gouado et al., 2007) which causes childhood blindness in many tropical and subtropical countries. *C. papaya* is widespread throughout tropical Africa; it belongs to the family Caricaceae. The plant is usually short-lived, but can produce fruit for over twenty years (Gross, 2003). The *papaya* has a complicated means of reproduction, it can be monoecious, diecious or hermaphroditic (Bruce and Peter, 2008). Hermaphrodite *papaya* trees (flowers with male and female parts) produces pear shaped fruits that are self pollinated (Jari, 2009). *Papaya* has been reported in folklore medicine to be effective in treating blindness, but the mechanism with which it does this is not clearly understood; hence, the aim of this study.

The fruits, leaves, seeds and latex are used medicinally (Beckstrom et al., 1994). The folklore medicinal use of *C. papaya* is as a digestive agent. The latex from the trunk of the tree is also applied externally to enhance ‘quick’ healing of wounds, ulcers, boils and warts. The seed is used to expel worm, the flower may be taken in an infusion to induce menstruation (Reed, 1976; Morton, 1977; Duke, 1984). *Annonaceous acetogenins* derived from the extracts of the twigs of *C. papaya* tree may be good chemotherapeutic agents for cancer as these compounds inhibit enzymes necessary for metabolism in tumor cells (Rupprecht et al., 1986; Hui et al., 1989a, b; Zhao et al., 1992, 1995; Reiser et al., 1992).

The superior colliculi form the rostral two bumps (one on each side) on the dorsal aspect of the midbrain (Marc et al., 2007). The caudal two bumps are the inferior colliculi and together they (inferior and superior colliculi) comprise the tectum or roof of the midbrain (Marc et al., 2007). In contrast to the inferior colliculus, which is an auditory centre, the superior colliculus is usually described as a visual reflex centre (Chevalier and Mana, 2000). It is a highly laminated (layered) structure. The dorsal-most three layers receive visual information primarily from two sources, that is, the retina and the visual cortex (area 17) (Marc et al., 2007). In contrast to the exclusively visual nature of the superficial layers, the intermediate and deep layers receive projections from many functionally different areas of the brain (Marc et al., 2007). These inputs are both “motor” and “sensory”. Since the latter category includes visual, auditory and somatosensory inputs, it can be seen that the superior colliculus is not exclusively related to visual function. Instead, it plays a role in helping to orient the head and eyes to all types of sensory stimuli (Lunenburger et al., 2001).

The lateral geniculate bodies are the ovoid protuberances lateral to the pulvinar of the thalamus in the diencephalon of the forebrain and into which the fibre of the optic tract synapse on their way to the visual cortex (Carlson, 2007). However, because of the semi-decussation of the optic nerve fibres in the optic chiasma, the lateral geniculate body in the right thalamus receives the fibres originating on the temporal retina of the right eye and the nasal fibres of the left (Carlson, 2007). Each body appears, in cross-section, to consist of alternating white and grey areas. The white areas are formed by the medullated nerve fibres of the optic tract, while the grey areas consist largely of the nerve cells in which the fibres of the optic tract terminate (synapses) and from which arises the fibres of the optic radiations (Goodale and Milner, 2004). There are six grey areas or layers of cells, with layer 1 being the most ventral and layer 6 the most dorsal (or posterior). Layers 1, 4 and 6 receive the crossed or nasal fibres from the contralateral retina, while layers 2, 3 and 5 receive the uncrossed or temporal fibres of the ipsilateral retina. The neural components of the lateral geniculate body (LGB) without the blood vessels and covering layer form the lateral geniculate nucleus (LGN) (Carlson, 2007).

Despite the folkloric, wide and historical use of *C. papaya* in the traditional management of many diseases, the scientific validation of its implication on the visual relay system is lacking. In view of this, this preliminary study was designed to evaluate the seed and pulp of *C. papaya* on the visual relay centres in rats.

MATERIALS AND METHODS

Collection of plant and preparation of plant extracts

Unripe fruit of *C. papaya* (Honey dew variety) were obtained from Imam Ahmad Garden, Asa-Dam, Ilorin, Kwara State, Nigeria. It was taken to the Department of Plant Science, University of Ilorin, Nigeria for identification and authentication. The pulp was sliced, seeds were removed and both air-dried separately. The dried samples were pulverized separately into powdery form with a Maxwell blender (Model W55110V).

Animal treatment

Thirty adult Wistar rats (first filial generation) of both sexes (6 to 7 weeks old) were used for this study. The rats were purchased from the Pharmacy Department of the Obafemi Awolowo University, Ile-Ife, Osun state, Nigeria. The rats were acclimatized for 24 h in the Animal Holding of Anatomy Department, University of Ilorin. Ethical approval was obtained from the Ethical Committee of the College of Health Sciences of the University of Ilorin on the use of animals for scientific studies and was strictly adhered to. The rats were grouped into three groups designated as A, B and C. The protocol of treatment is as follows: Group A: each rat was fed with 8.50 g of standard rat chow purchased from Bethel Feed Mill, Sawmill, Ilorin, twice daily; Group B: each rat was fed with 8.50 g of 1:1 mixture of *C. papaya* pulp and standard rat chow, twice daily; Group C: each rat was fed with 8.50 g of 1:1 mixture *C. papaya* seeds and standard rat chow, twice daily.

All rats were allowed free access to water *ad libitum*. The body weight of the rats was taken at 07:30 h of every day throughout the study. The duration of treatment was for 30 days.

Histopathologic study

Twenty-four hours after the last administration, the rats were sacrificed by cervical dislocation; the head and cranium were carefully removed, avoiding pressure on the underlying brain. The head and exposed brain were immersion-fixed in 10% formol calcium fixative solution. The brain remained *in situ* for seven days before its removal from the skull to avoid the development of neuronal hyperchromatosis. Whole brains were then removed and immersed in fresh 10% formol calcium fixative. Brains were blocked transversely at the caudal aspect of the pons, immersed in daily changes of the fixative, the superior colliculus (SC) and LGB were carefully excised, dehydrated in ascending grades of ethanol, cleared in three changes of xylene, vacuum-paraffin-infiltrated, and embedded. A rotary microtome was used to cut and collect 3 µm serial sections, three sections per slide. Sections for immunohistochemical technique were picked with charged slides.

Serial sections sets of the SC and LGB were stained with standard Heamatoxylin&Eosin (H&E), Cresyl Fast Violet (CFV), and Marsland, Glees and Erikson's technique for Nerve cells (MGE).

Immunohistochemical analysis

Clone SP 11 (Synaptophysin), a neuroendocrine marker was used to access and quantify the safety of the plant to the cells of the visual pathway. This antibody recognizes a protein of 38 kDa, identified as synaptophysin. It labels normal neuroendocrine cells of human adrenal medulla, carotid body, skin, pituitary gland, thyroid, lung, pancreas, gastrointestinal tract (GIT) mucosa. Neurons in the brain, spinal cord, and retina were also labeled. Antibody to synaptophysin is very useful in the identification of normal neuroendocrine cells (Thiel, 1993).

Slides were examined and evaluated microscopically for neuronal chromatolysis and other evidence of cellular pathology in the target organs. Photomicrographs of corresponding region of interest in serial sections were obtained using an Olympus (XSZ-107BN, No. 071771) binocular light microscope with 10× objective and a DXM1200 digital camera for histological analysis.

Histochemical study

The fraction of the SC and LGB for quantitative enzyme histochemistry were homogenized with mortar and pestle in cold 0.50 M sucrose solution (Collins et al., 2005), and were centrifuged at 1000 rpm for 5 min. The supernatant were collected and assayed for lactate dehydrogenase (LDH) (Wei, 1975), glucose-6-phosphate dehydrogenase (G6PDH) and acetylcholinesterase (AchE) (Lohr et al., 1974).

Statistical analysis

Data was analyzed using Statistical Package for Social Science (SPSS) software (version 16.0) by one way analysis of variance (ANOVA) and t-test. The results were expressed as the mean ± standard deviation (SD) and differences were considered significant for $P<0.05$.

RESULTS

Physical observations

During acclimatization, all rats appeared presumably healthy, with smoothly laid hairs on their skin, pinkish eyes, and normal skin color. There were considerable body weight gain in the rats of groups A and B, and this was consistent throughout the duration of treatment. On the other hand, the rats in group C lost weight progressively, the rate of weight loss is more pronounced in male than female rats. The loss observed diminished at the last 10 days of treatment and the statistical analysis of the weight loss in group C indicated a significant difference at $P<0.05$. A reduction of food consumption and body weight was observed in the rats in group C treated with 8.50 g of 1:1 mixture of *C. papaya* seeds and standard rats' chow twice daily. Figures 3 and 4 show the body weight of the male and female rats in groups A, B and C before and after 30 days of treatment. Since food consumption was reduced, similar reductions of body weight have been observed during the 30 days of study. All the rats in the control group had an increase in body weight gain when compared with the rats in groups B and C (Figures 3 and 4).

Histopathological observations

From the processed histological sections of the SC and LGB of the rats in the control group, it was observed that the rats in this group displayed a well preserved cyto-architectural profile as compared to the rats in groups B and C (Figures 1 and 2). The histological profile of the SC and LGB of the rats in group B has mild neuropathological disruptions when compared with the rats in groups C and A (Figures 1 and 2). The SC and LGB of the rats in group C had neuropathological alterations. The characteristic changes in the affected neurons were chromatolysis and neuronal degeneration characterized by loss of Nissl substances, perikaryonal swelling, margination of the nucleus, nucleolar changes, and increased perikaryonal eosinophilia with occasional clumping of eosinophilic debris. At the administered dose, the SC and LGB of the rats were affected and the neuropathological alterations were prominent (Figures 1 and 2).

Histochemical observations

Statistical analysis of quantitative data for the activities of LDH, G6PD and AChE in the SC and LGB acquired in this study showed that there were significant differences between the groups (Figures 5 to 7). Within the SC, the activity of LDH was of significant lowest mean level in the pawpaw seed treated group (C), whereas the significant highest mean level of the activities of LDH in SC was observed in the control group; although, the activities of LDH in the pawpaw pulp treated group was not significant (Figure 5). Within the LGB, the activity of LDH was of significant high level in the control rats, whereas it was insignificant in rats in groups B and C. In Figure 6, the

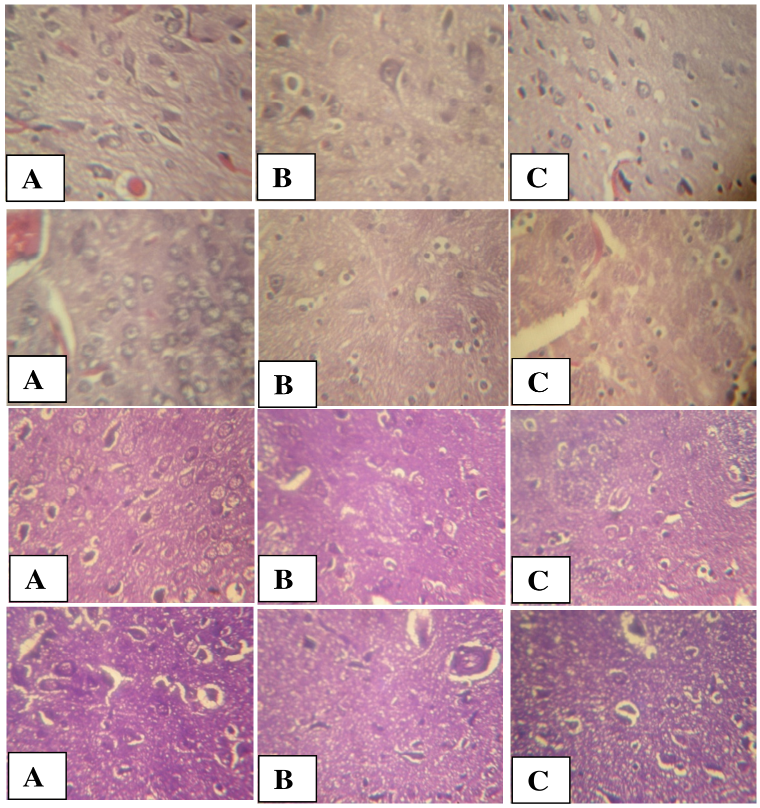

Figure 1. Panels 1 and 2 showing representative H&E stained micrographs of the SC and LGB (respectively) of rats in groups A, B and C. Panels 3 and 4 showing representative CFV stained micrographs of the SC and LGB (respectively) of rats in groups A, B and C. Panel 1: H&E stained section of the SC of representative rats in groups A, B and C, respectively (10x). Panel 2: H&E stained section of the LGB of representative rats in groups A, B and C, respectively (10x). Panel 3: CFV stained section of the SC of representative rats in groups A, B and C, respectively (10x). Panel 4: CFV stained section of the LGB of representative rats in groups A, B and C, respectively (10x)

activities of G6PD was observed to be insignificant in the SC of rats treated with pawpaw pulp (B) and pawpaw seed (C), but was significantly low in the control group (A). Similar characteristic was also observed in the activities of G6PD in the LGB of rats in all the treatment groups. The result in Figure 7 showed that, the level of AChE increased in the SC of rats in the control group, whereas the activities were significantly low in the pawpaw seed treated group. The activities of AChE in the LGB of rats in the pawpaw seed treated group was significantly low, but was insignificant in the control and pawpaw pulp treated group.

DISCUSSION

The literature on the effect of *C. papaya* on the visual relay centre appears very scarce. Though there were lots of phytochemical studies done in different parts of the world, which revealed that the plant pulp contains various beneficial elements (Oloyede, 2005) and that seeds are toxic to some body organs of various experimental animals in dose dependent manner (Chinoy et al., 2006). In this study, the physical changes recorded in group C rats during administration could be as a result of composition of the seeds, which has been shown to induce variable responses depending on the dose, duration, and route of administration in laboratory animals (Udoh, 1999; Chinoy et al., 2006). The reduced level of AChE can also be linked to the said inactivity. The slight weight gain in both male and female rats in group B supported the fact that *C. papaya* pulp contains some vital growth elements (Oloyede, 2005); though, according to this work, *C. papaya* pulp alone cannot supply the required balance diet for rats, as the weight gain in group A (the control group) was higher than that of B group. Significant weight loss ($P<0.05$) experienced by group C rats may be due to the fact that they did not feed well as compared to other groups, or may be as a result of the likely toxic effects of the *C. papaya* seeds, as it has been said that the seed is

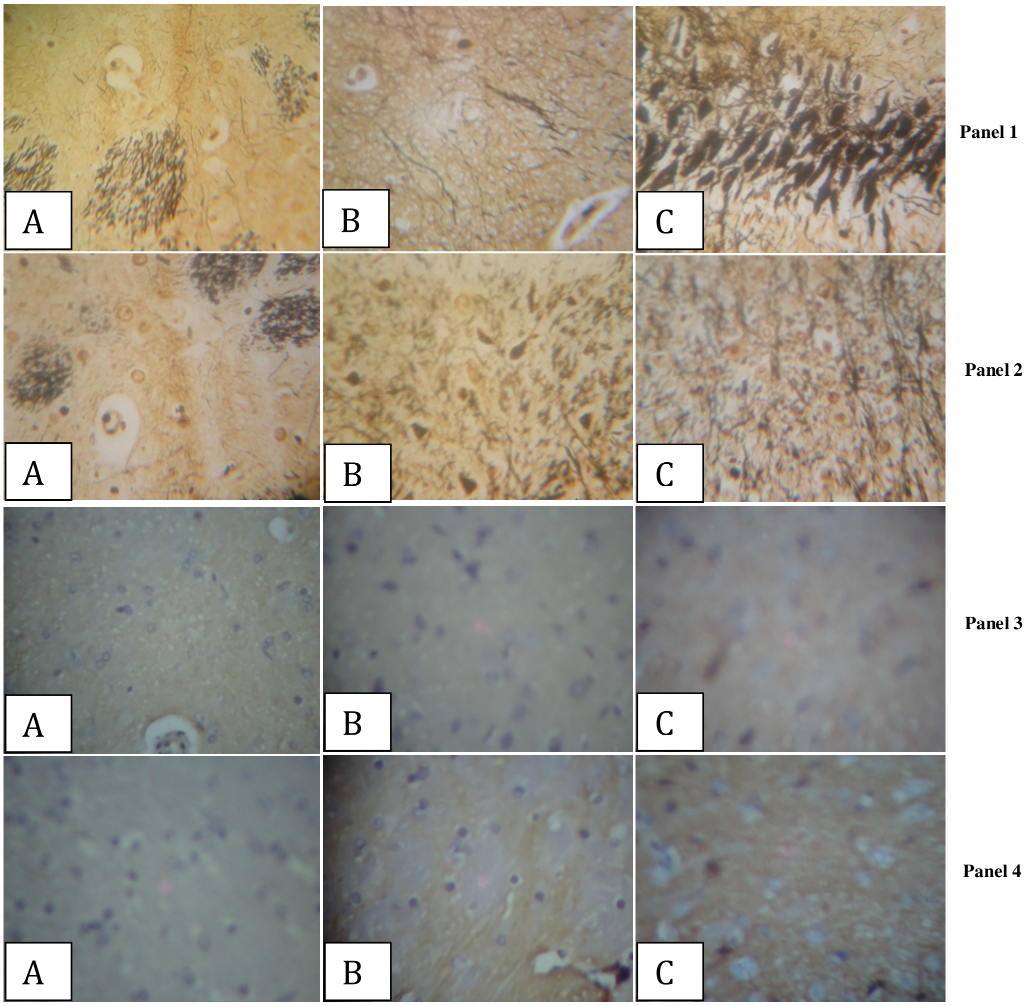

Figure 2. Panels 1 and 2 showing representative MGE stained micrographs of the SC and LGB (respectively) of rats in groups A, B and C. Panels 3 and 4 showing representative immunohistochemical identification (Clone SP 11 (Synaptophysin)) of normal neurons in the SC and LGB (respectively) of rats in groups A, B and C. Panel 1: MGE stained section of the SC of representative rats in groups A, B and C, respectively (10x). Panel 2: MGE stained section of the LGB of representative rats in groups A, B and C, respectively (10x). Panel 3: immunohistochemical identification of the SC of representative rats in groups A, B and C respectively (10x). Panel 4: immunohistochemical identification of the LGB of representative rats in groups A, B and C respectively (10x).

the seed is toxic to some body organs (Chinoy et al., 2006). This latter view is more likely, because of the gross reduction of the effect of AChE enzyme in the organs of the study considering the result of quantitative histochemistry.

The hyperchromic histological appearance of group C animals signified high activity level and this may be excited by the seeds composition, this corresponds with high level of G6PDH observed in the group. Since G6PDH is the first enzyme in the hexose monophosphate pathway that produces ribose-5-phosphate (R-5-P) and nicotinamide adenine dinucleotide phosphate (NADPH), R-5-P is the precursor for nucleotide and nucleic acid synthesis; this usually leads to cell proliferation, resulting in hyperchromic appearance. The normochromic histological appearance of group B cells on the other hand, still supports a normal metabolic activity of the animals in the group as could be seen as well in the group A (the control).

Though the cellular architecture of the cells in all the groups were preserved; the presence of numerous vacuolations (degeneration), and large perinuclear spaces in the group C are enough evidence to infer that *C. papaya* seeds could cause deleterious effects on the visual organs. Gross chromatolysis was noticed in the distribution of Nissl substances (the intracytoplasmic clumps

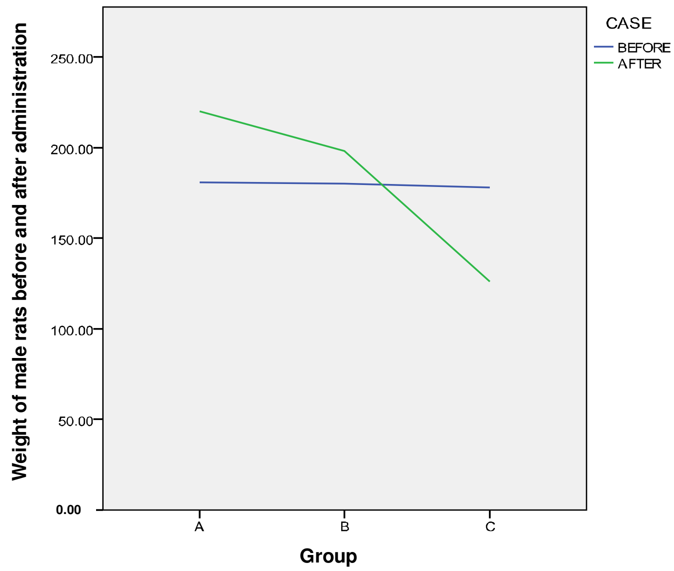

Figure 3. Body weights of male rats before and after the study.

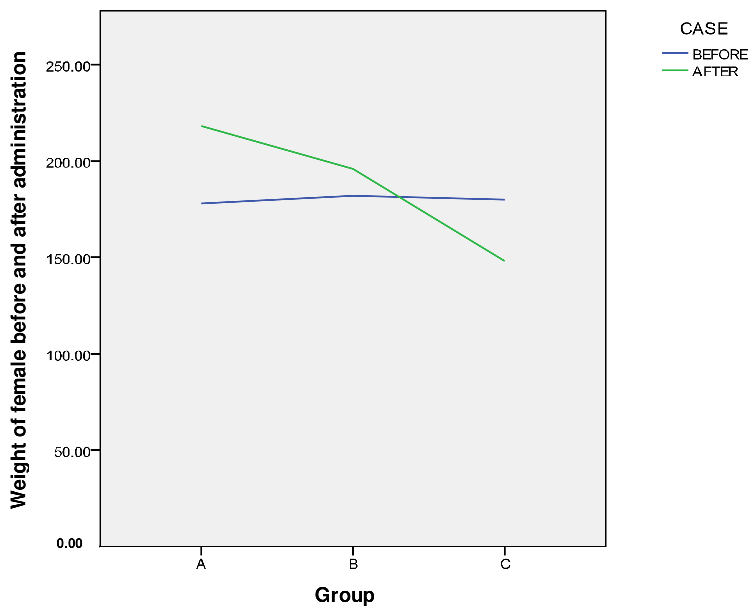

Figure 4. Showing body weights of female rats before and after the study.

or granule that represent the granular endoplasmic reticulum) in the neurons of group C rats. This provides a valuable microscopic assessment of the condition of the cells (Carleton, 1979). Groups A and B cells however shared common normal distribution. It can be inferred invariably from this point that *C. papaya* pulp did not affect the protein synthesis necessary for the visual functions of the rats, while the seed likely does. The hyperchromic nerve cells and axons of group C animals were clearly appreciated in the silver technique of the study by Marsland et al. (1954).

The stereological count (the SC and LGB) of the synaptophysin marked neurons of group C rats gave a reduced value that is statistically significant when compared with both counts of groups A and B. This is a pointer to the fact that *C. papaya* seeds could be neurotoxic.

It was also observed in this study that both *C. papaya* pulp and seeds did not increase adenosine-5'-triphosphate (ATP) production in the visual pathway through

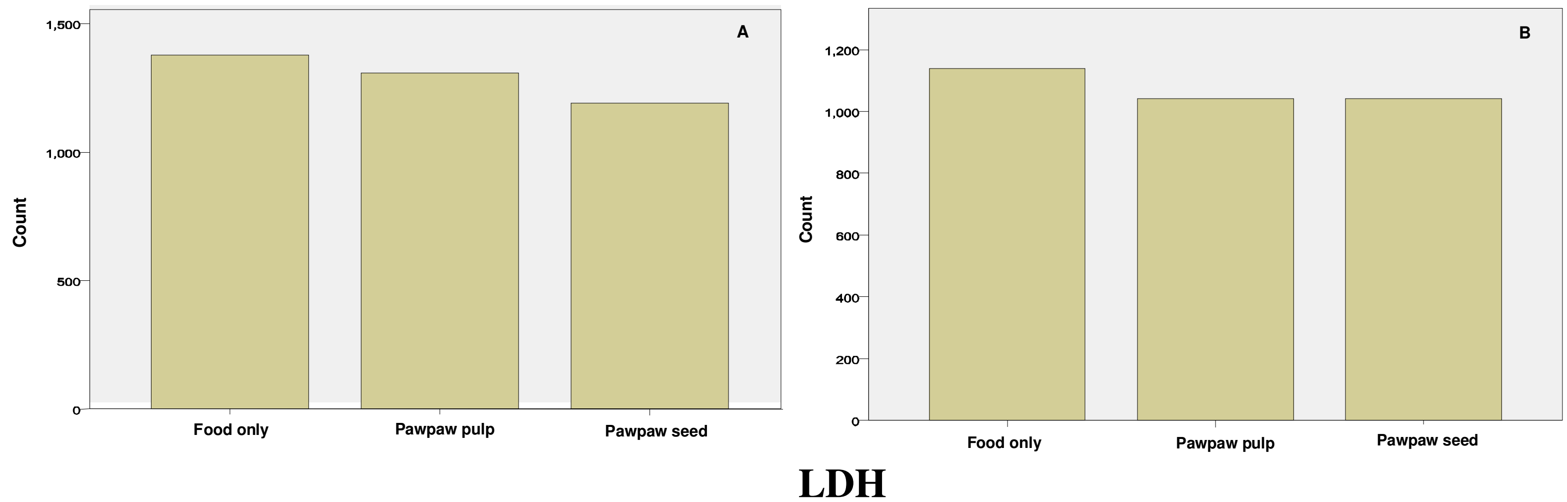

Figure 5. Activities of LDH in the SC and LGB of the experimental rats. A: LDH level of superior colliculus; B: LDH level of LGB.

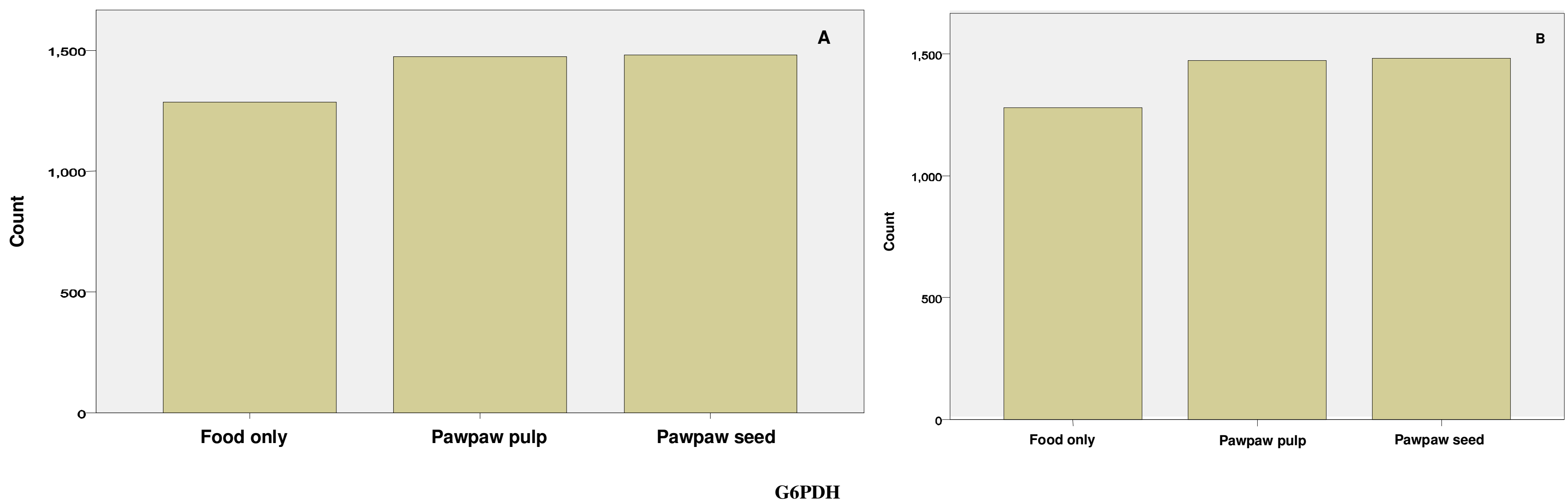

Figure 6. Activities of G6PDH in the SC and LGB of the experimental rats. A: G6PD level of superior colliculus; B: G6PD level of LGB.

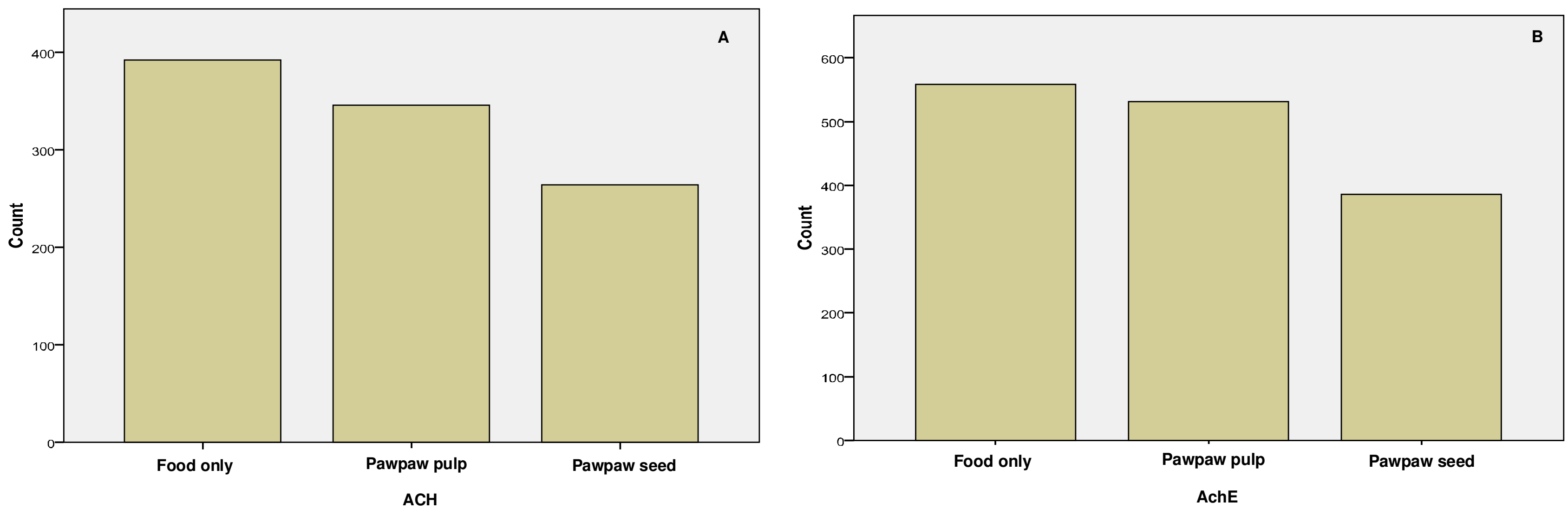

Figure 7. Activities of AchE in the SC and LGB of the experimental rats. A: ACH level of superior colliculus; B: AchE level of LGB.

the Embden Meyerhof's pathway, this is because the LDH levels remained below the normal values taking from the group A (the control group) throughout the experimental period. According to Zhang et al. (2000), LDH is present in almost all tissues of the body and is used to detect tissues alterations. It is responsible for the conversion of pyruvate to lactate and is the key enzyme in anaerobic breakdown of glucose to pyruvate to glycolysis. Because of its wide distribution throughout the body, cellular damage causes an elevation in the total serum and tissue levels of LDH, such that when there is an injury to the tissue, the cells increase in LDH and thus releasing it into the bloodstream, where it is identified in higher than normal value (Pagana, 1998).

G6PD is a cytosolic enzyme in the pentose phosphate pathway, a metabolic pathway that supplies reducing energy to cells by maintaining the level of the co-enzyme NADPH. The NADPH in turn maintains the level of glutathione in these cells that helps protect the cells against oxidative damage (Corpas et al., 1998).

However, it is observed in this investigation that most of the visual energy requirements of the rats are derived through the Hexose monophosphate pathway (HMP); this can be seen from the statistical activities of G6PD, which is consistently higher than that of the control group. The increased levels of G6PD suggest the generation of oxidative stress following administration of the *C. papaya* pulp and seed; this is higher in group C than B. G6PD is the first enzyme in the HMP that produces ribose-5-phosphate (R-5-P) and NADPH. R-5-P is the precursor for nucleotide and nucleic acid synthesis, eventually leading to cell proliferation. It is a cytoplasmic enzyme that plays a protective role during oxidative stress in eukaryotic animals, since they provide co-enzymes and substrates to the primary antioxidant enzymes (Algarwal and Abdelhaq, 2003). By virtue of its ability to produce NADPH along with glutathione reductase, G6PDH is conventionally regarded as a supporter of the antioxidant system (Idietzian et al., 1994). The antioxidant enzymes regulate free radicals by scavenging, repairing, quenching and chain-breaking reactions (Leite and De Barretto, 1998).

There is a gross reduction in the activities of AChE in both groups B and C, but the reduction in group C is the only one that is statistically significant ($P<0.05$). This may indicates that *C. papaya* seeds components may cause visual alteration, because the reduction is obvious for both SC and LGB. According to Preetee et al. (2008), papain, one of the active components of *C. papaya* significantly inhibited the AChE activity in the nervous tissue of *Lymnaea acuminata* at both *in vivo* and *in vitro* exposure.

AChE is an enzyme that degrades the acetylcholine, producing choline neurotransmitter and an acetate group. It is mainly found at neuromuscular junctions and cholinergic nervous system, where its activity serves to terminate synaptic transmission. It has a very high catalytic activity as each molecule of AChE degrades about 25000 molecules of acetylcholine per second. The choline produced by its action is recycled and transported, through reuptake, back into nerve terminals where it is used to synthesize new acetylcholine molecules (Dale et al., 2008).

It is obvious from this research finding that *C. papaya* pulp confers no deleterious effect on the visual pathway of rats treated with the *C. papaya* pulp. However, on the other hand, *C. papaya* seed inhibits the action of AChE and significantly reduced the neural population of the SC and LGB of the treated rats.

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