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Preliminary Assessment of DNA Profile Patterns of Neuronal Differentiation Factor (*NeuroD1*) Gene in the Hippocampus of Wistar Rats (*Rattus norvegicus*) Ingested Hyoscyamine Fraction of *Datura stramonium* Seeds

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ABSTRACT

The study was designed to find out how oral ingestion of hyoscyamine fraction of *D. stramonium* seeds affect the DNA profiles patterns and expression of *NeuroD1* gene in the hippocampus of adult Wistar rats. Fresh *D. stramonium* seeds were procured, identified, macerated and fractionated. Thirty-two (32) male adult Wistar rats weighed 200-250 grams were procured and divided into four groups comprised of control and three treatment groups for the study. The control received an equivalent bodyweight of normal saline, while the treatment groups received 200, 400 and 800 mg/kgbwt of hyoscyamine fraction for three weeks respectively. The animals were sacrificed, tissues harvested for histology, gel electrophoresis and gene expression studies. The fold changes were calculated and DNA profiles were determined using *LabImage* bio-imaging software. One-way ANOVA followed by Fisher's multiple comparisons post-hoc test was used to obtain the difference in the expression of the gene across the groups using *Minitab* 17 (LLC., U.K.) statistical package software. P < 0.05 was considered statistically significant. There was a statistically significant downregulation ($p = 0.001$) in the *NeuroD1* expression, increased DNA fragment size, decreased DNA migration distance and band volume, and necrosis of the dentate gyrus (DG) region in the hippocampus of the treated animals. In conclusion, oral ingestion of hyoscyamine fraction of *D. stramonium* seeds is toxic to the DNA profile patterns and expression of the *NeuroD1* gene in Wistar rats.

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Introduction

NeuroD1 is a basic Helix-Loop-Helix (bHLH) transcription factor. It involves the differentiation of peripheral neurons. It plays an essential role in the maintenance of neural precursor cells in adult neurogenesis: inducible stem cell-specific deletion of *NeuroD1* causes fewer newborn neurons in the hippocampus and the olfactory bulb. *NeuroD1* over-expression promotes neuronal differentiation of neural stem cells in vitro. *NeuroD1*-deficient mice exhibit neuronal cell loss in the inner ear, perturbed amacrine cell differentiation and decrease in the number of bipolar interneurons. *NeuroD1* deficiency decreased the survival of maturing neurons, which may be the consequence of defects in dendrite development and integration into the hippocampal circuitry. Expression of *NeuroD1* in the adult neuronal lineage is both positively regulated by GABA-induced signalling. DNA profiling or fingerprinting is a forensic technique in criminal investigations that compares criminal suspects' profiles to DNA evidence to assess the likelihood of their involvement in the crime. In clinical settings DNA profiling is used to settle paternity dispute, diagnosis of inherited disorders, developing cures for inherited disorders, tender as biological evidence and personal identification [1-11]. Variation in the DNA pattern is caused due to loss in one or more segments (mutation) or difference in individuals genetic-up, such that the gel moves at varying distances. This yield band patterns often described as bar code. *Datura stramonium* (*D. stramonium*) is a widespread annual plant that is known for its medicinal and toxic properties due to the presence of tropane alkaloids, which include atropine, hyoscyamine, and scopolamine. Pieces of literature have implicated tropane alkaloids in alteration of neurogenesis, however, little was reported on the mechanisms of its actions. *Datura* species are abundantly available in a wasteland and nearly

all habitations in Nigeria thereby, making it one of the easiest sources of poisonous intoxicants, dyes and medicine. Although accidental contamination of *D. stramonium* with the cereals, wheat and legumes have been the major sources of toxicity, deliberate consumption by the adolescent youth to provide hallucinogenic sensation is a cause of concern. We aimed to find out how oral ingestion of hyoscyamine fraction of *D. stramonium* seeds affect the DNA profiles patterns and expression of NeuroD1 gene in the hippocampus of adult Wistar rats [12-18]. Our study focused on the DNA fragment size, migration distance and band volume. The study could serve as biomarkers to assess the impact of deliberate consumption of neurotoxic herbs such as *D. stramonium* seeds on the neurogenesis in the living system.

Materials & Methods

We obtained approval from the ethics committee of Ahmadu Bello University Committee on Animal Use and Care (ABUCAUC/2018/042) that is in line with Helsinki Declaration, 1975 as revised in 2000 (http://www.who.int/bulletin/archives/79(4)373.pdf).

Collection of Plant Materials, Extraction & Fractionation

Fresh *D. stramonium* seeds were procured from Sharada residential area of Kano Municipal Local Government, Kano State, Nigeria. The seeds were identified and a voucher number (VN108) was issued at the herbarium of the Botany Department, Faculty of Life Sciences, Ahmadu Bello University, Zaria, Kaduna state, Nigeria. The seeds were separated from the pods, washed thoroughly with clean tap water and air-dried under shade for a week. Two thousand grams of the dried seeds were weighed using a digital weighing machine, grounded to a pulp using an electronic blender. The powdered sample was collected into a sterile cellophane bag and kept in a cool dry place for extraction. The seeds were extracted using cold maceration according to Djilani et al. The 200g of pulverized seeds was extracted in 1, 500 ml of 70% (v/v) ethanol at room temperature for 72 hrs. The extract was filtered and the solvent was evaporated in a water-bath at 40 °C. The residue, dissolved in H_2O and acidified with H_2SO_4 to pH 3-4, was extracted with petroleum ether and diethyl ether to remove lipophilic, acidic and neutral material. After basifying the aqueous solution to pH 9-10 with NH_4OH (25%, m/m), it was extracted with chloroform, the extract washed with distilled water to neutral pH, dried with Na_2SO_4 and concentrated to dryness under reduced pressure to obtain crude alkaloids. The fractionation was carried out according to Salamah and Ningsih method. Five grams of the viscous extract was dissolved in 10 ml of water [19,20]. The solution was then poured into a separating funnel, added with 10 ml of chloroform, and shaken to solve with two phases, namely water and chloroform. These two phases were separated and collected. This was repeated until the chloroform phase had the same colour as the chloroform solvent. The chloroform was then evaporated and recrystallized to obtain the hyoscyamine fraction. The alkaloid was analyzed with UV-Vis spectrophotometric method. The extraction and fractionation were carried out at the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Ahmadu Bello University Zaria.

Animals Procurement, Handling and Acute Toxicity Testing

A total of thirty-two (32) male adult Wistar rats were procured from the Animal House of the Anatomy Department, Faculty of Basic Medical Sciences, Bayero University Kano. The animals were transported by road to the Animal House of the Pharmacology Department, Faculty of Pharmaceutical Sciences, Ahmadu Bello University (ABU) Zaria. The male was separated from the female, housed and allowed to acclimatize for two weeks at ambient temperature, with alternate day and night cycles natural condition. Rat chow (Vital feeds®) and tap water were made available to the animal's *ad libitum*. The median lethal dose (LD_{50}) of hyoscyamine fraction was determined using Lorke's method [21].

Animal Grouping, Mating and Dose Administration

The animals were randomly selected and divided into four (4) groups; control and three treatment groups. The first group received an equivalent bodyweight of normal saline whereas the treatment groups received 200, 400 and 800 mg/kgbwt of hyoscyamine fraction of *D. stramonium* seeds orally as low, medium and high doses respectively for three weeks.

Animal Sacrifice and Dissection

The rats were euthanized using 75% Ketamine (10 mg/ml USP) anaesthesia. The brain tissues were dissected and preserved in Bouin's fluid and Phosphate Buffered Saline (p^H 7.4 PBS) for gel electrophoresis and gene expression analyses. The hippocampus was dissected following Hagihara et al. protocol. The brain tissues were placed in a Petri dish containing ice-cold PBS and cut longitudinally along the fissure of the cerebrum using a surgical knife [22]. The midbrain, hindbrain, and cerebellum were cut off. The diencephalon was carefully removed by placing the cerebral hemisphere medial side upward using forceps, the exposed hippocampus was isolated and removed. The isolated hippocampus was picked up with a needle-tip and placed it in a sample tube that contained RNALater solution, and frozen at -20°C for gel electrophoresis and gene expression analyses.

Primer Design

The primer for the targeted gene, Neuronal differentiation 1 (*NeuroD1*) and house-keeping gene, β – Actin, were designed from the National Centre for Biotechnology Information (NCBI) website and placed for order from the USA, through the DNA labs limited at No. 5, Danja Road, Unguwar Sarki, Kaduna State, Nigeria.

Table 1: Primers for Quantitative Real-Time PCR Analysis

Genes	Forward primer (+)	Reverse primer (-)	Size	Accession Number
**Neuro D1*	CGTGTGCAAG GAATGAATCG	ACGTCAGTTT CACCATTCCC	20	NM_0192118.2
***β-Actin*	GGTGTGATGG TGGGTATGGG	CAGTTGGT GACAATGCCGTG	20	NM_031144.3

Note: *Target gene, **Reference gene.

Quantitative real-time PCR of cDNA

The RT–PCR experiment was performed using a *Lightcycler* 2.0 rapid thermal cycler system (Roche). PCR reactions were set up per sample using 3µL cDNA, 1µmol/L each primer (Table 3.2), 50µL Quantitect SYBR Green PCR Master Mix (Qiagen). The conductions of the PCR machine were; pre - denaturation 1 cycle of 95°C for 10min, denaturation 35–40 cycles of 94°C for 15 sec, Annealing: 45°C for 10sec, 1 cycle of extension, 72°C for 5 minutes. The data generated were analyzed for cycle threshold and fold change using (2–ΔΔCT) Livak and Schmittgen. The resulting amplicons were checked for specificity and size in agarose gels. Beta-actinin was used as endogenous controls (i.e. House Keeping Gene) [23].

Agarose Gel Electrophoresis

The purified DNA extracts together with a marker, 1kb DNA-Marker, was loaded on a non-denaturing 1.5% agarose gel in

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Tris- Buffer (10 mM HCl acid, 1.25 mM EDTA, 100 mM Tris-EDTA, pH 8.5). About 8µl Ethidium bromide was added to each sample before its loading into each gel to mark the migration of these extracts. Gel electrophoresis was carried out at 70 V for around 1 hr using a horizontal mini gel electrophoresis system (VWR, UK). The migrating DNA was observed by staining the gel for about 40 min in the dark in 200 ml TAE buffer and 8µl SYBR Green I. The stained gels were captured by using Gene Snap Version 4.01.00 of Syngene. The migration distance of the DNA molecules from the top of the gel was used as a measure of DNA damage. The gel-image was imported into *LabImage* bio-imaging software (www.kapelandbio.com) and used to estimate the band sizes and Rf values of the DNA-Marker. *Aperio ImageScope* [v12.4.0.5043] (http://www.aperio.com) was used to measure the band migration after taking an average mean of three consecutive measurements from the origin to the centre of each fragmented DNA. A standard curve was plotted using the DNA-Marker fragment sizes and their respective migration distances. The fragment of the sizes of the experimental groups was extrapolated from the curve using their migration distances.

Statistical Analyses

Data obtained were expressed as Mean ± SEM. One-way ANOVA followed by Fischer's exact post-hoc test was carried out to compare the mean differences between the groups for the DNA fragment size, migration distance, band volume and fold changes of the gene expression using *Minitab* 17 (LLC, United Kingdom Inc. www.minitab.com) software. $P < 0.05$ was considered statistically significant. Figures were plotted with Graph Pad Prism 8 (San Diego, California USA, www.graphpad.com) and Microsoft excel sheet.

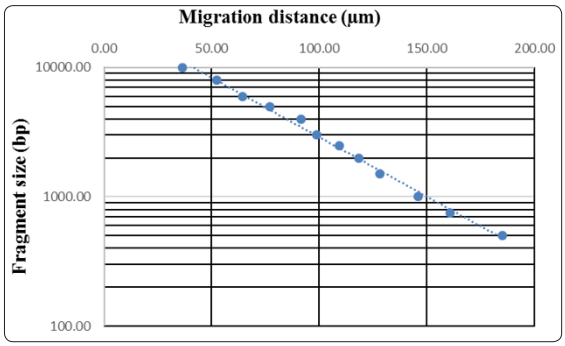

Figure 1: A standard curve DNA-Marker: Fragment size vs. Migration distance

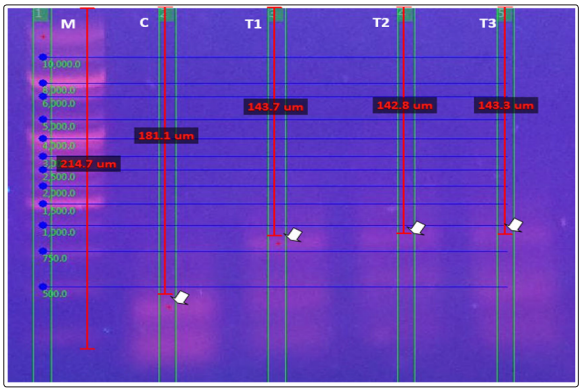

Figure 2: DNA marker (L) and the DNA extracted from the Adult Wistar rats: C: control group: received normal saline, and T1, T2 & T3: received 200, 400 and 800 mg/kgbwt hyoscyamine fraction of *D. stramonium* seeds respectively for three weeks.

Figure 2 shows the gel image of the DNA in the hippocampus of Wistar rats. The DNA size of the control group was 463.00±0.07 bp whereas those of the treated groups were 1031.40±0.05, 1031.30±0.04 and 1031.40±0.05 bps for the least, medium and high dose respectively. There was a gradual disappearance of bands from the medium and high dose treated groups compared to the least and control groups respectively. The mean DNA migration distance covered by the control group was 180.82±0.35 µm, whereas the average mean of the distance migrated by the treated group was 142.84±0.32, 144.48±0.30 and 143.79±0.05 µm for the least, medium and high doses respectively.

Table 2 shows the DNA profiles indices in Wistar rats. There was a statistically significant increase in the DNA fragments [$F(3, 16) = 2.93 \times 10^7$, $p < 0.001$] in the treated groups as compared to the control. There was a statistically significant decrease [$F(3, 16) = 4.32 \times 10^3$, $p < 0.001$] in the DNA migration distance of all the treated groups when compared to the control. A significant increase in the migration distance was however observed between the least and medium doses treated group ($p=0.001$), and also between the least and high doses treated groups ($p=0.032$). The band volume of the treated groups also statistically decreased [$F(3, 16) = 6.12 \times 10^7$, $p < 0.001$] in comparison to the control.

Table 2: DNA Profile Indices in Wistar Rats Treated with Hyoscyamine Fraction of D. stramonium Seeds

Variable	Mean±SEM				*p*-value
	Control	T1	T2	T3	
DNA Fragment (bp)	463.00±0.07*	1031.40±0.05*	1031.30±0.04*	1031.40±0.05*	<0.001
Migration distance (µm)	180.82±0.35*	142.84±0.32*, a, b	144.48±0.30*, a	143.79±0.05*, b	<0.001
Band volume (μm^3)	26223±0.15*	21901±0.25*	21901±0.24*	21901±0.41*	<0.001

T1= 200mg/kgbwt, **T2**= 400 mg/kgbwt, **T3**= 800 mg/kgbwt
*p<0.001, [a]p=0.001, [b]p=0.032,
SEM: Standard Error of Mean

Figure 3 shows comparisons of foldchanges of the neuronal differentiation1 (*NeuroD1*) gene expression in the hippocampus of adult Wistar rats between a control and hyoscyamine fraction of *D. stramonium* seeds treated groups. There was a statistically significant down-regulation [$F(3, 16) = 454.94$, $p < 0.001$] in the expression of the gene between the groups. Although the downregulation showed a partial non-monotonic dose responses fashion, a significant difference in the expression ($p < 0.008$) was equally observed between the treated groups ($p < 0.001$).

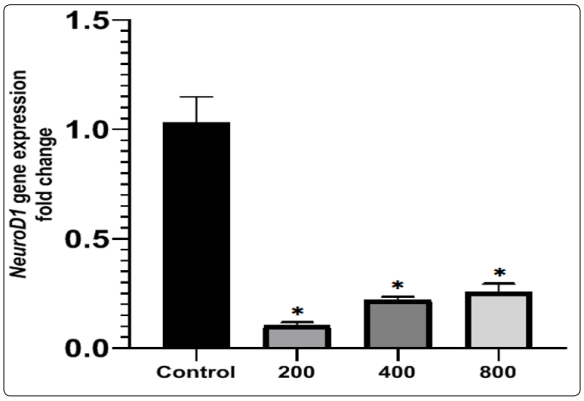

Figure 3: The fold changes of Neuronal differentiation factor 1 (NeuroD1) gene expression

Figure 4 shows photomicrographs of the dentate gyrus (DG) in the hippocampus in the (figure 4a) normal saline, (figure 4b) 200, (figure 4c) 400 and (figure 4d) 800 mg/kgbwt hyoscyamine fraction of *D. stramonium* seeds, as low, medium and high doses of hyoscyamine treated groups respectively. Normal histology of organized granular cell layer (GCL), molecular layer (ML), subgranular zone (SGZ) and polymorphic layer (PM) was seen in the control group compared to the treated groups where the ML and SGZ and GCL regions appeared denser with more hyperchromic granule cells (black arrows) and cytoplasmic vacuolations (black asterisk) in the group that received the least dose when compared to the control group. The medium and high dose exposures showed similar features except for the fact that the granule cells were denser and more hyperchromic (black arrows), and the ML was featured by patches of cytoplasmic vacuolations (black asterisk) when compared to the control group. Also, the high dose exposure (figure 4d) showed for patches of necrotic cells (black arrows) when compared to the control group.

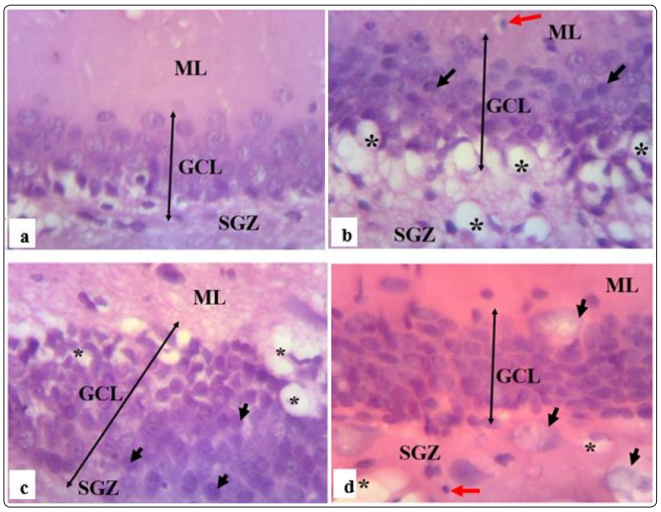

Figure 4: Photomicrographs of dentate gyrus (DG) of the hippocampus in adult Wistar rat at 12th week after being treated with an equivalent bodyweight of normal saline (a), 200 (b), 400 (c) and 800 (d) mg/kgbwt hyoscyamine fraction of *D. stramonium* L. seeds from the PND 56 – 77 (H&E, x400). (H&E, x400). GCL = granular cell layer, ML = molecular layer, SGZ = subgranular zone, ➞= hyperchromic granule cells, * = cytoplasmic vacuolation, ➧ = oligodendrocyte, ➡ = necrotic granule cells

Discussion

In this study, we aimed to provide baseline information regarding oral ingestion of hyoscyamine fraction *D. stramonium* seeds on the expression of *NeuroD1* gene and its DNA profiles based on the fact that tropane alkaloids alter neurogenesis, and that a few works of literature were available to that effect [15,16]. We, therefore, focused on DNA fragments, band length or migration distance and band volume as DNA profile indices. We observed a significant increase in the DNA fragments size among all the treated groups. This has suggested lesions in the hippocampus tissues analyzed caused by the ingestion of the hyoscyamine fraction of the seeds. *D. stramonium* seeds extract was reported to cause atrophy of the axons and fibres, cytoplasmic vacuolation, cell necrosis as well as cell losses in the pyramidal cells of the hippocampus. Necrosis is a form of cell death that destroys many contiguous parenchymal cells and may involve an entire tissue or organ. Studies have shown that apoptotic cells as well as necrotic cells produce circulating nucleic acids (cNA) that vary in fragment lengths and sizes. The DNA migration distance, however, decreased significantly in the treated groups. This may not be a surprise perhaps, as an increase in the fragment size earlier observed might indirectly add to the weight of the DNA through the circulating nucleic acids (cNA) of the apoptotic cells which in turn decreased the migration speed. DNA crosslinking occurs when exogenous or endogenous agents react with one or two nucleotides of DNA, forming a covalent linkage between them. DNA damage due to increased cross-linking proportionally reduces electrophoretic DNA migration. Other factors probably responsible for the delay in speed migration include; the DNA size, the voltage used, the ionic strength of the buffer, and the concentration of intercalating ethidium bromide used. DNA band volume is one of the indices used to assess estimate the amounts of DNA in gel electrophoresis [24-30]. The decrease in band volume observed may suggest a loss of DNA materials of the *NeuroD1* gene in the sample, probably due to damage caused by the cell losses on the pyramidal cells of the hippocampus [24].

The brain ability to generate new neurons provides it with plasticity for maintaining cellular homeostasis and potentials to respond to injury. The proneural basic helix-loop-helix (bHLH) transcription factors are essential for embryonic neurogenesis. The neuronal factor 1 (Neurod1), also known as *NeuroD or Beta2*, is predominantly expressed in the nervous system late in development and is, therefore, more likely to be involved in terminal differentiation, neuronal maturation and survival. Hyoscyamine fraction caused significant downregulation of the *NeuroD1* gene expression surprisingly in a partial non-monotonic dose-response manner in all group when groups when compared to control [31-35]. This has suggested that hyoscyamine fraction has impaired the expression of the gene by probably restricting the development of or altering the newborn glial and neuronal cells, which might lead to a reduction in neuroblasts and immature neurons formation. Hyoscyamine and atropine are racemic isomers with similar modes of action. Hyoscyamine is unstable and is racemized rapidly to atropine. [36] Tropane alkaloids have been shown to alter neurogenesis, because of its affinity for rapidly dividing cells, hence, being used in China as alternative chemotherapy [15,16,37-43]. The partial non-monotonic patterns observed could be attributable to the presence of so many factors such as the presence of phenolic compounds, heavy metals and ionic liquids either as an individual or as mixtures probably associated with the fraction. It is suggestive that the hyoscyamine fraction could have also exerted similar influence in the expression of the *NeuroD1* gene [44-46].

The hippocampal dentate gyrus (DG) is critical to neuronal plasticity with relevance to memory, anxiety disorders, and depression. Manipulations targeted specifically to DG impair memory acquisition, modulate anxiety levels, and block some effects of antidepressant drugs in clinically relevant models, leading to speculation that DG is a promising target for therapeutic manipulations. Cellular hyperchromasia, cytoplasmic vacuolations and necrosis were observed in the treated groups. This is indicative that hyoscyamine fraction is toxic to the hippocampus and impaired the expression of *NeuroD1* gene [47-56].

Conclusion

The study established that oral ingestion of hyoscyamine fraction of *D. stramonium* seeds causes increase DNA fragment size, decrease DNA migration distance and decrease DNA band volume; which could lead to the downregulation *NeuroD1* gene. We, therefore, recommend that caution should be exercised while using hyoscyamine as conventional drug or *D. stramonium* seeds in traditional herbal medicines. Deliberate consumption of the seeds should also be avoided as it could lead to neurodegenerative

diseases such as Alzheimer's and Parkinson's diseases.

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