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Sub-chronic toxicological evaluation of genetically modified *AVP1* sugarcane in albino rabbits

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Abstract

Genetically modified crops require biosafety evaluation for approval from the regulatory competent authorities prior to commercialization. Genetically modified (GM) sugarcane expressing the *Arabidopsis* Vacuolar H^{+-} pyrophosphatase gene (*AVP1*) which confers drought tolerance has been developed at NIBGE. The risks associated with GM sugarcane containing the *AVP1* gene were evaluated in a sub-chronic feeding study on rabbits. Various parameters including body weight, food consumption, serum biochemistry, haematology, absolute and relative organ weight were compared between rabbits fed on GM and non-GM sugarcane after consumption for 90-day. In addition, genotoxicity was conducted among treatment groups and mutagenic potential was determined with AVP1 purified protein in an Ames assay. No adverse effects related to GM and normal diet were detected. The results obtained did not reveal any statistically significant differences in the micronuclei frequency or any DNA damage in rabbit's peripheral blood cells. Furthermore, AVP1 purified protein showed no mutagenic activity at the concentration of 512µg/plate. This is the first report on biosafety of *AVP1* sugarcane in Pakistan and scientific data generated through this research will be helpful for the commercial release of *AVP1* GM sugarcane.

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Introduction

Sugarcane is an essential crop in the production of sugar for further usage in food and beverages (Kinkema *et al.*, 2014). Pakistan shares 56.8 million tons from 1.04 million ha with a yield of 55.8 ton/ha out of the worldwide production of 1.83 billion ton of stalk from 26.1 million ha with an average yield of 71 ton/ha (Ghumman, 2017). In Pakistan, salinity and drought stress are the major constraints affecting the lower yield of sugarcane (Raza *et al.*, 2016). Water deficiency may limit the sugarcane production and yield up to 50% or more throughout the world (Venkataramana *et al.,* 1986).

Biotechnology has a great potential to resolve such problems through recombinant DNA technology to incorporate the specific genes that can overcome the drought and salinity stress (Singh *et al.*, 2013). The global hectarage of biotech crops has increased more than 110-fold from 1.7 to 185.1 million ha in last two decades *i.e.* 1996 to 2016, this makes biotech crops the fastest adopted crop technology in recent times (James, 2016). Plants with abiotic stress tolerance have been developed by introducing stress tolerant genes (James, 2015). In the era of the developing world, approximately onebillion people suffer from poverty and hunger. On the other hand, a constant increase in the population rate declined the agricultural land area that seems to worsen these issues. Biotech crops with novel traits produce high yield quality and better adaptability. GM crops are more specific and fast in development as compared to conventionally bred lines (Lucht, 2015).

The *Arabidopsis* vacuolar proton-pump pyrophosphatase (*AVP1)* gene exhibits more tolerance to drought and salinity. The transgenic sugarcane expressing *AVP1* gene has been developed at National Institute for Biotechnology and Genetic Engineering (NIBGE) by Raza *et al.*, 2016. The genetically modified plants are strictly regulated and there is need for biosafety and risk assessment to be performed by an appropriate competent authority before proceeding to commercial release (Craig *et al.*, 2008; Domingo, 2016; Smart *et al.*, 2017; Delaney *et al.*, 2017).

Methods have been documented to identify the risks and adverse effects associated with GM crops (EFSA, 2008). Sub-chronic (90-day) rodent feeding studies of genetically modified crops have been recommended to determine the harmful effects on animals' health. (FAO/WHO, 2003; WHO, 1995). Various genetically altered crops have been assessed including tomato (Noteborn *et al.*, 1995), soybean (Appenzeller *et al.*, 2008), rice (Schrøder *et al.*, 2007), maize (Arjó *et al.*, 2012), canola (Delaney *et al.*, 2014) and cotton (Dryzga *et al.*, 2007). At the end of 90-day animal feeding trials with GM food, biochemical analyses are performed as reported by Séralini *et al.*, 2007 (maize); He *et al.*, 2008 (maize) and Delaney *et al.*, 2014 (canola).

The current biosafety assessment studies address the toxicity/genotoxicity evaluation which is directly related to the genetic modification and potential unintended adverse effects associated with GM *AVP1* sugarcane. The risk assessment studies have been carried out prior to commercialization.

Genotoxicity assays have been conducted to identify DNA reactive molecular compounds (Maluszynska and Juchimiuk, 2005) through both *in vitro* and *in vivo* studies. These assays are designed to recognize those substances that cause mutation, DNA damage and chromosomal aberrations (Auffan *et al.*, 2006; Colognato *et al.*, 2008 and Fenech, 2008). These assays have also been performed for biosafety evaluation studies of GM crops. (The comet assay (single-cell gel electrophoresis) is a technique for the evaluation of direct DNA damage by the exposure of any chemical compound, radiation or recombinant protein in any eukaryotic organism. Jaszczak *et al.* (2008) performed a micronucleus test and comet assay in mice fed on GM triticale (bar transgene). The *in vitro* reverse mutation (amino-acid requiring) detection Ames test is most widely used to detect a mutation in the bacterial strains of *Salmonella typhimurium* that is related to the frameshift mutations of a single or a couple of DNA base pairs (Ames *et al.*, 1975). The micronucleus test is another tool for genotoxicity assessment of a potentially toxic chemical or a compound (carcinogen) caused by genetic damage both *in vivo* and *in vitro* (OECD, 2010).

The micronucleus test is applicable to various cell types such as peripheral blood, bone marrow and epithelial cells (Heddle, 1990, Konopacka *et al.* 1998 and Krishna *et al.* 1991).

Biotech crops with novel traits are being approved for commercialization that would be beneficial to the consumers and the farmer. The biotech crops are used as food such as golden rice (beta-carotene enriched) tested for field trials in Bangladesh and Philippines; banana (bunchy top virus resistant) in Uganda; banana (*Fusarium* wilt resistant) and wheat (drought tolerant, disease resistance, grain composition and modified oil content) in Australia; wheat (high yield and biomass) in UK; potato (late blight resistant var. Desiree and Victoria) in Uganda and potato (late blight and nematode resistant var. Maris Piper with less bruising and less acrylamide) in the EU; chickpea, pigeon pea and mustard (insect resistant) in India; sugarcane (drought tolerant) in India and Indonesia and camelina (omega-3 enriched) in the EU (ISAAA, 2015). To date, the first GM (drought tolerant) sugarcane was commercialized by Product Biosafety Commission KKHPRG Indonesia (James, 2013). Recently, the second GM (Bt, insect resistant) sugarcane is released for commercial purpose by CTNBio National Biosafety Technical Commission Brazil (ISAAA, 2017).

According to a survey report based on 147 agronomical studies, the benefits of GM crops to the farmers has been increased up to 68% and crop yield has risen by 22% and these profits were higher for the developing countries compared to the developed countries. Regardless of the high seed cost for GM varieties, the farmers get extensive profits and that was the main reason for selecting GM crops over conventional ones (Klümper and Quaim, 2014). The adoption of biotech crops in the USA has reached more than 95% of GM food for animal consumption. There is the need to get more information and knowledge about biotech crop and biosafety evaluations for the benefit of the consumer and the farmer which makes this study more appreciated (Lucht, 2015).

The present research work evaluates the potential risks of GM *AVP1* sugarcane by using various approaches and the protocol and design of experiments were approved and reviewed by Institutional Biosafety Committee (IBC, NIBGE) and National Biosafety Committee (NBC, Pakistan).

A sub-chronic toxicological evaluation was conducted via feeding trial of albino rabbits using leaves of *AVP1* GM and non-GM sugarcane and a control with standard rabbit feed only. Genotoxicity evaluations based on the AVP1 purified protein through the Ames test (mutagenicity), the comet assay (DNA damage) and the micronucleus test (cytotoxicity). The biosafety assessment data generated through the current research work will be helpful for the commercialization of *AVP1* GM sugarcane.

Materials and methods

Purification of Transgenic AVP1 and non-Transgenic Leaf Protein

Purification of transgenic (*AVP1*) and non-transgenic sugarcane leaf protein was performed according to the methods described by (Gaxiola *et al.*, 2001; Pasapula *et al.*, 2011) from the selected transgenic (Trans 1 and 4) and non-transgenic sugarcane cultivars. Total soluble protein (TSP) was determined with BSA using Bradford (1976) standard method. The concentration of *AVP1* protein was 0.7 mg/1g leaf tissue by using spectrophotometer at optical density 595 nm. Protein samples of 50 µg were loaded onto each well on the SDS-PAGE gel. Furthermore, immunodetection was performed by using chromogenic Western hybridization immunodetection kit (Invitrogen, USA). The specific AVP1 protein from GM sugarcane leaf was quantified (0.2 µg/uL) with the help of ImageJ software (Raza *et al.*, 2016).

Expression and Purification of Recombinant AVP1 Protein

The bacterial vector pET-32 was used for transformation (*AVP1* clone) and *E. coli* cells strain BL21 (DE3) were used as an expression host. The bacterial strain contains a lacUV5 promoter that controls the T7 RNA polymerase gene which is transformed with pET-32.

The purification (McCleary and Harrington, 1988) of the expressed protein was performed by using Ni-NTA agarose column in accordance with the manufacturer's instruction (His-bind protein purification kit (Novagen, cat# 70239-3). The quality of AVP1 purified protein was tested on 10% SDS-Page gel and quantification was performed through Bradford (Bradford, 1976; Kiaie *et al.*, 2012).

Quantification of AVP1 Gene Transcript

For quantitative relative expression of the *AVP1* GM sugarcane lines (trans 1 and trans 4), total RNA was used to synthesize cDNA and amplified with cDNA specific primers in reverse transcription PCR (RT-PCR). RNA was extracted by using a plant RNA purification reagent (Invitrogen USA) and cDNA was synthesized by using Revert Aid cDNA synthesis kit (Fermentas, USA) and confirmed by qPCR. Sequence information (Accession No. M81892) from the NCBI gene bank was used to design the quantitative primers and probe (Table 1). Syber Green® (Sigma-Aldrich) reaction mixture was used for real-time quantitative PCR (RT-qPCR) which contained 7µL Syber Green PCR master mixture and 5µL of diluted cDNA in a final volume of 12µL. The internal control gene was 18S rRNA (Allmann *et al.*, 1993; Köppel *et al.*, 1998). Samples were placed in an automated fluorometer (Multicolour Real-Time PCR Detection System, iQ5 Bio-Rad). The primers sequences are given in Table 1.

Table 1. Primers sequences.

Gene	Primer Description 5' to 3'	Reference
*AVP*1 gene-F	GACGAATTCGTGGCG CCTGCTTTGTTA	Designed and used
*AVP*1 gene-R	GACGCGGCCGCTTAG AAGTACTTGAAAAGG	Designed and used
*AVP*1 cDNA-F	CTTTGTTTTCCTCGGC TCTG	Designed and used
AVP1 cDNA-R	CAATAGACCACTCGC TGCAA	Designed and used
18 S rRNA-F	TCTGCCCTATCAACTT TCGATGGTA	Köppel *et al.*, 1998)
18 S rRNA-R	AATTTGCGCGCCTGC TGCCTTCCTT	Allmann *et al.*, 1993

Housing and Grouping of Laboratory Animals

Albino rabbits approximately 5-7 months of age (from National Institute for Health) were assigned to Five groups following randomization based on body weight. Animals were housed in the animal house (22±3°C with 12h light and dark and relative humidity of 50-60%) at NIBGE. Animals were acclimatized for 10 days with normal rabbit diet and water *ad libitum* prior to starting the treatments. The experimental design was completely randomized with 5 animals per treatment.

Dosing of Experimental Animals

During the test period, animals were fed on highly expressed transgenic (trans 4) and non-transgenic sugarcane leaves with different combinations of the normal diet for five treatment groups (Table 2). *AVP1* (Trans 4) and non-transgenic sugarcane cultivar CSSG-668 (Shakarganj Sugarcane Research Institute (SSRI), Jhang, Pakistan) were grown in the NIBGE field. Fresh leaves of both transgenic and non-transgenic sugarcane were harvested daily and cut down into small pieces and mixed with normal diet for dosing in five different treatment groups (Table 2). The normal feed was a nutritionally balanced and modified diet for rabbits as standard laboratory chow PMI® Nutrition International, LLC Certified Rabbit Lab-Diet®5322 (PMI® 5322). The nutritional evaluation of diets was performed for all dosed groups of rabbits (Table 3). The percentage values were calculated in different laboratories of NIBGE and Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad, Pakistan.

Table 2. Diet description of *AVP1* GM sugarcane non-GM, sugarcane and normal feed.

Treatment Groups	Animals	Dose Description
G1 (40% GM sugarcane)	5	80 g leaves +120 g normal feed
G2 (40% non-GM sugarcane)	5	80 g leaves +120 g normal feed
G3 (20% GM sugarcane)	5	30 g leaves +120 g normal feed
G4 (20% non-GM sugarcane)	5	30 g leaves +120 g normal feed
G5 (normal feed only)	5	120 g normal feed

Table 3. Nutritional composition of diets among different dosed groups.

Groups	G1	G2	G3	G4	G5
Minerals					
Na (%)	0.20	0.20	0.21	0.21	0.25
K (%)	2.2	2.1	2.1	2.0	2.5
Ca (%)	1.0	1.0	1.1	1.1	1.2
Mg (%)	0.25	0.25	0.24	0.27	0.3
Iron (%)	0.03	0.03	0.03	0.03	0.04
Zinc (%)	0.00	0.00	0.00	0.00	0.01
Chromium (%)	0.01	0.00	0.00	0.00	0.00
Lead (%)	0.00	0.00	0.00	0.00	0.001
Copper (%)	0.00	0.00	0.00	0.00	0.001
Manganese (%)	0.01	0.01	0.02	0.02	0.02
Moisture content (%)	9.8	9.8	9.9	9.9	10
Ash (%)	6	6	6	6	6.5
Crude fibre (%)	22	21.9	22.1	22.1	22
Crude fat (%)	2.0	2.1	2.3	2.3	2.9
Carbohydrates (%)	46.9	46.9	46.2	46	44.9
Fatty Acids					
Cholesterol, ppm	7	7.2	8	8	9
Arachidonic acid (%)	0.00	0.00	0.00	0.00	0.01
Omega-3 fatty acids (%)	0.41	0.41	0.43	0.42	0.44
Linoleic acid (%)	0.67	0.66	0.65	0.66	0.69
Linolenic acid (%)	0.27	0.27	0.26	0.27	0.28
Total fatty acids (%)	0.34	0.34	0.34	0.34	0.36
Protein (%)	13.3	13.3	13.5	13.7	13.7
Arginine	0.61	0.60	0.62	0.62	0.64
Glycine	0.52	0.52	0.53	0.52	0.56
Histidine	0.31	0.30	0.3	0.3	0.35
Cystine	0.2	0.22	0.21	0.21	0.24
Lysine	0.67	0.68	0.66	0.69	0.71
Leucine	1.0	1.0	1.0	1.0	1.01
Isoleucine	0.7	0.72	0.71	0.7	0.77
Tyrosine	0.37	0.36	0.33	0.35	0.4
Methionine	0.3	0.3	0.29	0.28	0.3
Phenylalanine	0.57	0.55	0.58	0.60	0.63
Threonine	0.50	0.52	0.50	0.50	0.52
Tryptophan	0.1	0.1	0.99	0.90	0.13
Aspartic acid	1.44	1.45	1.48	1.49	1.51
Valine	0.59	0.58	0.59	0.6	0.67
Serine	0.6	0.6	0.62	0.62	0.66
Proline	0.77	0.79	0.80	0.81	0.87
Alanine	0.67	0.69	0.70	0.73	0.75
Glutamic acid	1.80	1.81	1.88	1.90	2.0

Clinical Observations, Necropsy and Gross Pathology

Animals were observed daily to note any change in behavioural or autonomic activities (irritation, urination, salivation corneal reflex, spontaneous responses) and mortality. Body weight was recorded daily for 15 days then measured on a weekly basis for all animals in the treatment groups. The animals were euthanized at the end of the experiment and different organs included heart, lungs, liver, spleen, kidney, gonads and adrenal glands were carefully dissected out and weights were recorded and relative organ weight (%) to body weights were calculated.

Blood Haematology and Biochemistry

At the end of the experiment, blood samples were collected in vacutainers (EDTA and Heparin coated) for their biochemical and haematology analysis. Biochemical tests such as Blood Sugar, Blood Urea, Serum Creatinine, Serum Uric Acid, Cholesterol, Triglycerides, HDL Cholesterol, LDL Cholesterol, Total Bilirubin, Direct Bilirubin, Indirect Bilirubin, Alanine Aminotransferase (ALT or SGPT), Aspartate Aminotransferase (AST or SGOT), Alkaline Phosphatase, Serum Proteins, Serum Albumin, Serum Globulin, and Albumin to Globulin ratio (A/G ratio) were performed. In Haematology, various parameters were studied such as Haemoglobin, Erythrocyte Sedimentation Rate (ESR), Total Lymphocyte count (TLC), Differential Leukocyte count (DLC), Neutrophils, Lymphocytes, Monocytes, Eosinophils. These analyses were done by a clinical pathology Laboratory (PINUM, ISO 9001-2008 certified hospital, Faisalabad).

Genotoxicity and Mutagenicity Assessment

Genotoxicity was detected by performing the comet assay and the micronucleus test *in vitro* based mutagenicity was observed by the Ames test.

Alkaline Single Cell Gel Electrophoresis (Comet Assay)

After 90-day feeding trial, the comet assay (peripheral blood cells of rabbits) was performed with some modifications from previously used protocols (Singh *et al.*, 1988; Klaude *et al.*, 1996; (Collins, 2002; Klaude *et al.*, 1996) to detect DNA damage. Methyl Methane Sulphonate (MMS) was used as a positive control. Peripheral blood mononuclear cells (PBMC) were resuspended in PBS at a concentration of 1×10^7 cells/mL and 20µL of the suspension was mixed with 180 µL of low melting point agarose (0.5%). Two drops (100 µL) from the mixture were spread on each of the duplicated spots on frosted microscopic slides that were pre-coated with normal melting point agarose (0.6%).

The cell-gel layer (three layered) was covered with a cover slip and the gel was allowed to solidify for 10 min at 4°C. The cover slips were removed and slides were immersed in the cold fresh lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris pH 10 with 10 % DMSO and 1 % Triton X-100 added 30 min before lysing) for at least 1h at 4°C in the dark. The slides were then submerged in fresh cold alkaline electrophoresis buffer (300mM NaOH, 1 mM Na_2 EDTA, pH > 13) in a horizontal gel electrophoresis unit and left in the solution at 4°C for 20min for salt equilibrium and DNA unwinding. Electrophoresis (Brunborg, 2008) was carried out at 24-25 V (~ 0.73 V/cm), 300 mA for 25 min at 4°C under dark conditions. To remove excess alkali the slides were neutralized three times with 0.4 M Tris buffer (pH 7.5) and finally were stained with 20 µL of ethidium bromide (20 µg/mL). Then slides were observed under an epifluorescent microscope (Labomed Lx400, Labo America, Inc USA) equipped with an excitation filter 515-560 nm and emission filter 590 nm. A total of 100 individual peripheral blood cells were observed and the total score was counted visually according to the number of cells classified in five classes ranged from 0 to 4. The visual scoring was performed according to Collins *et al*. (2002). The arbitrary units (AUT) were calculated as AUT = N0 × 0 + N1 × 1 + N2 × 2 + N3 × 3 + N4 × 4

Where N is the number of nuclei scored in each category (Collins, 2002).

Micronucleus Assay

The *in vivo* micronucleus assay was performed from peripheral blood of rabbits after 90-day of feeding study. The blood samples were collected from both male and female animals then the slides were immediately prepared from blood smears and stained according to Jaszczak *et al.*, 2008. A total of about 1,000 normochromatic erythrocytes (NCE) and polychromatic erythrocytes (PCE) were observed for each animal under the light microscope (Labomed Lx400, Labo America, Inc USA, oil immersion lens, 100/1.25).

Mutagenicity Determination Test (Ames Test)

The standard plate incorporation method was used to check mutagenicity potential of AVP1 protein (Maron and Ames, 1983).

The recombinant AVP1 protein was tested in the Ames test with the most widely used strains of *Salmonella typhimurium* TA100 and TA98 with and without rat liver (Sprague-Dawley) S9 mixture (Sigma-Aldrich from St. Louis, MO, USA). The reversion from histidine (obligatory for growth) auxotrophy to prototrophy is the basic principle of the Ames test for the evaluation of mutagens/carcinogens. The optical density (OD) of test strains was 0.7-0.8 (containing approximately 3×10^7cells/mL) of 100µL of overnight grown cultures. Autoclaved glass tubes with 2mL top agar (0.5% agar, 0.5% NaCl in distilled water) were placed in a water bath at 45°C. Then histidine/biotin solution (0.5mM) and S9 mix (10% v/v S9) 500 µL were mixed and poured onto Vogel Bonner (VB) minimal medium plates for S9+ and S9- without S9 mixture. Then AVP1 protein various concentration such as 8, 16, 32, 64, 128, 256 and 512 µg/plate were used (with and without S9 mixture) and incubated for 48 to 72 h at 37°C. Plates for negative control with autoclaved distilled water were prepared. The positive control plates were prepared without S9 mixture. Potassium dichromate ($K_2Cr_2O_7$) for TA 98 (25 µg/plate) and the same concentration of sodium azide (NaN_3) for TA 100 and with S9 (2- Aminoanthracene, 2µg/plate) was used for both strains. Test results were compared with negative control (spontaneous mutation) plates. The test compound was considered mutagenic when the number of the revertant colonies increased by two over the negative control plates (Ames, 1975). Mutagenicity expressed as the mutagenic index (MI). A sample is considered mutagenic when MI is ≥ 2 for TA 98 and TA 100 strains. The MI was calculated by using the following formula!

$$\text{Mutagenic Index (MI)} = \frac{\text{No. of revertant colonies in test sample plate}}{\text{No. of revertant colonies in the negative control plate}}$$

If the mutagenic index (MI) of the test substance is less than 2 with TA 98 and TA 100 then the test material is considered to be non-mutagenic and vice versa.

Statistical Analyses

Statistical analyses were performed with SigmaPlot 13.0 (SYSTAT software). Data were analysed using ANOVA, to account for the differences in data distribution and variance. Pairwise comparison of mean values was made using (Tukey, 1993) test.

Results

Quantification of AVP1 Gene Transcription

The presence of the *AVP1* gene in GM sugarcane lines and absence in non-transgenic sugarcane (control) was detected by standard reverse transcription PCR (Fig. 1). The expression analysis of the *AVP1* gene in the transgenic sugarcane lines was confirmed by RT-qPCR analysis (Fig. 2).

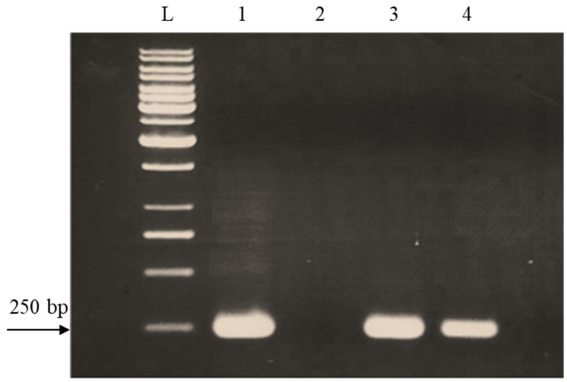

Fig. 1. Reverse transcription PCR (RT-PCR) for GM *AVP1* sugarcane cDNA with gene-specific primers. L for DNA ladder (1kb) Fermentas, lane (1): positive control (PC) with *AVP1* gene plasmid DNA; lane (2): negative control (NC) with water and lanes (3 and 4): *AVP1* GM sugarcane lines Trans 1 and Trans 4 respectively.

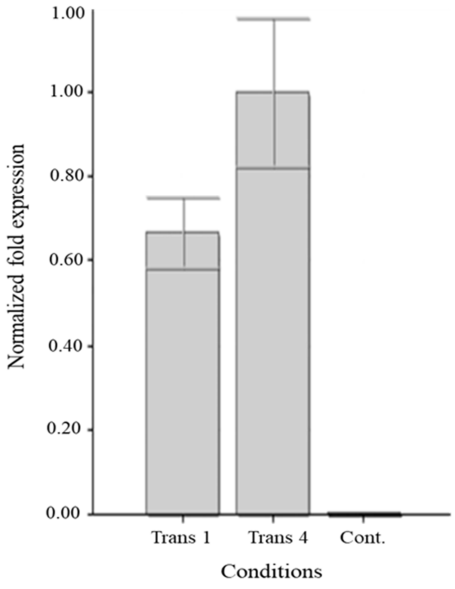

Fig. 2. RT-qPCR amplification of *AVP1* transgenic and non-transgenic sugarcane lines. Trans 1 and Trans 4 represent *AVP1* GM sugarcane lines fold expression and Cont. for control with non-GM sugarcane line.

Body Weight and Food Consumption Analysis

In the 90-day sub-chronic study, the body weight of rabbits significantly (P<0.05) increased with time in the animals of all treatment groups (Fig. 3).

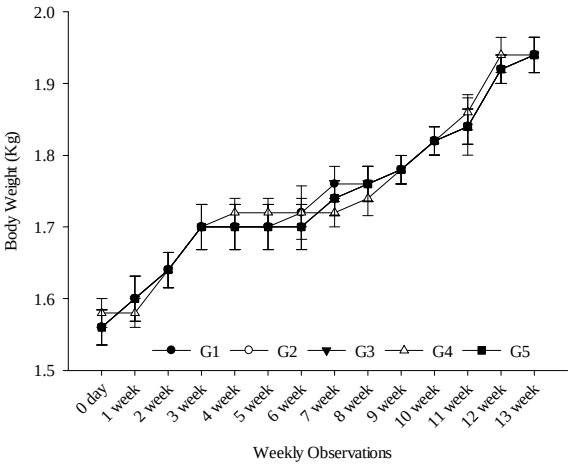

Fig. 3. Rabbits weekly body weight (Kg). Data represent weekly body weight (mean±SD) no significant differences at (P≤0.05) among the treatment groups. (G1) and (G2): 40% *AVP1* GM and 40% non-GM sugarcane fed group respectively; (G3) and (G4): 20% *AVP1* GM sugarcane and 20% non-GM sugarcane fed group respectively; (G5): normal diet fed group.

The average food consumption was 35-50g for G1 and G2 while it was 20-65 g for G3 and G4 and 85g for G5 dosed groups (Table 4). It was approximately equal to the aggregate food consumption (per week and after 90-day) as presented in Fig. 4. Weekly food consumption (Fig. 4) was recorded with non-significant differences among the treatment groups (P>0.05).

Table 4. Food consumption of *AVP1* GM sugarcane no-GM, sugarcane and normal feed.

Treatment Groups	Animals	Dose Description
G1 (40% GM sugarcane)	5	35 g leaves +50 g normal feed
G2 (40% non-GM sugarcane)	5	35 g leaves +50 g normal feed
G3 (20% GM sugarcane)	5	20 g leaves +65 g normal feed
G4 (20% non-GM sugarcane)	5	20 g leaves +65 g normal feed
G5 (normal feed only)	5	85 g

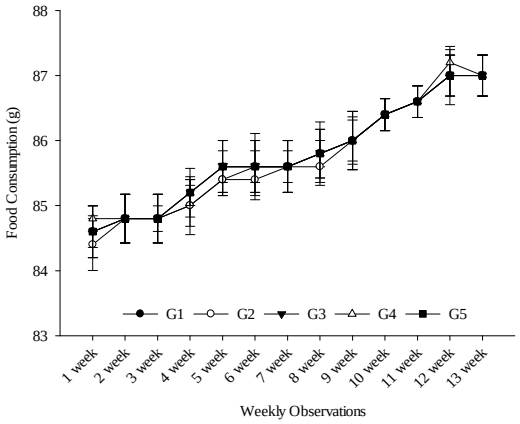

Fig. 4. Rabbits weekly food consumption (g). Data represent weekly food consumption (mean±SD) no significant differences at (P≤0.05) among the treatment groups. (G1) and (G2): 40% *AVP1* GM and 40% non-GM sugarcane fed group respectively; (G3) and (G4): 20% *AVP1* GM sugarcane and 20% non-GM sugarcane fed group respectively;(G5): normal diet fed group.

There were no physical toxic signs or symptoms recorded in terms of clinical or behavioural or necropsy observations after 90-day sub-chronic toxicity studies among all the treatment groups (data not shown). There were no significant differences (P>0.05) between GM *AVP1* sugarcane fed group and control (normal diet) observed in terms of body relative to weight organs weight such as heart, lungs, liver, spleen, kidney, gonads and adrenal glands weight (%) after 90-day feeding studies (Table 5). No anomalies were observed among all the treatment groups.

Table 5. Relative organ weight to body weight (%) of rabbits after 90-days sub-chronic study of *AVP1* GM sugarcane, non-GM sugarcane and normal feed groups.

Organs	G1	G2	G3	G4	G5
Heart	0.21± 0.02^a	0.21± 0.01^a	0.20± 0.03^a	0.21± 0.02^a	0.20± 0.04^a
Lungs	0.39± 0.04^a	0.39± 0.02^a	0.39± 0.05^a	0.38± 0.05^a	0.39± 0.06^a
Liver	3.39± 0.13^a	3.44± 0.28^a	3.35± 0.18^a	3.42± 0.17^a	3.39± 0.25^a
Spleen	0.03± 0.01^a	0.03± 0.00^a	0.03± 0.01^a	0.02± 0.01^a	0.02± 0.0^a
Kidney	0.60± 0.08^a	0.62± 0.07^a	0.60± 0.11^a	0.63± 0.09^a	0.66± 0.05^a
Male gonads	0.26± 0.01^a	0.25± 0.05^a	0.26± 0.03^a	0.26± 0.05^a	0.26± 0.02^a
Female gonads	0.02± 0.00^a	0.02± 0.00^a	0.02± 0.00^a	0.02± 0.00^a	0.02± 0.00^a
Adrenal	0.02± 0.00^a	0.01± 0.00^a	0.02± 0.01^a	0.02± 0.00^a	0.02± 0.00^a

Different organs weight relative to body weight (%) values (mean±SD) at the significance level (P≤0.05) among all the treatment groups. Similar letters represent non-significant differences. (G1) and (G2): 40% *AVP1* GM and 40% non-GM sugarcane fed group respectively; (G3) and (G4): 20% *AVP1* GM sugarcane and 20% non-GM sugarcane fed group respectively; (G5): normal diet fed group.

Blood Haematology and Biochemistry

Variation in the blood biochemical and haematological parameters were analysed to evaluate each diet effect on rabbits among which showed statistically non-significant (P>0.05) results (Table 5). The biochemical parameters such as blood sugar, the indicators of kidney functions (blood urea, serum creatinine, serum uric acid), the cardiovascular risk marker like cholesterol, HDL cholesterol, LDL cholesterol and triglycerides, the liver function indicators like total bilirubin, direct bilirubin, indirect bilirubin, Alanine Aminotransferase (ALT or SGPT), Aspartate Aminotransferase (AST or SGOT), alkaline phosphatase, the total proteins as a biomarker (immune system function) were examined such as serum proteins, serum albumin, serum globulin, albumin to globulin ratio (A/G ratio) were not significantly different (P>0.05) in treated transgenic and non-transgenic groups compared with control (normal diet).

Haematological parameters such as haemoglobin, erythrocyte sedimentation rate (ESR), Total Lymphocyte Count (TLC), Differential Leukocyte Count (DLC), neutrophils, lymphocytes, monocytes and eosinophils were also assessed to recognise the potential immune-toxicity or anaemia related to liver disease, iron deficiency, etc. The results showed non-significant differences (P>0.05) compared control reference values among all treatment groups. These results revealed that feeding of the GM *AVP1* sugarcane didn't show any toxic effect on the blood biochemistry and haematology in 90-day sub-chronic toxicity studies (Table 6).

Table 6. Haematological and biochemical analyses of rabbits fed with *AVP1* GM sugarcane, non-GM sugarcane and normal feed groups after 90-days sub-chronic study.

Blood Biochemistry						
Parameters	Units	G1	G2	G3	G4	G5
Blood Sugar	(mg/dL)	47.8± 6.4[a]	46.6± 3.1[a]	45.4± 5.0[a]	45.2± 4.7[a]	45.8± 5.3[a]
Blood Urea	(mg/dL)	65.0± 3.7[a]	65.4± 4.7[a]	65.2± 4.1[a]	65.0± 6.8[a]	65.2± 5.5[a]
Serum Creatinine	(mg/dL)	1.0± 0.2[a]	1.0± 0.1[a]	1.0± 0.1[a]	1.0± 0.0[a]	1.0± 0.3[a]
Serum Uric Acid	(mg/dL)	2.2± 0.2[a]	2.1± 0.1[a]	2.0± 0.1[a]	2.0±0. 0[a]	2.0± 0.0[a]
Cholesterol	(mg/dL)	65.0± 0.4[a]	65.0± 2.4[a]	65.0± 2.4[a]	65.0± 5.0[a]	65.0± 1.0[a]
Triglycerides	(mg/dL)	143.6 ±34.4[a]	143.6± 15.8[a]	143.6± 9.8[a]	144.0 ±6.0[a]	144.0± 13[a]
HDL Cholesterol	(mg/dL)	19.4± 3.0[a]	19.0± 1.0[a]	19.6± 3.4[a]	19.0± 3.0[a]	19.0± 5.0[a]
LDL Cholesterol	(mg/dL)	38.6± 2.5[a]	39.8± 3.1[a]	39.6± 2.8[a]	40.0± 4.0[a]	40.0± 3.0[a]
Total Bilirubin	(mg/dL)	1.2± 0.4[a]	1.1± 0.2[a]	1.1± 0.2[a]	1.0± 0.0[a]	1.0± 0.0[a]
Direct Bilirubin	(mg/dL)	0.4± 0.2[a]	0.4± 0.0[a]	0.3± 0.1[a]	0.3± 0.0[a]	0.3± 0.0[a]
Indirect Bilirubin	(mg/dL)	0.8± 0.2[a]	0.8± 0.1[a]	0.8± 0.2[a]	1.0± 0.0[a]	1.0± 0.0[a]
SGPT	(U/L)	38.4± 10.8[a]	38.2± 6.1[a]	38.8± 4.4[a]	38.0± 19.8[a]	38.0± 18.8[a]
SGOT	(U/L)	37.0± 2.4[a]	37.8± 1.6[a]	37.4± 3.2[a]	38.0± 3.0[a]	37.0± 6.0[a]
Alkaline Phosphatase	(U/L)	75.2± 3.4[a]	74.6± 2.3[a]	71.2± 3.7[a]	72.0± 3.0[a]	76.0± 6.6[a]
Serum Proteins	(mg/dL)	7.5± 0.0[a]	7.5± 0.2[a]	7.4± 0.2[a]	7.3± 0.3[a]	7.4± 0.2[a]
Serum Albumin	(mg/dL)	3.5± 0.1[a]	3.6± 0.2[a]	3.6± 0.1[a]	3.5± 0.6[a]	3.5± 0.2[a]
Serum Globulin	(mg/dL)	4.0± 0.1[a]	3.9± 0.2[a]	3.9± 0.1[a]	3.7± 0.2[a]	3.9± 0.3[a]
Blood Haematology						
A/G Ratio		0.9± 0.0[a]	0.9± 0.1[a]	0.9± 0.1[a]	0.9± 0.2[a]	0.9± 0.1[a]
Hemoglobin	(g/dL)	13.5± 1.5[a]	13.5± 0.6[a]	13.6± 0.6[a]	13.4± 1.7[a]	13.8± 0.5[a]
ESR	(mm/h)	2.8± 0.8[a]	2.8± 0.5[a]	3.2± 1.1[a]	2.8± 0.4[a]	3.4± 1.3[a]
TLC	(mcL)	9140± 517[a]	9120± 626[a]	9160± 805[a]	9120± 545[a]	9160± 923[a]
Neutrophils	(%)	26.2± 1.6[a]	26.4± 1.4[a]	26.4± 2.6[a]	24.6± 2.3[a]	26.4± 2.6[a]
Lymphocytes	(%)	63.4± 6.1[a]	61.4± 4.5[a]	60.8± 14.3[a]	63.2± 10.3[a]	60.8± 14.3[a]
Monocytes	(%)	3.3± 0.8[a]	3.4± 0.6[a]	3.3± 0.5[a]	3.5± 1.0[a]	3.2± 0.4[a]
Eosinophils	(%)	2.6± 0.4[a]	2.6± 0.4[a]	2.6± 0.3[a]	2.8± 0.4[a]	2.6± 0.5[a]

Biochemical and haematological data represent (mean±SD) at the significance level (P≤0.05). Similar alphabets show non-significant differences among all the treatment groups. (G1) and (G2): 40% *AVP1* GM and 40% non-GM sugarcane fed group respectively; (G3) and (G4): 20% *AVP1* GM sugarcane and 20% non-GM sugarcane fed group respectively; (G5): normal diet fed group.

Genotoxicity and Mutagenicity Assessment

After 90-day, comet assay (DNA damage test) was conducted with the peripheral blood of treated rabbits to detect DNA damage and scored visually according to their classes (Fig. 5). The arbitrary units (AUT) were calculated according to their scores, no significant difference (P>0.05) in DNA damage was observed among all treatment groups as represented in Fig. 5. However, level 1 damaged cells were observed in all treatment groups (non-significant P>0.05). Level 2, 3 and 4 damaged cells were not observed in any treatment group as compared to the positive control (methyl methanesulphonate or MMS) where class 2, 3 and 4 damaged cells were observed.

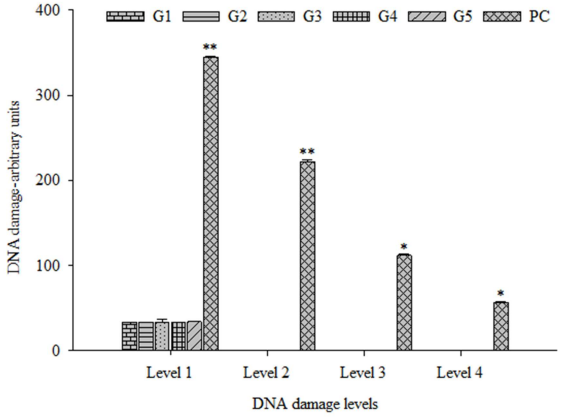

Fig. 5. Comet assay with rabbit's peripheral blood after 90-day sub-chronic study. DNA damage different levels (1, 2, 3 and 4) represent as mean±SD (arbitrary units) with non-significant (P≥0.05) differences among different dosed groups.(G1) and (G2): 40% *AVP1* GM and 40% non-GM sugarcane fed group respectively; (G3) and (G4): 20% *AVP1* GM sugarcane and 20% non-GM sugarcane fed group respectively;(G5): normal diet fed group. Positive control (PC) with methyl methanesulphonate (MMS). **, * represented significant difference.

The micronuclei frequency in the peripheral blood of rabbits after the 90-day feeding of transgenic sugarcane ranged from 1.93 to 2.03% and in non-transgenic sugarcane and normal diet control group ranged from 1.97 to 2.03 %. The micronuclei frequency in blood erythrocytes revealed non-significant (P>0.05) signs of toxicity among all the treatment groups (Table 7).

The results of the comet assay and micronuclei assays revealed that feeding *AVP1* GM sugarcane showed no genotoxicity or DNA damage in the peripheral blood of rabbits (Fig. 5 and Table 7).

Table 7. The frequency of micronuclei (%) in peripheral blood erythrocytes of rabbits after 90-day sub-chronic toxicity of *AVP1* GM sugarcane, non-GM sugarcane and normal feed groups.

Treatment Groups	Total cells	Erythrocytes	% age
G1 (40% GM sugarcane feed)	1000	19.3±3.1^a	1.93±0.31^a
G2 (40% non-GM sugarcane feed)	1000	19.7±2.5^a	1.97±0.25^a
G3 (20% GM sugarcane feed)	1000	20.0±2.6^a	2.0±0.26^a
G4 (20% Non-GM sugarcane feed)	1000	20.3±8.3^a	2.03±0.83^a
G5 (normal feed)	1000	20.3±8.3^a	2.03±0.83^a

Micronucleus assay data values (mean±SD) at significance level (P≤0.05). Similar letters indicate non-significant difference among all treatment groups.

The Ames test with AVP1 purified (pET 32 bacterial expression system) protein (0.9 mg/mL) at various concentrations (µg/plate) showed non-mutagenic activity in comparison with negative (water) control (Table 8). At maximum dose that was 512 µg/plate of purified protein showed mutagenic index (MI) 50% lower than negative control indicated that AVP1 protein was non-mutagenic (MI of AVP1 protein was less than 2 with TA 98 and less than 1.8 with TA 100). The positive controls were $K_2Cr_2O_7$ and NaN_3 (25 µg/plate).

Table 8. Ames test (AVP1 purified protein) using *Salmonella typhimurium* strains (TA 98 and TA 100) with and without metabolic activation mixture S9.

Dose µg/ plate	AVP1 (recombinant) purified protein			
	No. of revertant colonies/plate (Mean±SD)			
	TA 98		TA 100	
	-S9	+S9	-S9	+S9
NC	58.3±1.5^a	61.0±2.0^a	149.7±2.1^a	157.3±2.1^a
8	36.6±3.2^a	37.7±1.5^a	82.7±2.5^a	89.6±1.5^a
16	39.3±2.1^a	38.0±2.0^a	85.0±1.7^a	90.0±4.6^a
32	39.3±2.1^a	41.0±2.0^a	86.0±2.6^a	90.3±3.5^a
64	40.0 ±1.0^a	42.0 ±2.6^a	86.3±2.1^a	91.0±2.0^a
128	42.3±3.1^a	44.3±2.5^a	86.3±2.5^a	91.7±3.5^a
256	43.0±2.6^a	44.0±2.6^a	86.3±2.1^a	92.0±4.4^a
512	43.3±1.5^a	45.0±3.6^a	93.0±2.0^a	92.3±5.1^a
PC	395.6±2.1^c	428.3±3.1^d	445.7±4.2^e	423.3±4.5^f

Ames test indicating the number of reverse mutants values induced by AVP1 purified protein (Mean±SD). (PC): positive control ($K_2Cr_2O_7$ and NaN_3); (NC): negative control (water). Similar alphabets show non-significant difference among all treatment groups.

Discussion

Transgenic crops are strictly regulated and require biosafety evaluation for the approval from relevant competent authorities (Smart *et al.*, 2017) and must be thoroughly tested for the safety of animals and humans if consumed as a food and feed prior to commercialization (Garcia-Alonso, 2013). The aim of the current research work was to identify the expected adverse effects associated with GM *AVP1* sugarcane expressing the *AVP1* gene. The current 90-day animal feeding trial was performed according to the recommendations by FAO/WHO, 2000.

The expression of the *AVP1* gene in GM sugarcane lines and its absence in non-GM sugarcane was confirmed by gene-specific qualitative reverse transcription PCR (RT-qPCR). Similarly, Delaney *et al.* (2014) detected the presence of a specific trait in the test canola (73496 and 73496GLY) and its absence in control (091) and reference by performing event specific RT-qPCR analysis.

The rabbit's diet was nutritionally balanced and there was no obvious diet-related toxic effects observed in all treatment groups. The survival rate was 100% and no mortalities were found in the animals of all dosed groups. Mean body weight gain was persistent both in male and female rabbits of different dosed groups (fed with 40% and 20% *AVP1* GM and non-GM sugarcane respectively). There were no significant differences observed in the data regarding body weight and food consumption among all dosed groups. The data showed similarities with the previously published reports on 90-day animal feeding trial based on GM food such as GM maize (Arjó *et al.*, 2012), canola (Delaney *et al.*, 2014) and cotton (Dryzga *et al.*, 2007).

The GM *AVP1* sugarcane didn't show statistically significant or treatment-related differences in the mean values of biochemical, haematological parameters and organ weight among the animals of all treatment groups.

These findings were in accordance with already published research reports such as a 13-week rodent feeding trial by Delaney *et al.* (2014) on herbicide tolerant (DP-Ø73496-4) canola; Hammond *et al.* (2006) on corn borer-protected corn, corn rootworm-protected corn, glyphosate-tolerant corn and Bt-resistant (*Cry3Bb1*) MON 810 corn; MacKenzie *et al.* (2007) on maize grain event DAS-Ø15Ø7-1; Malley *et al.* (2007) on maize event DAS-59122-7; Appenzeller *et al.* (2008) on soybean DP-356Ø43–5 and GM stacked trait *Lepidopteran* and *Coleopteran* resistant DAS-Ø15Ø7-1xDAS-59122-7 maize grain and herbicide-tolerant maize DP-Ø9814Ø-6 respectively; He *et al.* (2008) on event DAS-59122-7 of maize; Healy *et al.* (2008) on corn rootworm-protected and glyphosate-tolerant (*CryBb1*) MON 88017 corn; Arjó *et al.* (2012) on genetically engineered multivitamin (β-carotene, ascorbate and folate) corn in mice.

No genotoxicity was observed in the micronucleus test and the comet assay with the peripheral blood of rabbits after feeding of *AVP1* GM sugarcane for 90-day. Similarly, Jaszczak *et al.* (2008) performed the micronucleus test and the comet assay on mice fed on a dose containing GM triticale (*bar* transgene) and reported that there were no statistically significant differences in the micronuclei frequency and DNA damage (comet assay) between the control and experimental groups of mice and the results didn't show any chromosomal damage or DNA breaks/ lesions. The micronucleus test with peripheral blood erythrocytes exhibited a useful insight of the risk of mutation (Hamada *et al.*, 2001; Bhilwade *et al.*, 2004).

There was no significant increase in the revertant colonies observed in the Ames test with AVP1 purified protein at the concentration of 512µg/plate both with TA 100 and TA 98 bacterial strains. Although the revertant colonies increased in a dose-dependent manner, however the mutagenic index was below the toxic range in all AVP1 protein concentrations even at the highest concentration used. The AVP1 protein was found to be non-mutagenic. Chen *et al.* (2003) performed the Ames test and the micronucleus test in rats feeding on GM varieties of tomato and sweet pepper with no signs of any toxicity.

This is the first report on biosafety assessment of GM sugarcane expressing *AVP1* gene. This research work revealed that under the conditions of sub-chronic toxicity evaluations, the *AVP1* GM sugarcane was found to be non-toxic to albino rabbits when they were administered orally for 90-day. Scientific data generated through this research work will be valuable and provide some important information that will support the commercialization of GM *AVP1* sugarcane.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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