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Risk assessment of genetically modified sugarcane expressing *AVP1* gene

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ABSTRACT

Biosafety is a multidisciplinary approach that encompasses social, societal, ethical issues and policies for the regulations of genetically modified (GM) organisms. The potential health risks associated with GM sugarcane containing *AVP1* gene confers resistance against drought and salinity were evaluated by animal feeding studies and some genotoxicity assays. Acute and sub-chronic toxicity examinations were carried out via oral dose administration of GM sugarcane juice supplemented with the normal diet (modified from certified rodent standard diet) on Wistar rats. AVP1 protein concentration in sugarcane juice was 1mg/1 mL. Biochemical, haematological blood analyses were performed and the results revealed that there were non-significant differences among all the treatment groups; GM sugarcane juice, non-GM sugarcane juice and the control group (normal diet and water). Genotoxicity assessment based on the comet assay and the micronucleus assay data exhibited that *AVP1* GM sugarcane was not genotoxic or cytotoxic in rat's peripheral blood. These research findings supported the conclusion that GM *AVP1* sugarcane was non-toxic in experimental animals. Therefore, data generated through this research work would be helpful for the commercial release of GM *AVP1* sugarcane.

1. Introduction

Sugarcane is one of the most important receptors of solar energy, which is converted to fermentable sugars and fibres (FAO, 1988). Although unprocessed sugarcane juice is normally used to produce crystalline sugars, sometimes sugarcane juice (extracted from pressed cane) is used as a beverage (owing to its nutritional importance) in many countries particularly where it has been commercially developed such as Pakistan, India, Southeast Asia, Egypt, and Latin America. The evaporated sugarcane juice as a component in prepared beverages and food represents a sugar (sweetener) derivative of cane syrup (FDA, 2009). Sugarcane juice is sold nationally among the street sellers in Indonesia and Malaysia, whereas in Singapore it is sold in food courts only (Satran, 2013). It is a national drink of Pakistan.

Genetically modified (GM) crop plants have been developed and grown on a commercial scale on approximately 1.96 billion hectares during the past 19 years in 28 countries of the world (James et al., 2015). Food and feed obtained from GM crops must be assessed for potential harm to animals, humans, and the environment prior to commercial release (Domingo, 2016). The methods of safety/risk assessment are referred to as comparative safety assessments (Bartholomaeus et al., 2013; Codex Alimentarius Commission, 2009). Biosafety assessments differentiate GM crops from their parental counterparts and this process is based on a "weight of evidence" law where new information is available regarding potential distinctions and their impact on food or feed safety (Cockburn, 2002; Herman et al., 2009; Herman and Price, 2013; König et al., 2004; Kuntz and Ricroch, 2012; Parrott et al., 2010; Ricroch, 2013). However, the variations do not automatically mean that any harmful effects would result. If deviations are identified then the likelihood that they would potentially harm the animal or human health must be thoroughly assessed (Garcia-Alonso, 2010).

Transgenic sugarcane expressing the *Arabidopsis* vacuolar proton-pump pyrophosphatase (*AVP1*) gene has been developed at the National Institute for Biotechnology and Genetic Engineering (NIBGE) and sugarcane plants expressing higher levels of *AVP1* are more tolerant to drought and salinity (Raza et al., 2016).

GM crops expressing a particular protein resulting from a genetic alteration are evaluated via acute (short-term) and chronic (long-term repeated dose) experiments on animals (Bartholomaeus et al., 2013; Delaney et al., 2008b; EFSA, 2008; Hammond and Cockburn, 2008; Rice et al., 2007). Acute (short-term) toxicity studies performed (OECD, 2001) with single or multiple doses administration of a purified protein in a short period of time not exceeding from 24 h. Sub-chronic (long-

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term repeated dose) rodent feeding studies of GM crops recommended (FAO/WHO, 1996) to determine if there are any harmful effects based on any toxic health effect, potential allergenicity/animal studies, nutritional compositional changes and inserted gene integrity (Codex Alimentarius Commission et al., 2003; WHO, 1995). Different ways to identify the risks/adverse effects associated with GM crops have been documented (EFSA, 2008). Various genetically altered crops have been assessed by these methods, such as tomato (Noteborn et al., 1995), soybean (Appenzeller et al., 2008; Zhu et al., 2004), rice (Poulsen et al., 2007; Poulsen et al., 2007a; Schrøder et al., 2007; Wang et al., 2002), maize (Hammond et al., 2004, 2006a; He et al., 2008; MacKenzie et al., 2007; Malley et al., 2007), and cotton (Dryzga et al., 2007).

Genotoxicity assays have been performed to identify DNA reactive molecular compounds (Maluszynska, 2005) through both *in vitro* and *in vivo* means. These assays are designed to recognize substances that cause mutation, DNA damage, and chromosomal aberrations. The substances have proven to be genotoxic (positive) after experimentation are considered carcinogens (may stimulate cancer cell proliferation) or mutated by interrupting DNA molecules (Auffan et al., 2006; Colognato et al., 2008; Fenech, 2008). The GM plants are strictly regulated in the form of an organised network. For example, in the United States of America, the Food and Drug Administration (FDA) works with federal agencies, including the Environmental Protection Agency (EPA) and the United States Department of Agriculture (USDA) that demand full risk assessment evaluation studies for newly discovered chemical active ingredients or GM food before their commercialization. In Pakistan, GM crops are regulated by the National Biosafety Committee (NBC) which emphasizes that genetically modified organisms (GMOs) should be properly evaluated both for toxicity and allergenicity following the Cartagena Protocols on Biosafety (World Health Organization, 2017).

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. When it was first introduced (Ostling and Johanson, 1984), the name “Comet” was used because the images produced from the assay resemble a comet with a discrete head consisting of intact DNA and a smear of damaged DNA giving the impression of the tail of a comet. The amount of DNA in the comet tail is directly proportional to the amount of DNA damage.

A micronucleus test is a tool for the genotoxicity assessment of a potentially toxic chemical or compound (a carcinogen) caused by genetic damage both *in vivo* and *in vitro* means (OECD, 2010). It is an inexpensive, simple, rapid, and reliable method. A micronucleus is a third (erratic) nucleus, which is produced during anaphase of mitosis or meiosis. Micronuclei were first used to measure chromosomal damage (Evans et al., 1959) in root tips of *Vicia faba* (Heddle, 1973; Schmid, 1975); thereafter, further researchers (Jaszczak et al., 2008) performed a micronucleus test and comet assay in mice fed on genetically modified (GM) triticale (bar transgene).

The present research was conducted to assess the risks associated with GM *AVP1* sugarcane based on acute and sub-chronic toxicity studies in Wistar rats. Biochemical, haematological analyses and genotoxicity assays were performed which revealed that GM *AVP1* sugarcane did not exhibit any toxic effect on Wistar rats.

2. Materials and methods

2.1. Maintenance of laboratory animals

Wistar albino rats (male and female) aged 6–8 weeks were obtained from the National Institute for Health (NIH) and acclimatized (22 ± 3°C; 12 h light and 12 h dark; 60% relative humidity) for one week in properly labelled polypropylene cages in the animal room at NIBGE prior to the start of experiment. They were fed on a modified pelleted diet (modified from certified rodent diet 5002*) and drinking water *ad libitum*. Paddy husk (Al-Murtaza Wood Crafts, Jhang Road, Faisalabad, Pakistan) was used as bedding material and was changed twice a week. The selection of rats was made because of their similarity in various cellular, enzymatic functions, and 95% genome identity with human beings.

2.2. Nutritional evaluation of GM and non-GM sugarcane juice

The nutritional evaluation was performed for both non-transgenic and transgenic *AVP1* sugarcane juice. The percentage values of proximates were calculated in different laboratories of NIBGE and Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad, Pakistan.

2.3. Extraction, elution and quantification of AVP1 protein from AVP1 GM sugarcane juice

AVP1 protein was extracted from *AVP1* sugarcane juice according to the procedure of Cullis et al. (2014). The specific AVP1 protein from the crude mixture was run in an SDS-PAGE (10% and stained with Coomassie Brilliant Blue) and the exact 85 kDa band of AVP1 protein was excised from the gel and eluted by following the Thermo Fisher Scientific procedures (Thermo Fisher Scientific, 2009). The eluted specific AVP1 protein was quantified using the standard Bradford's method, where the concentration (1 mg/mL) of GM *AVP1* protein in sugarcane juice was determined (Bhattacharyya et al., 2007; Bradford, 1976).

2.4. Acute toxicity studies of GM AVP1 sugarcane juice

Male and female Wistar albino rats (200–250 g) were selected for a two-week study with GM *AVP1* sugarcane juice. Animals were randomly distributed in three treatment groups consisting of 10 animals per each group (5 male and 5 female). Five animals were maintained per cage. They were supplied with standard rodent feed (5002*) and water *ad libitum* (Bhattacharyya et al., 2007). Animals of all the treatment groups were administered to repeated doses (after 24 h) of 0, 15 mL (30% juice), and 30 mL (60% juice)/body weight/day or 0, 1050 and 2100 mg/kg body weight respectively with *AVP1* GM and non-GM sugarcane juice for 14-day. Group 1 (G1) animals were dosed with GM *AVP1* sugarcane juice, animals of group 2 (G2) were dosed with non-GM sugarcane juice and group 3 (G3) animals were fed with normal rodent diet and water, serving as the control. GM and non-GM sugarcane fresh juice were mixed in distilled water according to their daily consumption (35 mL water + 15 mL juice for 30% and 20 mL water + 30 mL juice for 60%). The animals were observed for 48 h carefully, after hourly intervals, and after every 12 h till the day of sacrifice. The following physical examinations such as skin and eye irritation, urination, salivation corneal reflex, spontaneous responses in behavioural or autonomic activities mortality were observed (OECD, 2001).

2.5. Sub-chronic toxicity studies of GM AVP1 sugarcane juice

A 90-day sub-chronic study on Wistar albino rats (male and female) was conducted according to OECD guidelines 408 to assess the effect of fresh GM *AVP1* sugarcane fresh juice (harvested and crushed daily). Wistar rats approximately 200–250 g of weight were assigned to three groups of 10 animals (5 males and 5 females). The juice of GM *AVP1* sugarcane plants was used throughout this study. One selected dose of 12 mg or 12 mL (24% juice)/body weight/day or < 5000 mg/kg body weight and two control treatments, one with non-GM sugarcane (12 mL/kg body weight/day) and the other with distilled water were administered orally. GM and non-GM sugarcane fresh juice were mixed in distilled water according to their daily consumption (38 mL water + 12 mL juice). Fresh rodent pelleted modified diet was provided daily as required. Animals were observed daily to note any changes in behavioural or autonomic activities (irritation, urination, salivation corneal reflex, spontaneous responses and mortality).

2.6. Body weights and food consumption

Body weight and food consumption (g) were recorded daily during the first week of the exposure time and weekly thereafter.

2.7. Haematology and blood biochemistry

Blood samples were collected from the external jugular vein at the end of the experiment (EDTA-coated and Heparin vacutainers for biochemical and haematological, evaluations respectively). The blood samples were sent to a clinical pathology laboratory (PINUM, ISO 9001–2008 certified hospital, Faisalabad) for analyses. Haematological parameters such as haemoglobin, erythrocyte sedimentation rate (ESR), total lymphocyte count (TLC), differential leukocyte count (DLC), neutrophils, lymphocytes, monocytes and eosinophils were observed. Biochemical parameters 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, albumin to globulin ratio were analyzed.

2.8. Relative organ weight

At the end of the experiment, the animals were euthanized. Various organs such as the heart, lungs, liver, spleen, kidneys, gonads and brain were carefully dissected out and weights were recorded. The relative organ weight of each animal was calculated as

$$\text{Relative organ weight (\%)} = \frac{\text{Absolute organ weight (g)}}{\text{Body weight of rat on sacrifice day (g)}} \times 100$$

2.9. Histopathological examination

Rat liver and kidney tissue samples were fixed in 15% neutral formalin and organised for histopathological examination. Risen embedded sections were stained with toluidine blue in 1% boric acid for microscopic examination at 10X and 100X magnification.

2.10. Genotoxicity assessment

Genotoxicity was tested by performing the comet assay (DNA damage) and the micronucleus test (cytotoxicity).

3. Statistical analyses

Statistical analyses were performed with SigmaPlot 13.0 (SYSTAT software). Data were analyzed using ANOVA, to account for differences in data distribution and variance. The pairwise comparison was made using Tukey's test (Tukey, 1993).

4. Results

The rat's diet was nutritionally balanced and there were no obvious diet-related toxic effects observed in all treatment groups. GM and non-GM sugarcane juice proximate nutritional evaluations showed non-significant differences as presented in Table 1.

4.1. Weekly body weight in acute toxicity test

No mortalities were observed during the period of 14-day acute toxicity evaluations. There were no statistically significant differences in the mean body weights and food consumption data of both male and female rats after 14-day oral administration of GM *AVP1* sugarcane juice at the dose of 2100 mg/kg body weight (Fig. 1A and B respectively).

Table 1
Nutritional composition analyses of *AVP1* GM and non-GM sugarcane juice.

Proximates	GM sugarcane juice	Non-GM sugarcane juice
Protein (%)	0.45 ± 0.01	0.45 ± 0.02
Ash (%)	0.43 ± 0.02	0.43 ± 0.01
Moisture content (%)	78.67 ± 1.53	79.00 ± 1.00
Crude fat (%)	0.81 ± 0.02	0.81 ± 0.01
Crude fibre (%)	6.20 ± 0.20	6.17 ± 0.12
Brix content (%)	23.93 ± 1.01	24.00 ± 0.20
Total dry matter (%)	19.97 ± 0.15	20.10 ± 0.20
pH	5.60 ± 0.10	5.60 ± 0.10
Sucrose (%)	32.10 ± 0.20	32.03 ± 0.15
Glucose (%)	1.59 ± 0.02	1.57 ± 0.03
Fructose (%)	2.41 ± 0.01	2.40 ± 0.01
Sodium (%)	0.27 ± 0.06	0.27 ± 0.06
Potassium (%)	0.03 ± 0.01	0.02 ± 0.01
Calcium (%)	0.02 ± 0.01	0.02 ± 0.01
Magnesium (%)	0.02 ± 0.01	0.01 ± 0.01
Iron (%)	1.10 ± 0.10	1.07 ± 0.06
Zinc (%)	1.17 ± 0.06	1.13 ± 0.06
Chromium (%)	0.03 ± 0.01	0.03 ± 0.01
Lead (%)	0.01 ± 0.01	0.01 ± 0.01
Copper (%)	0.17 ± 0.01	0.18 ± 0.01
Manganese (%)	1.43 ± 0.01	1.43 ± 0.02

Values presented (Mean ± SE) for GM and non-GM sugarcane juice.

Values presented (mean ± SD) body weight of male rats. (G1): rats fed with GM sugarcane juice, (G2): rats fed with non-GM sugarcane juice, (G3): rats fed with water and normal diet. Significance checked at $P \leq 0.05$.

4.2. Weekly body weight in sub-chronic toxicity studies

The male and female maximum body weight gains were up to 364.6 g and 270 g respectively and there were non-significant variations ($P \geq 0.05$) among all the treatment groups under sub-chronic (90-day) toxicological evaluations. The overall weights of male rats were greater than the female that was considered negligible regarding any adverse effect (Fig. 2A and B respectively).

Values presented (mean ± SD) body weight of male rats. (G1): rats fed with GM sugarcane juice, (G2): rats fed with non-GM sugarcane juice, (G3): rats fed with water and normal diet. Significance checked at $P \leq 0.05$.

4.3. Weekly food consumption during sub-chronic toxicity study

Weekly food consumption of male and female rats under sub-chronic toxicity studies was increased with time and correlated with body weight in all treatment groups (Fig. 3A and B respectively) with non-significant variations ($P \leq 0.05$). Male and female rats consumed an average of 27.08 and 25.7 g of fresh pelleted diet, respectively.

Values presented (mean ± SD) body weight of male rats. (G1): rats fed with GM sugarcane juice, (G2): rats fed with non-GM sugarcane juice, (G3): rats fed with water and normal diet. Significance checked at $P \leq 0.05$.

4.4. Weekly water consumption during sub-chronic toxicity study

Weekly water consumption was consistent throughout the sub-chronic toxicity studies both in male and female Wistar rats (supplementary data).

4.5. Biochemical and haematological analyses after acute and sub-chronic toxicity

There were no statistically significant ($P \geq 0.05$) differences observed in the mean values of measured clinical chemistry variables

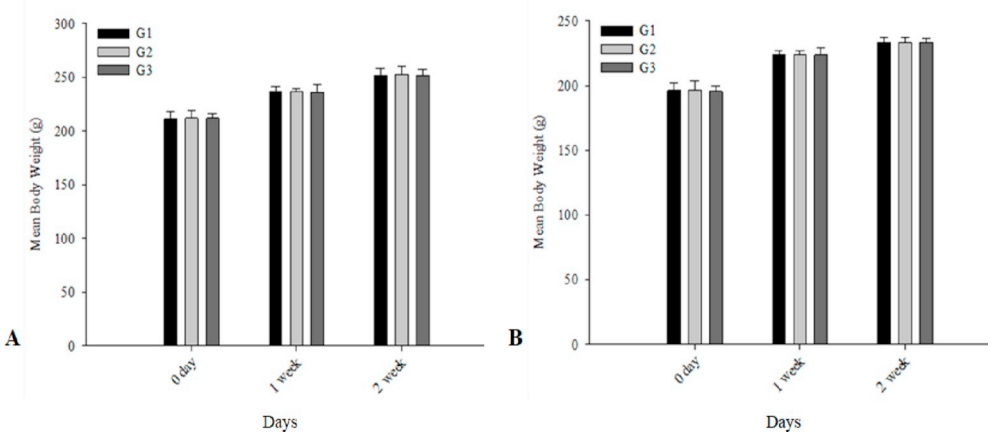

Fig. 1. Weekly body weight (g) male (A) and female (B) Wistar rats after acute toxicity studies.

(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, albumin to globulin ratio (A/G ratio), in both acute (male and female Tables 2 and 3 respectively) and sub-chronic (male and female, Tables 4 and 5, respectively) studies on Wistar rats (male and female). Similarly, blood haematological parameters (Haemoglobin, erythrocyte sedimentation rate (ESR), total lymphocyte count (TLC), differential leukocyte count (DLC), neutrophils, lymphocytes, monocytes, eosinophils showed non-significant differences ($P \leq 0.05$) among all the groups in sub-chronic studies both in male and female rats (Tables 4 and 5 respectively) and acute toxicity (Tables 2 and 3 respectively) studies on Wistar rats (male and female). Rats fed with GM sugarcane did not show significant variations in comparison with its non-GM comparator. Therefore, GM *AVP1* sugarcane did not show any toxic effect in the blood regarding biochemical and haematological analyses.

4.6. Relative organ weight after acute and sub-chronic toxicity

In acute and sub-chronic toxicity studies, there were no significant differences ($P \leq 0.05$) observed in the animals fed with the *AVP1* GM and non-GM sugarcane juice and a control group in terms of the relative organ weight of heart, lungs, liver, kidney, spleen, gonads and brain to body weight (at the day of sacrifice). Significant ($P \geq 0.05$) differences were observed in gonads of male and females in all dosed groups (Tables 6 and 7 respectively) because there was a difference between male and female reproductive systems. Transgenic *AVP1* sugarcane juice did not exhibit any difference to its non-GM comparator and animals fed with normal feed and drinking water both in the acute and sub-chronic toxicity studies.

4.7. Histopathology

No treatment-related differences observed in the day of sacrifice. All microscopic images of the liver and kidney tissues were normal for both male and female Wistar rats, after the acute and sub-chronic toxicity evaluations (supplementary data).

4.8. Genotoxicity assessment after acute and sub-chronic toxicity

Genotoxicity related to the comet assay (DNA damage test) with peripheral blood of rats after 14-day acute toxicity studies showed that there were no damaged cells observed in any treatment group, whereas the 90-days sub-chronic studies exhibited DNA damage at the comet class 1 but with non-significant ($P \geq 0.05$) variations among the treatment groups of male and female rats. Damaged cells up to level 2, 3 and 4 were not observed in any treatment group as compared to the positive control (methyl methanesulfonate or MMS) where significantly higher ($P \leq 0.05$) DNA damage was observed up to level 4 with positive control. The comet assay revealed that *AVP1* GM sugarcane was not related to DNA damage in the blood after the 90-day sub-chronic toxicity study (supplementary data).

The micronuclei frequency in the peripheral blood of rats after the 14-day (acute toxicity) feeding of transgenic sugarcane juice ranged

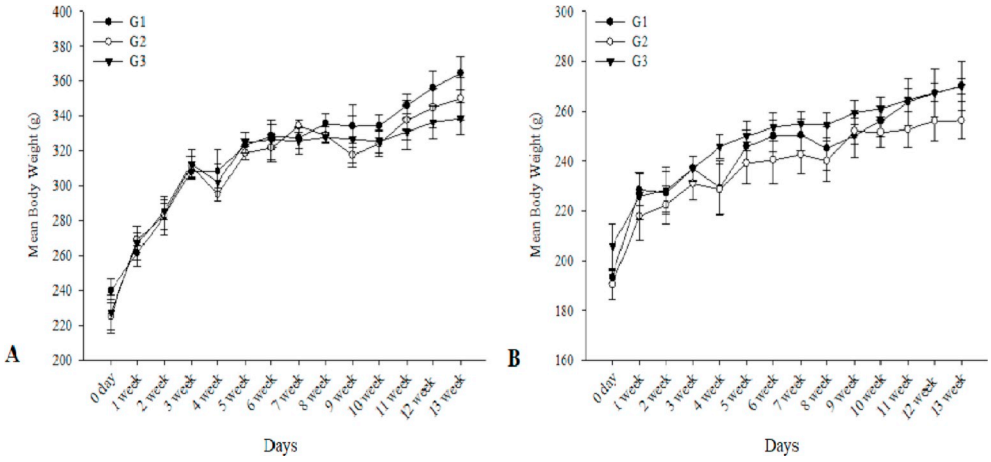

Fig. 2. Weekly body weight (g) male (A) and female (B) Wistar rats after sub-chronic toxicity studies.

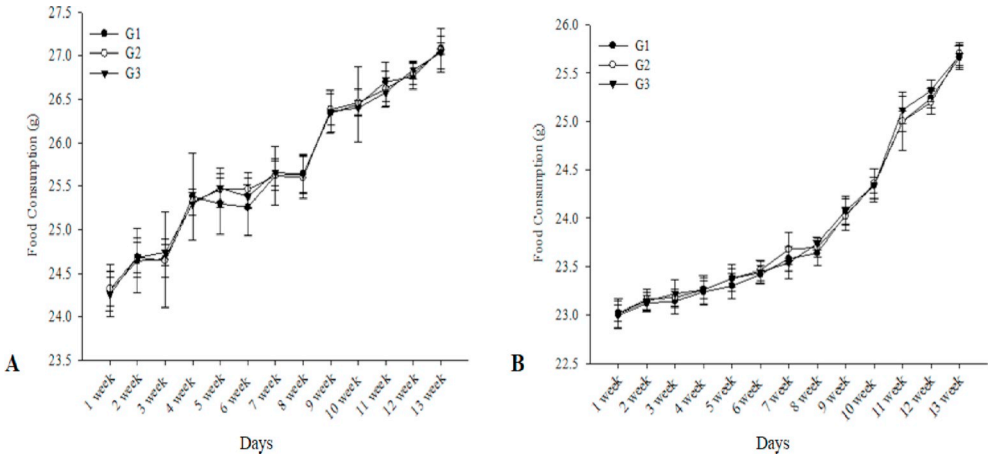

Fig. 3. Weekly food consumption (g) male (A) and female (B) Wistar rats after sub-chronic toxicity studies.

Table 2
Biochemical and haematological analyses for male Wistar rats after 14-day acute toxicity.

Biochemical Parameters				
	Units	G1	G2	G3
Blood Sugar	(mg/dL)	70.20 ± 5.54[a]	70.60 ± 12.22[a]	69.80 ± 9.44[a]
Blood Urea	(mg/dL)	40.00 ± 3.81[a]	41.40 ± 2.88[a]	40.10 ± 0.74[a]
Serum Creatinine	(mg/dL)	0.60 ± 0.07[a]	0.64 ± 0.05[a]	0.66 ± 0.09[a]
Serum Uric Acid	(mg/dL)	1.66 ± 0.29[a]	1.63 ± 0.38[a]	1.61 ± 0.07[a]
Cholesterol	(mg/dL)	64.60 ± 4.34[a]	65.40 ± 4.51[a]	62.80 ± 0.84[a]
Triglycerides	(mg/dL)	73.00 ± 16.64[a]	74.00 ± 2.45[a]	73.80 ± 5.93[a]
HDL Cholesterol	(mg/dL)	23.20 ± 1.30[a]	22.40 ± 1.62[a]	22.90 ± 4.72[a]
LDL Cholesterol	(mg/dL)	38.00 ± 6.20[a]	38.60 ± 3.05[a]	39.50 ± 0.50[a]
Total Bilirubin	(mg/dL)	1.02 ± 0.19[a]	1.08 ± 0.19[a]	1.17 ± 0.08[a]
Direct Bilirubin	(mg/dL)	0.22 ± 0.08[a]	0.22 ± 0.04[a]	0.25 ± 0.05[a]
Indirect Bilirubin	(U/L)	0.77 ± 0.15[a]	0.80 ± 0.18[a]	0.78 ± 0.08[a]
SGPT	(U/L)	5.20 ± 0.84[a]	5.40 ± 0.89[a]	5.20 ± 0.84[a]
SGOT	(U/L)	3.20 ± 0.84[a]	3.200.45[a]	3.52 ± 0.48[a]
Alkaline Phosphatase	(mg/dL)	483.60 ± 94.95[a]	481.00 ± 88.99[a]	482.80 ± 63.82[a]
Serum Proteins	(mg/dL)	7.08 ± 0.43[a]	7.02 ± 0.13[a]	7.05 ± 0.11[a]
Serum Albumin	(mg/dL)	3.24 ± 0.09[a]	3.18 ± 0.22[a]	3.12 ± 0.04[a]
Serum Globulin	(mg/dL)	3.86 ± 0.34[a]	3.88 ± 0.19[a]	3.87 ± 0.16[a]
A/G Ratio		0.78 ± 0.04[a]	0.78 ± 0.10[a]	0.77 ± 0.04[a]
Haematological Parameters				
Haemoglobin	(g/dL)	16.60 ± 0.35[a]	16.18 ± 0.19[a]	16.32 ± 0.31[a]
ESR	(mm/h)	2.60 ± 0.55a	2.02 ± 0.04[a]	2.00 ± 0.00[a]
TLC	(mcL)	8300.00 ± 692.82[a]	8360.00 ± 1566.21[a]	8360.00 ± 1757.27[a]
Neutrophils	(%)	15.80 ± 3.03[a]	15.20 ± 0.84[a]	15.86 ± 3.57[a]
Lymphocyte	(%)	77.80 ± 7.19[a]	77.40 ± 4.22[a]	77.60 ± 3.58[a]
Monocytes	(%)	1.12 ± 0.16[a]	1.16 ± 0.47[a]	1.22 ± 0.44[a]
Eosinophils	(%)	1.66 ± 0.65[a]	1.66 ± 0.30[a]	1.60 ± 0.38[a]

Values presented mean ± SD. Significance checked at $P \leq 0.05$. (G1): group 1 rats fed with GM sugarcane juice, (G2): rats of group 2 fed with non-GM sugarcane juice, (G3): rats of group 3 fed with water and normal diet (n = 10). Similar alphabets represented non-significant variation among different treatment groups.

from 6.72% to 6.74% whereas in non-transgenic sugarcane juice and the control (normal feed) it was ranged from 6.72 to 6.74% both in female and male rats respectively. The micronuclei frequency after the 90-day feeding of transgenic sugarcane juice was ranged from 7.70% to 7.72% whereas in non-transgenic sugarcane and in the control (normal diet) it was 7.72% both in male and female Wistar rats respectively. The micronuclei frequency in blood erythrocytes revealed that there were non-significant ($P \geq 0.05$) signs of toxicity among all the treatment groups (supplementary data).

The results of the comet assay and micronuclei assays revealed that feeding *AVP1* GM sugarcane didn't show any cytotoxicity or DNA damage in the peripheral blood of Wistar rats fed with transgenic sugarcane juice.

5. Discussion

GM crops are subjected to risk assessment for the safety of animals and humans owing to their consumption as feed and food respectively (Delaney et al., 2018; Ferrante and Conti, 2018). The methods and data have been developed over many years for the safety assessment studies by various international environmental protection authorities such as the Organization for Economic Cooperation and Development (OECD), Food and Agriculture Organization (FAO) and World Health Organization (WHO) (Garcia-Alonso, 2013). In the current research, potentially toxic and genotoxic risks effects associated with transgenic *AVP1* sugarcane were assessed. Similarly, Badawy et al. (2008) carried out risk assessment studies on GM sugarcane (insect resistant) by following the international standard protocols of testing.

Although, there are some intended benefits of GM crops, their safety

Table 3
Biochemical and haematological data for female Wistar rats after 14-day acute toxicity.

Biochemical Parameters				
	Units	G1	G2	G3
Blood Sugar	(mg/dL)	75.80 ± 10.08[a]	74.00 ± 5.05[a]	75.80 ± 1.30[a]
Blood Urea	(mg/dL)	41.20 ± 3.35[a]	41.00 ± 4.47[a]	41.00 ± 1.58[a]
Serum Creatinine	(mg/dL)	0.66 ± 0.05[a]	0.64 ± 0.05[a]	0.66 ± 0.05[a]
Serum Uric Acid	(mg/dL)	1.82 ± 0.13[a]	1.82 ± 0.28[a]	1.82 ± 0.14[a]
Cholesterol	(mg/dL)	72.003.67[a]	73.00 ± 4.12[a]	72.00 ± 1.58[a]
Triglycerides	(mg/dL)	74.00 ± 5.24[a]	74.60 ± 2.88[a]	73.80 ± 0.84[a]
HDL Cholesterol	(mg/dL)	23.40 ± 2.07[a]	22.40 ± 2.79[a]	22.40 ± 3.44[a]
LDL Cholesterol	(mg/dL)	39.00 ± 1.41[a]	39.00 ± 6.04[a]	38.30 ± 0.84[a]
Total Bilirubin	(mg/dL)	1.14 ± 0.25[a]	1.20 ± 0.16[a]	1.44 ± 0.21[a]
Direct Bilirubin	(mg/dL)	0.28 ± 0.08[a]	0.28 ± 0.08[a]	0.29 ± 0.03[a]
Indirect Bilirubin	(U/L)	0.92 ± 0.26[a]	0.92 ± 0.18[a]	0.92 ± 0.18[a]
SGPT	(U/L)	5.20 ± 0.45[a]	5.28 ± 1.22[a]	5.20 ± 0.84[a]
SGOT	(U/L)	3.40 ± 0.55[a]	3.40 ± 0.55[a]	3.40 ± 0.38[a]
Alkaline Phosphatase	(mg/dL)	506.00 ± 103.05[a]	509.80 ± 98.96[a]	509.10 ± 20.50[a]
Serum Proteins	(mg/dL)	7.12 ± 0.50[a]	7.14 ± 0.09[a]	7.16 ± 0.10[a]
Serum Albumin	(mg/dL)	3.42 ± 0.08[a]	3.30 ± 0.25[a]	3.30 ± 0.28[a]
Serum Globulin	(mg/dL)	3.92 ± 0.23[a]	3.92 ± 0.29[a]	3.93 ± 0.05[a]
A/G Ratio		0.83 ± 0.04[a]	0.80 ± 0.16[a]	0.80 ± 0.04[a]
Haematological Parameters				
Haemoglobin	(g/dL)	15.40 ± 0.34[a]	15.44 ± 0.59[a]	15.40 ± 0.89[a]
ESR	(mm/h)	2.42 ± 0.53[a]	2.40 ± 1.14[a]	2.42 ± 0.58[a]
TLC	(mcL)	8620.00 ± 829.46[a]	8616.00 ± 2739.03[a]	8675.00 ± 1719.74[a]
Neutrophils	(%)	15.60 ± 3.91[a]	15.60 ± 0.89[a]	15.80 ± 3.56[a]
Lymphocyte	(%)	82.00 ± 9.51[a]	83.40 ± 3.21[a]	80.40 ± 82.20[a]
Monocytes	(%)	1.42 ± 0.53[a]	1.42 ± 0.53[a]	1.42 ± 0.58[a]
Eosinophils	(%)	1.60 ± 0.55[a]	1.62 ± 0.57[a]	1.62 ± 0.40[a]

Values presented mean ± SD. Significance tested at $P \leq 0.05$. (G1): group 1 animals fed with GM sugarcane juice, (G2): animals of group 2 fed with non-GM sugarcane juice and (G3): animals of group 3 fed with water and normal diet (n = 10). Similar letters represented non-significant variation among different treatment groups.

Table 4
Biochemical and haematological data for male Wistar rats after 90-days sub-chronic toxicity.

Biochemical Parameters				
	Units	G1	G2	G3
Blood Sugar	(mg/dL)	63.60 ± 4.56[a]	63.80 ± 6.83[a]	63.50 ± 4.18[a]
Blood Urea	(mg/dL)	46.40 ± 2.41[a]	46.40 ± 3.85[a]	46.00 ± 5.70[a]
Serum Creatinine	(mg/dL)	0.62 ± 0.04[a]	0.60 ± 0.06[a]	0.60 ± 0.03[a]
Serum Uric Acid	(mg/dL)	1.84 ± 0.42[a]	1.85 ± 0.43[a]	1.84 ± 0.04[a]
Cholesterol	(mg/dL)	103.60 ± 36.05[a]	102.40 ± 5.68[a]	102.00 ± 8.28[a]
Triglycerides	(mg/dL)	111.20 ± 7.50[a]	110.60 ± 21.89[a]	111.50 ± 34.18[a]
HDL Cholesterol	(mg/dL)	29.60 ± 3.05[a]	28.80 ± 2.28[a]	29.30 ± 0.84[a]
LDL Cholesterol	(mg/dL)	60.40 ± 17.13[a]	60.00 ± 8.63[a]	60.50 ± 4.53[a]
Total Bilirubin	(mg/dL)	0.51 ± 0.02[a]	0.50 ± 0.14[a]	0.50 ± 0.09[a]
Direct Bilirubin	(mg/dL)	0.23 ± 0.04[a]	0.22 ± 0.03[a]	0.22 ± 0.04[a]
Indirect Bilirubin	(U/L)	0.32 ± 0.03[a]	0.34 ± 0.13[a]	0.32 ± 0.11[a]
SGPT	(U/L)	5.14 ± 0.77a	5.14 ± 0.74[a]	5.13 ± 0.61[a]
SGOT	(U/L)	7.62 ± 0.52[a]	7.60 ± 2.17[a]	7.66 ± 1.15[a]
Alkaline Phosphatase	(mg/dL)	659.60 ± 81.01[a]	659.80 ± 122.12[a]	659.80 ± 36.55[a]
Serum Proteins	(mg/dL)	7.56 ± 0.30[a]	7.58 ± 0.26[a]	7.57 ± 0.27[a]
Serum Albumin	(mg/dL)	3.52 ± 0.33[a]	3.52 ± 0.22[a]	3.52 ± 0.32[a]
Serum Globulin	(mg/dL)	4.14 ± 0.11[a]	4.14 ± 0.20[a]	4.14 ± 0.09[a]
A/G Ratio		0.82 ± 0.07[a]	0.82 ± 0.06[a]	0.82 ± 0.12[a]
Haematological Parameters				
Haemoglobin	(g/dL)	15.70 ± 0.97[a]	15.68 ± 1.90[a]	15.68 ± 1.51[a]
ESR	(mm/h)	2.04 ± 0.15[a]	2.00 ± 0.00[a]	2.05 ± 0.54[a]
TLC	(mcL)	12000 ± 4773[a]	12160 ± 3051[a]	12060 ± 378.15[a]
Neutrophils	(%)	25.00 ± 2.12[a]	25.00 ± 1.58[a]	24.60 ± 3.58[a]
Lymphocyte	(%)	73.20 ± 9.31[a]	73.80 ± 5.26[a]	73.90 ± 5.73[a]
Monocytes	(%)	4.24 ± 0.43[a]	4.24 ± 0.45[a]	4.24 ± 0.78[a]
Eosinophils	(%)	3.000.21[a]	3.07 ± 0.69[a]	3.08 ± 0.73[a]

Data presented mean ± SD. Significance tested at $P \leq 0.05$. (G1): group 1 animals fed with GM sugarcane juice, (G2): animals of group 2 fed with non-GM sugarcane juice and (G3): animals of group 3 fed with water and normal diet (n = 10). Similar alphabets represented non-significant variation among different treatment groups.

Table 5
Biochemical and haematological data for female Wistar rats after 90-days sub-chronic toxicity.

Biochemical Parameters				
	Units	G1	G2	G3
Blood Sugar	(mg/dL)	71.00 ± 5.87[a]	70.40 ± 4.62[a]	70.00 ± 1.87[a]
Blood Urea	(mg/dL)	49.20 ± 11.26[a]	49.60 ± 2.41[a]	48.82 ± 2.93[a]
Serum Creatinine	(mg/dL)	0.62 ± 0.04[a]	0.60 ± 0.01[a]	0.60 ± 0.01[a]
Serum Uric Acid	(mg/dL)	2.26 ± 0.22[a]	2.21 ± 0.53[a]	2.23 ± 0.28[a]
Cholesterol	(mg/dL)	102.00 ± 22.31[a]	101.20 ± 7.60[a]	103.40 ± 5.03[a]
Triglycerides	(mg/dL)	112.60 ± 1.67[a]	113.40 ± 5.13[a]	113.95 ± 2.66[a]
HDL Cholesterol	(mg/dL)	31.40 ± 4.88[a]	30.00 ± 1.58[a]	30.30 ± 1.57[a]
LDL Cholesterol	(mg/dL)	54.00 ± 12.92[a]	55.00 ± 3.32[a]	55.10 ± 3.47[a]
Total Bilirubin	(mg/dL)	0.75 ± 0.14[a]	0.74 ± 0.13[a]	0.74 ± 0.04[a]
Direct Bilirubin	(mg/dL)	0.23 ± 0.04[a]	0.23 ± 0.04[a]	0.23 ± 0.04[a]
Indirect Bilirubin	(U/L)	0.52 ± 0.11[a]	0.52 ± 0.09[a]	0.52 ± 0.11[a]
SGPT	(U/L)	5.37 ± 0.41[a]	5.38 ± 1.06[a]	5.37 ± 0.55[a]
SGOT	(U/L)	7.40 ± 2.19[a]	7.42 ± 1.53[a]	7.40 ± 0.38[a]
Alkaline Phosphatase	(mg/dL)	645.60 ± 20.96[a]	646.20 ± 171.99[a]	645.30 ± 31.22[a]
Serum Proteins	(mg/dL)	7.69 ± 0.26[a]	7.68 ± 0.24[a]	7.67 ± 0.06[a]
Serum Albumin	(mg/dL)	3.57 ± 0.38[a]	3.56 ± 0.50[a]	3.57 ± 0.07[a]
Serum Globulin	(mg/dL)	4.13 ± 0.59[a]	4.12 ± 0.44[a]	4.13 ± 0.04[a]
A/G Ratio		0.92 ± 0.07[a]	0.91 ± 0.08[a]	0.92 ± 0.05[a]
Haematological Parameters				
Haemoglobin	(g/dL)	15.02 ± 0.23[a]	15.04 ± 0.88[a]	15.00 ± 0.81[a]
ESR	(mm/h)	3.37 ± 0.71[a]	3.33 ± 0.41[a]	3.26 ± 0.54[a]
TLC	(mcL)	10120 ± 3455[a]	10108 ± 678.47[a]	10145 ± 1218.40[a]
Neutrophils	(%)	20.40 ± 2.07[a]	20.40 ± 1.82[a]	21.80 ± 3.11[a]
Lymphocyte	(%)	73.00 ± 0.71[a]	73.20 ± 1.48[a]	73.60 ± 6.11[a]
Monocytes	(%)	4.56 ± 1.04[a]	4.56 ± 0.43[a]	4.54 ± 0.36[a]
Eosinophils	(%)	3.29 ± 0.85[a]	3.26 ± 0.43[a]	3.28 ± 0.81[a]

Data presented mean ± SD. Significance tested at $P \leq 0.05$. (G1): group 1 animals fed with GM sugarcane juice, (G2): animals of group 2 fed with non-GM sugarcane juice and (G3): animals of group 3 fed with water and normal diet (n = 10). Similar letters represented non-significant variation among different treatment groups.

assessment related to nutritional and toxicological evaluations are required prior to commercial release. A survey report (summarising 147 agronomical studies) has been published based on the performances of GM crops at various geographical regions in different countries predict that the benefits of biotech crops have increased the farmer's profit by 68% and the yield has been risen up to 22%. Although the seed cost of GM varieties can be higher, the farmer gets extensive benefits and that is the major reason for selecting biotech crops over the conventionally bred lines (Klümper and Quaim, 2014). So, there is a necessity to increase crop yield by 70% especially in the developing countries to fulfil the demand of food for increasing world's population, So, there is the need to cultivate the crops with novel traits and better performances (Delaney, 2015). The Product Biosafety Commission (KKHPRG), Indonesia (James, 2013) and the National Biosafety Technical Commission (CTNBio), Brazil (ISAAA, 2017) approved the GM drought tolerant and insect resistant sugarcane respectively, for commercialization.

The main goal of the risk assessment studies of GM *AVP1* sugarcane was to provide the scientific information that demonstrates it would not cause any toxic effect when consumed as a food. This enables the risk managers to determine if any additional measures are needed when making a well informed decision. Proximate compositional analyses from sugarcane juice revealed that the GM *AVP1* sugarcane was substantially equivalent to its non-GM counterpart. Pelleted rodent diets were nutritionally balanced for all dosed groups as reported by Delaney et al. (2014). The measured quantity of dose was orally administered for 90-days to the experimental animals according to the 90-day sub-chronic (OECD, 1988) and 14 days sub-acute toxicity guidelines (OECD, 2001), in accordance with studies recommended in research areas (FAO/WHO, 1996).

The Implementing Regulation (EU) needs a mandatory 90-day rodent feeding study as a part of the toxicological assessment in order to identify potential risks associated with GM food, even when no particular risk hypothesis might be expected. The European Food Safety Authority (EFSA) incorporate the recommendations into a legal text (EFSA, 2011). The results obtained from the current sub-acute and sub-chronic toxicological assessment in Wistar rats indicated that there

Table 6
Relative organ weights (%) of rats (male and female) after two weeks of oral administration of *AVP1* GM sugarcane juice, non-GM sugarcane juice and normal diet treatments.

Groups	G1		G2		G3	
Organ	Male	Female	Male	Female	Male	Female
Heart	0.29 ± 0.02[a]	0.31 ± 0.03[a]	0.28 ± 0.03[a]	0.30 ± 0.03[a]	0.27 ± 0.02[a]	0.30 ± 0.03[a]
Lungs	0.57 ± 0.07[a]	0.60 ± 0.08[a]	0.56 ± 0.03[a]	0.60 ± 0.06[a]	0.55 ± 0.11[a]	0.61 ± 0.08[a]
Liver	2.92 ± 0.11[a]	3.01 ± 0.31[a]	2.86 ± 0.28[a]	3.03 ± 0.48[a]	2.74 ± 0.27[a]	2.97 ± 0.70[a]
Spleen	0.16 ± 0.02[a]	0.19 ± 0.03[a]	0.16 ± 0.04[a]	0.18 ± 0.02[a]	0.16 ± 0.02[a]	0.17 ± 0.03[a]
Kidney	0.65 ± 0.05[a]	0.69 ± 0.14[a]	0.65 ± 0.07[a]	0.69 ± 0.11[a]	0.63 ± 0.05[a]	0.67 ± 0.04[a]
Gonads	1.07 ± 0.08[a]	0.07 ± 0.01[b]	1.03 ± 0.12[a]	0.07 ± 0.01[b]	0.94 ± 0.18[a]	0.07 ± 0.01[b]
Brain	0.65 ± 0.02[a]	0.71 ± 0.02[b]	0.65 ± 0.08[a]	0.70 ± 0.06[b]	0.63 ± 0.07[a]	0.68 ± 0.05[b]

Data presented mean ± SD values for each organ relative to body weight (%). Similar superscripts for each organ relative weight presented the non-significant differences ($P \geq 0.05$) and vice versa among all the treatment groups (male and female). (G1): group 1 animals fed with GM sugarcane juice, (G2): animals of group 2 fed with non-GM sugarcane juice and (G3): animals of group 3 fed with water and normal diet.

Table 7
Relative organ weights (%) of rats (male and female) after 90-days oral administration of *AVP1* GM sugarcane juice, non-GM sugarcane juice and normal diet treatments.

Groups	G1		G2		G3	
Organ	Male	Female	Male	Female	Male	Female
Heart	0.21 ± 0.04[a]	0.28 ± 0.02[a]	0.21 ± 0.01[a]	0.28 ± 0.05[a]	0.21 ± 0.04[a]	0.28 ± 0.03[a]
Lungs	0.56 ± 0.01[a]	0.76 ± 0.08[a]	0.55 ± 0.11[a]	0.75 ± 0.04[a]	0.55 ± 0.09[a]	0.75 ± 0.05[a]
Liver	3.05 ± 0.32[a]	4.18 ± 0.30[a]	3.05 ± 0.26[a]	4.17 ± 0.10[a]	3.05 ± 0.17[a]	4.16 ± 0.16[a]
Spleen	0.15 ± 0.02[a]	0.21 ± 0.01[a]	0.15 ± 0.01[a]	0.21 ± 0.01[a]	0.15 ± 0.01[a]	0.20 ± 0.01[a]
Kidney	0.53 ± 0.04[a]	0.71 ± 0.03[a]	0.53 ± 0.02[a]	0.71 ± 0.02[a]	0.52 ± 0.01[a]	0.72 ± 0.02[a]
Gonads	0.74 ± 0.03[a]	0.06 ± 0.01[b]	0.74 ± 0.02[a]	0.06 ± 0.00[b]	0.73 ± 0.01[a]	0.07 ± 0.01[b]
Brain	0.46 ± 0.02[a]	0.63 ± 0.01[b]	0.46 ± 0.01[a]	0.62 ± 0.02[b]	0.45 ± 0.01[a]	0.62 ± 0.01[b]

Values presented mean ± SD values for each organ relative to body weight (%). Similar superscripts for each organ relative weight presented the non-significant differences ($P \geq 0.05$) and vice versa among all the treatment groups (male and female). (G1): group 1 animals fed with GM sugarcane juice, (G2): animals of group 2 fed with non-GM sugarcane juice and (G3): animals of group 3 fed with water and normal diet.

were not statistically significant or treatment-related differences based on daily physical behaviour or signs, body weight, feed consumption, serum biochemistry, haematology, relative organ weight, and histopathology. The GM *AVP1* sugarcane juice exposure at a rate of 2100 mg/kg/body weight (OECD, 2001) for the acute toxicity evaluation and more than 5000 mg/kg/body weight (OECD, 1988) for the sub-chronic toxicity determination to rats was found to be non-toxic and safe. Similarly, Delaney et al. (2014) observed that there was no statistically significant or treatment-related differences after a 13-week (sub-chronic) exposure of herbicide tolerant (DP-Ø73496-4) canola on rats (male and female). These findings were similar to previously published reports on 13-week (sub-chronic) and 2-week (acute) rodent feeding studies for GM glyphosate-tolerant corn, corn borer-protected corn and corn rootworm-protected corn (Hammond et al., 2004, 2006a, 2006b) maize grain event DAS-Ø15Ø7-1 (Malley et al., 2007) maize grain event DAS-59122-7 (Appenzeller et al., 2008, 2009) (soybean DP-356Ø43–5, 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) event DAS-59122-7 of maize (Healy et al., 2008) corn rootworm-protected, glyphosate-tolerant MON 88017 corn (Arjó et al., 2012) genetically engineered multivitamin (β-carotene, ascorbate and folate) corn in mice (Harrison and Bailey, 1996); glyphosate-tolerant soybean and Bt-resistant corn (Huang, 2017; Juberg et al., 2009), Bt rice (Cao et al., 2010; Huang, 2017) proved that there were no adverse health effects.

AVP1 GM sugarcane juice did not show any cytotoxicity based on the results of the micronucleus test in the peripheral blood of rats after 90-day and 14-days toxicity experiments. The comet assay results revealed that *AVP1* GM sugarcane juice was not related to DNA damage in the blood (< 5000 mg/kg/body weight) in sub-chronic and in the acute (< 2000 mg/kg/body weight) studies. Similarly, Jaszczak et al. (2008) performed a micronucleus test and comet assay on mice fed with a dose containing GM triticale and reported that there were no statistically significant differences in the micronuclei frequency and DNA damage between the control and experimental groups of mice in all the treatment groups. They concluded that the diet based on GM triticale (*bar* transgene) did not reveal any chromosomal damage and had no role in the creation of DNA breaks or lesions.

This is the first report on biosafety assessment of GM sugarcane expressing the *AVP1* gene. This research work revealed that under the conditions of acute and sub-chronic toxicity evaluations, the *AVP1* (drought tolerant) GM sugarcane juice was found to be non-toxic in Wistar albino rats (male and female) when they were administered orally for 14-day and 90-day. Scientific data generated through this research work would be valuable and provides some important information that will support the safety assessment of GM *AVP1* sugarcane a pre-requisite for commercialization.

6. Conclusions

The present research was conducted to evaluate the potential risks associated with GM *AVP1* sugarcane juice via different biosafety assessment protocols. The results exhibited that *AVP1* (drought tolerant) GM sugarcane juice was non-toxic to Wistar rats after the acute and the sub-chronic toxicity studies. The data generated through this research will be valuable for the commercialization of *AVP1* GM sugarcane.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Conflicts of interest

We all the authors declare no conflict of interests either financial or personal relationships regarding this manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2019.05.034.

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