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EVALUATION OF DETRIMENTAL FACTORS OF DRINKING WATER AND THE EFFECT OF BLOOD GLUCOSE LEVELS AND ANXIETY STATUS: AN EXPERIMENTAL STUDY IN WISTAR ALBINO RATS USING ELEVATED PLUS MAZE

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Keywords:

Blood glucose level, Body weight, Drinking water, Elevated plus maze, Faecal bacteria

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ABSTRACT: This study is focused on evaluating the detrimental factors of drinking water and its relation to blood glucose level and anxiety status of non-stressed experimental rats. The study was conducted on 30 adult Wistar albino rats. The control group was treated with filtered water by reverse osmosis unit, and test 1, test 2, test 3, and test 4 groups were treated with well water from rural area 1, rural area 2, urban area 1, and pipeline water from urban area 2 respectively. Each week, body weight and fasting blood glucose (FBG) levels were measured. From the 11th week onwards, animals' anxiety response was measured using an elevated plus maze (EPM). On the 80th day of the experiment, all the animals were sacrificed, blood was collected and the pancreas was removed for further investigations. On 9th week of the experiment, FBG level became 83 ± 3.89 mg/dL, 94.6 ± 10.05 mg/dL, 79.8 ± 2.54 mg/dL, 79.5 ± 4.51 mg/dL, 107 ± 6.14 mg/dL for control, test 1, test 2, test 3 and test 4 groups respectively. The anxiety status of the control group in expressed mean and standard error which was 205 ± 22.64 seconds in the closed arm of EPM. The control group showed better responses in the FBG level and EPM test. The study revealed that there is a significant difference between the anxiety status of the study groups during the experimental period.

INTRODUCTION: Disease origin and its causes are concomitant with daily lifestyle and are not limited. Many studies are there on non-communicable diseases that resulted from changes in lifestyles. In the past scenario, most of the study was focused on drinking water and the risk of cardiovascular diseases.

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Studies were there on the origin of blood pressure and cardiovascular diseases (CVDs) from different drinking water sources. Many epidemiological studies on water hardness and cardiovascular disease mortality were conducted throughout the world and reported an association between cardiovascular mortality and water hardness [1].

A study was conducted on coastal areas in Bangladesh and they reported that pregnant women who have been using their drinking water are the reason for preeclampsia and hypertension in pregnancy [2]. The continuation of this study has been done in non-pregnant adults of coastal Bangladesh, which has shown that sodium

concentrations in drinking water are strongly associated with blood pressures [3]. Another study has reported drinking water containing even low-to-moderate inorganic arsenic may act as a sympathetic nervous system trigger for hypertension risk [4]. Another study has focused hardness of drinking water, and this characteristic feature of drinking water releases adequate magnesium from drinking water which reduces hypertension [5]. A similar report has been observed by Momeni *et al*., that calcium and magnesium content of drinking water may have a protective role against CVDs [6]. Earlier studies have reported that arsenic in drinking water is associated with type 2 diabetes [7-9]. Another study was conducted in animal models suggesting that arsenic impairs beta-cell function [10]. An earlier study strongly associates the relationship of acidity of drinking water and type 1 diabetes and another study is on elevated zinc in tap water lowered the risk of type 1 diabetes [11]. Evidence from a study suggested that factors in drinking water implicated in the environmental exposures contribute to the development of type 1 diabetes [12].

Anxiety is more prevalent in diabetic patients and is one of the mental health disorders. Gupta and associates reported that anxiety is increased in diabetic conditions; in their study, long-term diabetes resulting in significant anxiety-like behavioural problems in mice [13]. Drinking plenty of water is recommended by health care practitioners for the health management of various disorders like obesity, depression. This is explained by the water transport mechanisms which remove toxins and transport nutrients to tissues. Plenty of water facilitates brain function by signaling pathways. There is an inverse relation between plain water consumption and psychological status [14]. But, so far a link between metabolic mood disorder and the quality of drinking water is not yet established. So, we studied the quality of drinking water and its factors associated with diabetes and related anxiety in the present scenario.

However, no sufficient studies were done evaluating the association of drinking water and the effect of blood glucose level and anxiety behaviour. Hence, the present study tried to evaluate the effect of daily used drinking water and its relation to blood glucose level and anxiety status of non-stressed experimental healthy rats.

MATERIALS AND METHODS: All animal experiments were performed after the approval of the Institutional Animal Ethical Committee, Medical College, Thiruvananthapuram of the Committee for Control and Supervision of Experiments on Animals (CPCSEA). The study was conducted at the Animal House of Government Medical College, Thiruvananthapuram (IAEC NO.01/10/2018/MCT) in an experimental design on inbred rats. Adult Wistar albino rats of either sex were used for the experiments. Animals were housed in polypropylene cages with metallic mesh. Weighing scale (Docbell Co.), measuring jar, sterilized lancet, One Touch On Call® Plus glucometer (REF G133-117, *ACON* biotech Hangzhou Co. Ltd.) (variability value no greater than 20%), and other miscellaneous laboratory materials such as sterile water, 0.9% saline, 70% alcohol, cotton, coplin jar, 10% phosphate-buffered formalin and sample collecting tubes for blood collection and stool pellets. The animals were provided with standard laboratory animal feed and water *ad libitum*. Elevated plus-maze was manufactured in-house based on the information from a study [15]. Thirty rats weighing 140-230 grams were used for the experiment, which were divided into five groups having six animals in each group and were grouped as follows:

Experimental group:

1. Control group (n=6) healthy group were continued to be fed on filtered drinking water by reverse osmosis unit from the Animal House, Govt. Medical College, Thiruvananthapuram District.
2. Test I (n=6) were fed on drinking water from rural area 1 - well water from Thiruvananthapuram District.
3. Test 2 (n=6) were fed on drinking water from rural area 2 - well water from Thiruvananthapuram District.
4. Test 3 (n=6) were fed on drinking water from urban area 1 - well water from an urban area in Thiruvananthapuram District.

5. Test 4 (n=6) were fed on drinking water from urban area 2 - pipeline water from an urban area in Thiruvananthapuram District.

Water Sample Collection: Water samples of both well and pipeline water were collected from rural and urban areas of Thiruvananthapuram District of Kerala state. The well water was collected directly from the well using a bucket and rope and not used pumped water. A proforma was filled with a detailed history of the well including how long they used the well for drinking purposes, chlorination status, natural method of drinking water purification, distance of the well from a septic tank. Early morning freshwater samples were collected from each source. Before the collection of water it was confirmed that the selected water sample was purely used for drinking and household purposes and was sampled using 500 ml and 100 ml sterile plastic bottles for general and microbial analysis, respectively. Before the collection of a water sample, the container was rinsed with the respective water sample which was to be collected in it. All collected water samples were analyzed from the Water Analysis Centre, Kawadiyar of Thiruvananthapuram District.

Experimental Design:

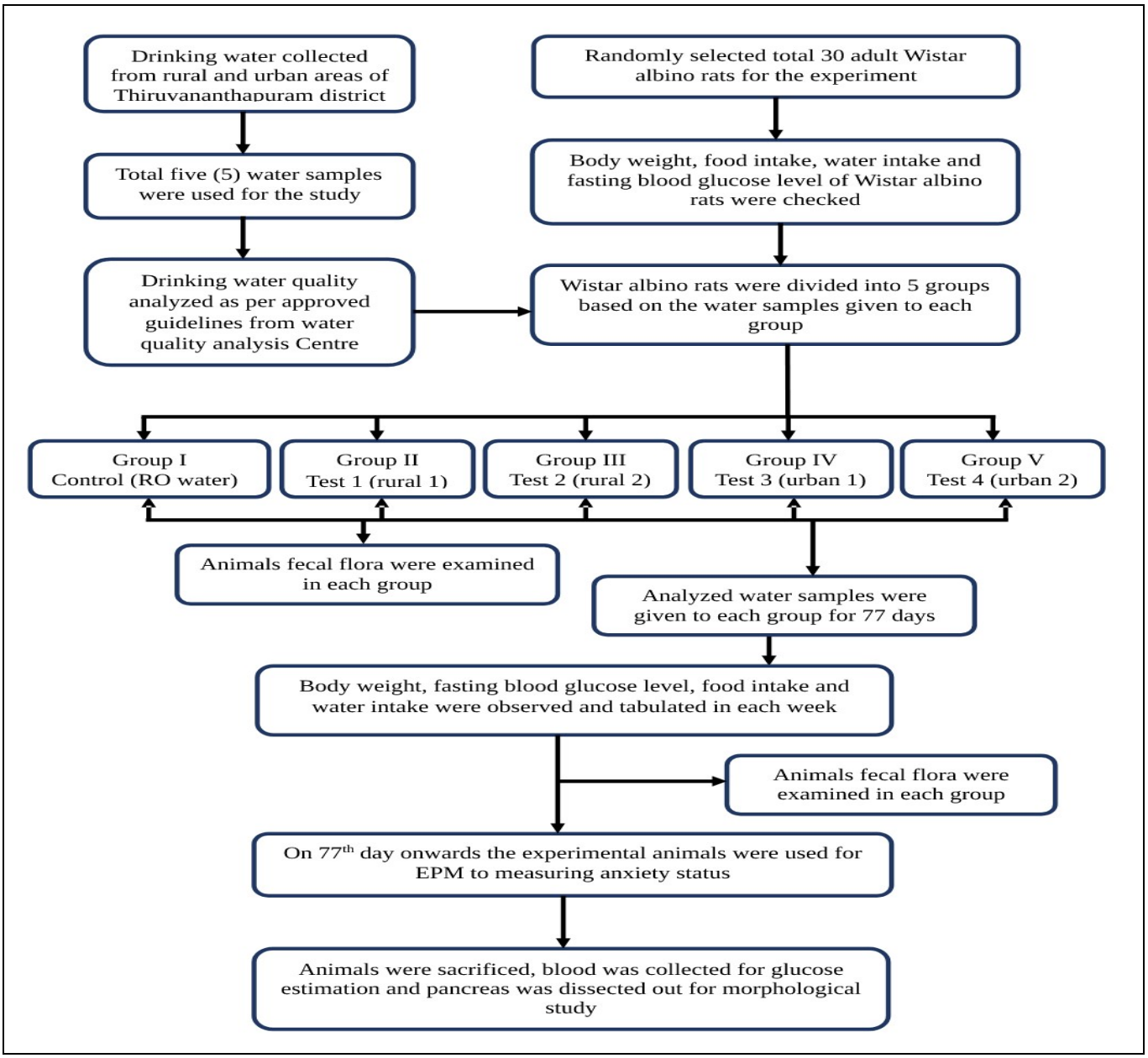

FIG. 1: SCHEMATIC REPRESENTATION OF THE STUDY PLAN

The total experimental period was 80 days. Schematic representation of the experiment is described as in **Fig. 1**. All the animals were acclimatized to the experimental conditions for a two-week observation period. Animals were randomly selected and housed in polypropylene cages. Before starting the experiment, the animal's body weight was measured using a calibrated weighing scale. Animals´ fasting blood glucose (FBG) level were measured using a calibrated On Call® Plus blood glucose meter (REF G133-117, *ACON* biotech Hangzhou Co. Ltd.) to confirm that the animal was not diabetic. On the same day, animal stool culture was examined using a wet stool pellet. On the first day of the experiment, all the animals were grouped according to their age, weight, food intake and water intake. Food and water were measured as per the number of rats accommodated in the cages (10g pellet/100g/ body weight for food and 15 ml/100g/body weight). Food and water were measured by weighing scale and measuring jar respectively. During the water administration period, any kind of external stress like force-feeding or use of an intubation tube was not used in the experimental procedure. Total food intake and water intake were measured daily throughout the experimental period. Body weight and blood glucose were checked every week, and it was continued to the eleventh week of the experiment. On the 77th day onwards, animals' anxiety was measured using an elevated plus-maze in a dark room to avoid distractions. Each animal trailing the floor of the maze was cleaned. The total duration of the EPM test was 6 minutes (360 seconds).

Animal Anxiety Measurement: Elevated plus maze is a commonly used anti-anxiety effect of pharmacological agents [16]. Here it was used to compare the anxiety level in rats after 80 days period of drinking water exposure and its relationship to the blood glucose of animals without any external stimuli. The maze was constructed using a wooden board. It was cross-shaped or configuration of a plus platform, consisting of two open and two closed arms [17] placed 50 cm above the ground. The dimensions of EPM is with two open arms (25 × 5 × 0.5 cm) across from each other and perpendicular to two closed arms (25 × 5 × 16 cm) with a centre platform (5 × 5 × 0.5 cm) as previously described [18]. The open arms have small (0.5 cm) walls to decrease falls, whereas the closed arms have a high (16 cm) wall to enclose the arm. The test is performed as mentioned in the study [19] that animal entry is counted when the four paws of the rat are placed in the respective arms. On the 80th day of the experiment, the early morning stool pellet was collected from each fasted animal. Subsequently, the animals were sacrificed using a carbon dioxide chamber. Blood samples were collected in an anti-coagulated (sodium fluoride and potassium oxalate) bottle, and blood glucose levels were measured analytically. A small portion of the pancreas was quickly removed from all the groups and immersed separately in 10% phosphate-buffered formalin in a separate coplin jar for morphological analysis of the tissue.

Stool Collection and Investigation: Stool was collected and examined two times, one at initial, and the other was done at the end of the experiment. Rats were individually selected and third pellet of the stool was collected in a common sterile bottle for each group. Pooled stool samples of each group were separately taken for inoculating in Selenite F broth and alkaline peptone water as liquid media and subculturing in Mac Conkey agar, xylose lysine dextrose agar and deoxycholate citrate agar as solid media and incubated for 48 hours and examined microscopically. All the procedures were set in aseptic conditions.

Blood Investigation: Collected blood samples were centrifuged at a speed of 3000 rpm. The obtained supernatant was used for the estimation of glucose by the hexokinase method [20] using Roche C311 (SL.No.115B820) biochemical analyzer.

Histology of Pancreas: Dehydrated and embedded tissues in paraffin were sectioned at a thickness of 5 µm. Prepared slides were stained with Haematoxylin and Eosin (H&E) [21] and observed by light microscopy using LABOMED (SL. No.091126825).

Statistical Analysis: The present study data were expressed in mean and standard error of the mean (SEM). Data were evaluated statistically and analyzed multiple comparisons of the study groups by Tukey's HSD (honestly significant difference) test which was significantly different from each other using software SPSS version 16.0.

RESULTS:

TABLE 1: WATER QUALITY PARAMETERS OF DIFFERENT WATER SAMPLES

Parameters	Components with expressing unit	Requirement (Acceptable limit) (mg/L)	Sample				
			1	2	3	4	5
General	pH	6.5 - 8.5	6.6	5.7	6.2	7.8	7.5
	Total dissolved solids (mg/L)	500	12	75	102	110	43
	Electrical conductivity (mmho/cm)	-	22	125	170	183	72
	Turbidity (NTU)	1	0	0	2	0	1
	Hardness	200	10	25	20	70	20
	Alkalinity	200	12	16	24	48	16
Chemical	Calcium (mg/L)	75	2	4	6	20	4
	Magnesium (mg/L)	30	1.2	3.7	1.22	4.9	2.4
	Sodium (mg/L)	-	0.5	15.9	28	12.2	8.2
	Potassium (mg/L)	-	0.1	0.46	5.5	1.6	1
	Carbonate (mg/L)	-	0	0	0	0	0
	Bicarbonate (mg/L)	-	15	20	29.2	58	19.5
	Sulphate (mg/L)	200	1	1.8	20	2.0	3
	Chloride (mg/L)	250	5	28	28	26	14
	Fluoride (mg/L)	1	Not detected	Not detected	Not detected	Not detected	Not detected
	Iron (mg/L)	0.3	0	Not detected	0.25	0.03	0.05
	Nitrate – N (mg/L)	10	0.2	1.4	0.7	1.0	1.4
	Lead (mg/L)	-	Not detected	0.4136	Not detected	Not detected	Not detected
Biological	Total coliform (MPN/100 ml)	Nil	Nil	>1100	>1100	29	Nil
	Fecal coliform (MPN/100 ml)	Nil	Nil	>1100	>1100	29	Nil

Sample 1 – filtered water from animal house, sample 2 - well water from rural area 1, sample - 3 well water from rural area 2, sample - 4 well water from an urban area and sample - 5 pipeline water from an urban area. '-'indicates not defined, mg/L – milligram in a litre, mmho/cm – millimhos in a distance in cm NTU-nephelometric turbidity unit, MPN- most probable number.

Table 1 shows the drinking water quality parameters of collected drinking water from the rural and urban areas. Sample 1 from filtered water of the animal house, sample 2 well water from rural area 1, sample 3 well water from rural area 2, sample 4 well water from an urban area and sample 5 pipeline water from an urban area. From the results, the rural area's sample pH is slightly acidic when compared with urban areas. Urban well water and urban pipeline water pH are slightly alkaline, as shown in the above table. Water sample of the animal house was also slightly acidic and it is under limited range, may be due to the effect of the filter unit for the purpose of regular use in animal house.

TABLE 2: COMPARISON OF FAECAL BACTERIA IN POOLED SAMPLES IN EACH GROUP

Animal groups with numbers	Species identified from the faecal sample before treatment	Species identified from the faecal sample after treatment
Control (n=6)	*Escherichia coli* – approximate 74 colonies	*Escherichia coli* – approximate 23 colonies
Test 1 (n=6)	*Escherichia coli* – approximate more than 10^3 colonies	*Klebsiella species* - approximate 60 colonies *Staphylococcus aureus* – approximate 32 colonies *Escherichia coli* – approximate 30 colonies
Test 2 (n=6)	*Escherichia coli* – more than 10^3 colonies	*Escherichia coli* – approximate 10^4 CFU/mL *Staphylococcus aureus* – approximate 10^5 CFU/mL
Test 3 (n=6)	*Escherichia coli* - approximate 23 colonies	*Escherichia coli* - approximate 100 colonies
Test 4 (n=6)	*Escherichia coli* – approximate 73 colonies	*Escherichia coli* – approximate 10^4 CFU/mL *Staphylococcus aureus* – approximate 10^3 CFU/mL

Control- water sample 1 treated, Test 1- water sample 2 treated, Test 2- water sample 3 treated, Test 3 – water sample 4 treated and Test 4 – water sample 5 treated group. CFU/mL – Colony-forming unit in a milliliter.

Water samples of well sample 2, sample 3 and sample 4 contain coliform bacteria but it was not seen in pipeline waters, sample 1 and sample 5, respectively. This may be due to the effect of chlorination in the pipeline water. **Table 2** shows the comparison of faecal bacteria in each group; bacteria *Escherichia coli* and *Staphylococcus aureus* are the commonest bacteria in the majority groups, but total colonies per milliliter varied due to the effect of various factors of the drinking water sample. Before treating with respective water samples in the experimental animals, the total number of bacteria in faecal samples was decreased when compared with after treatment with drinking water. *Klebsiella sp.* is observed only in test 1 group rats.

TABLE 3: COMPARISON OF BODY WEIGHT IN VARIOUS GROUPS

Body weight in gm per Week	Groups (n=6)				
	Control	Test 1	Test 2	Test 3	Test 4
0	147.5± 6.92	188.3± 10.69	150± 2.88	229.2± 5.97	209.2± 10.91
1	153.3± 6.54	200.8 ± 12.14**	155.8± 3$^{@@}$	235.8± 5.38 $^{\wedge\wedge\ \%\ \#\#}$	220± 9.04$^{++\&\&}$
2	160.8± 8.21	214.2 ± 15.99**	162.5± 2.81$^{@@}$	240.8 ± 5.38$^{\wedge\wedge\ \#\#}$	225.8± 7.23$^{++\&\&}$
3	168.3± 9.88	215.8 ± 15.35 **	169.2± 3.51$^{@}$	252.5± 5.43$^{\wedge\wedge\ \#\#}$	232.5± 7.04$^{++\&\&}$
4	175.8± 10.19	214.2± 17.29 **	171.6± 2.47$^{@}$	255.8± 5.68$^{\%\#\#}$	239.2± 7.35$^{++\&\&}$
5	178.3± 10.38	225.8± 21.92	176.6± 2.47	260± 5.16$^{\wedge\wedge\#\#}$	247.5± 8.92$^{++\&\&}$
6	183.3± 10.05	229.2± 21.61	180± 1.82	256.6± 4.22$^{\wedge\wedge\#\#}$	260.8± 13.92$^{++\&\&}$
7	189.2± 10.52	230.8± 26.72	182.5± 2.5	252.5± 4.23$^{\wedge\#}$	263.3± 14.35$^{+\&\&}$
8	194.2± 10.12	228.3± 27.71	184.2± 2.38	250.8± 4.54$^{\#}$	265.8± 15.07$^{+\&\&}$
9	199.2± 10.12	225± 28.22	186.6± 3.8	245.8± 3.27	267.5± 15.42$^{+\&\&}$
10	201.6± 9.97	225± 29.74	190± 4.65	245± 3.42	267.5± 14.53$^{+\&}$
11	205.8 ± 10.19	226.6± 29.87	196.6 ± 5.72	245.8± 4.54	270 ± 14.94 $^{\&}$

*****-denotes control group compared with test 1, ^-denotes control group compared with test 3, **+**-denotes control group compared with test 4, **@**-denotes test 1 group compared with test 2, **%**-denotes test 1 group compared with test 3, **#**-denotes test 2 group compared with test 3, **&**-denotes test 2 compared with test 4. Every single symbol represents significance at 5% level and the double symbol represents significance at 1% level. Values are expressed in Mean ± Standard Error Mean (SEM).

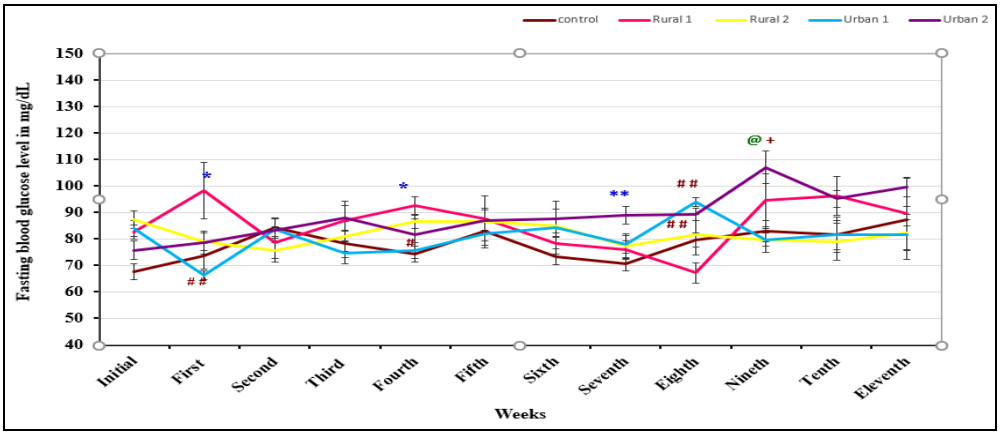

FIG. 2: COMPARISON OF FASTING BLOOD GLUCOSE LEVEL OF VARIOUS GROUPS IN THE EXPERIMENTAL PERIOD
Fasting blood glucose level in mg/dL (milligram per deciliter) is expressed as the mean ± standard error of the mean. Statistical significance difference between groups as denoted as symbols as *, #, @ and +. *- Compared with the control group, there is significance between test 1 or well water sample from rural area 1 treated group at 5% ($p < 0.05^{*}$) level, # - compared with test 1 group, significance between test 3 or well water sample from urban area 1 treated group at 1% $p < 0.01^{\#\#}$) level, @ -compared with test 2 group, significance between test 4 or pipeline water sample from urban area 2 treated group at 5% ($p < 0.05^{@}$) level and + - compared with test 3 group, significance between test 4 or pipeline water sample from urban area 2 treated group at 5% ($p < 0.05^{+}$) level.

From multiple comparison tests by Tukey's HSD, there was a significant difference between the control group and the test group's body weight. It is clear from **Table 3** that the *p*-value is less than 0.01 ($p<0.01$) or 0.05 ($p<0.05$) which is considered as statistically significant. From the analysis there was a statistically significant difference between in the animals body weight and is represented as control group with test 1** ($p<0.01$) at 1%, control group with test 3$^{\wedge\wedge}$($p<0.01$) 1 % or $^{\wedge}$ ($p<0.05$) 5 %, control group with test 4 $^{++}$ ($p<0.01$) 1 %, test 1 group with test 2 $^{@@}$ ($p<0.01$) 1 %, test 1 group with test 3 $^{\%}$ ($p<0.05$) 5 %, test 2 group with test 3 $^{\#\#}$ ($p<0.01$) 1 % and test 2 group with test 4 $^{\&\&}$ ($p<0.01$) 1 % level.

Fig. 2 shows fasting blood glucose level is expressed as the mean and standard error of the mean of all the animals at the onset of the study was 67.6 ± 2.9 mg/dL, 82.6 ± 2.71 mg/dL, 87.3 ± 3.48 mg/dL, 84 ± 3.04 mg/dL, 75.8 ± 3.43 mg/dL for control, test 1, test 2, test 3 and test 4 respectively. After nine weeks of continuous daily non-stressful treatment of drinking water, it became 83 ± 3.89 mg/dL, 94.6 ± 10.05 mg/dL, 79.8 ± 2.54 mg/dL, 79.5 ± 4.51 mg/dL, 107 ± 6.14$^{@+}$mg/dL for control, test 1, test 2, test 3 and test 4 respectively as shown in the above figure (See **Fig. 2**) indicating that there is a significant difference between the fasting blood glucose level of animal groups especially test 1 and test 4 groups.

TABLE 4: COMPARISON OF FASTING BLOOD GLUCOSE LEVEL USING SERUM

Groups (n=6)	Fasting blood glucose level in mg/dL	Comparison between groups (n=6)	Tukey HSD Q statistics	Tukey HSD *p*-value	Significant
Control	80.6 ± 4.84	Test 1	1.1774	0.899995	Nil
		Test 2	1.201	0.899995	Nil
		Test 3	1.4835	0.809628	Nil
		Test 4	1.5895	0.768829	Nil
Test 1	97.3 ± 15.87	Test 2	0.0235	0.899995	Nil
		Test 3	0.3061	0.899995	Nil
		Test 4	0.4121	0.899995	Nil
Test 2	97.6 ± 19.89	Test 3	0.2826	0.899995	Nil
		Test 4	0.3885	0.899995	Nil
Test 3	101.6± 14.10	Test 4	0.106	0.899995	Nil
Test 4	103 ± 11.46	Test 3	0.106	0.899995	Nil

Fasting serum blood glucose level in unit mg/dL (milligram per decilitre) are expressed as Mean ± Standard Error Mean (SEM). Nil – Statistically no significant difference between the groups.

Table 4 shows the fasting blood glucose (FBG) concentration of test 3 and test 4 groups were slightly elevated when compared with the other groups. It means that there is a minute change in the FBG level of animals that may be due to the effect of used drinking water. **Table 4** also shows a comparison of each group statistically analyzed by Tukey's HSD test of fasting blood glucose level, but there is no statistical significance between the groups.

Anxiety Response Study - using Elevated Plus Maze Experiment (See Fig. 3 - Fig. 7): Fig. 3 and **4** represents a comparison of the number of open arm entry and closed arm entry in the elevated plus-maze, respectively. From **Fig.3** symbol represents ##-significance at 1% ($p < 0.01$) level between test 3 or well water sample from urban area 1 treated group and test 2 or well water from rural area 2 and test 3 or well water sample from urban area 1 treated group and test 4 or pipeline water sample from urban area 2 treated groups. Compared with the control group, test 1 and test 4 showed a lesser entry to the closed arm. The pipeline water sample treated group showed poor responses than the other water sample treated groups (see **Fig. 4**).

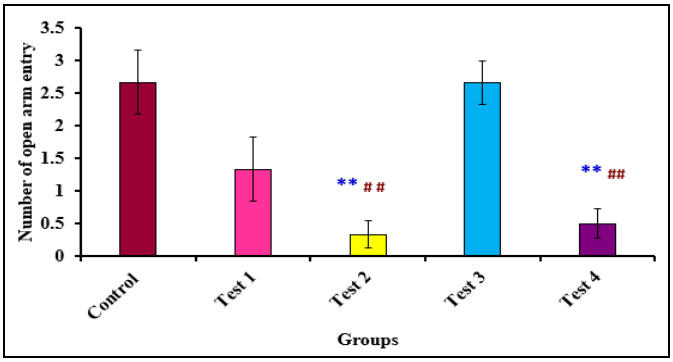

FIG. 3: COMPARISON OF NO. OF OPEN ARM ENTRY IN THE EPM. The number of entries into the open arm is expressed as mean ± standard error of the mean. **-significance at 1 % ($p < 0.01$) level compared with the control group, ##-significance at 1 % ($p < 0.01$) level compared with the test 3 group.

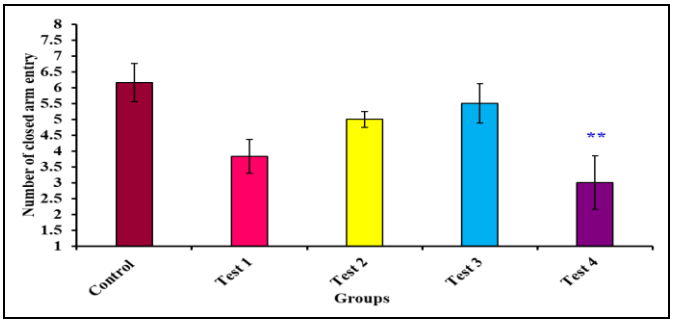

FIG. 4: COMPARISON OF NO. OF CLOSED ARM ENTRY IN THE EPM. The number of entries into the closed arm is expressed as mean ± standard error of the mean. **-significance at 1% ($p < 0.01$) level compared with the control group.

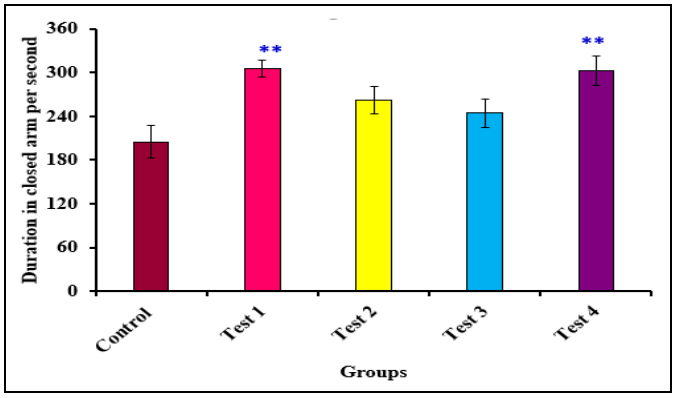

FIG. 5: COMPARISON OF TOTAL DURATION IN THE CLOSED ARM OF THE EPM. The duration of stay in the closed arm is expressed as mean ± standard error of the mean. **-significance at 1 % ($p < 0.01$) level compared with the control group.

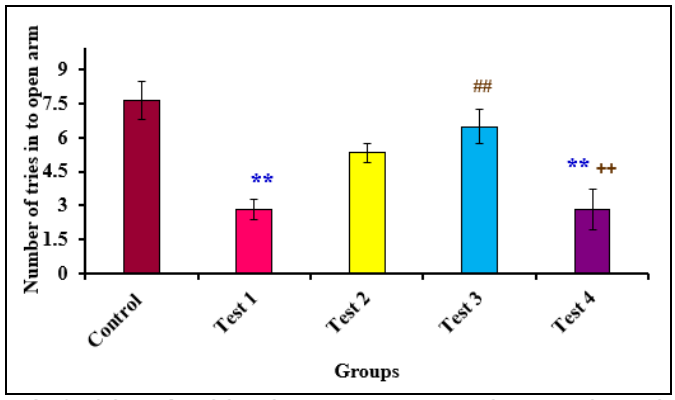

FIG. 6: COMPARISON OF THE NUMBER OF TRIES INTO THE OPEN ARM OF THE EPM. The number of tries into the open arm is expressed as mean ± standard error of the mean. **-significance at 1 % ($p < 0.01$) level compared with the control group, ##-significance at 1 % ($p < 0.01$) level compared with the test 1, ++-significance at 1 % ($p < 0.01$) level compared with the test 3 group.

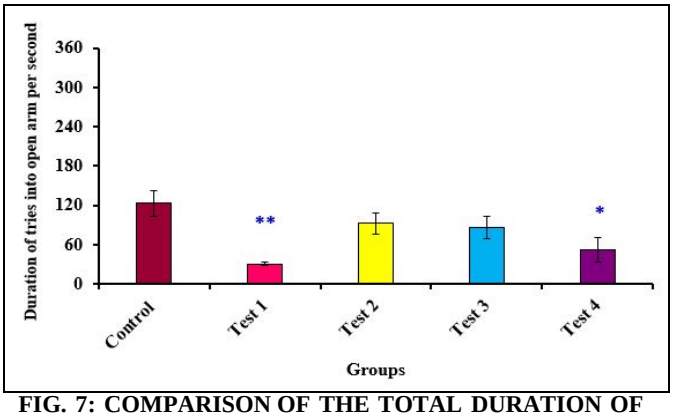

FIG. 7: COMPARISON OF THE TOTAL DURATION OF TRIES TO OPEN ARM OF THE EPM. The total duration of tries into the open arm is expressed as mean ± standard error of the mean. **-significance at 1% ($p < 0.01$) level and *-significance at 5% ($p<0.05$) level compared with the control group.

The control group spent less time (205 ± 22.64 seconds) in the closed arm of EPM indicating that there is a significant reduction in their anxiety status. Other groups showed that duration 305.2 ± 11.43 sec, 262.2 ± 18.82 sec, 244.2 ± 19.88 sec and 302.5 ± 19.6 sec for test 1, test 2, test 3 and test 4 respectively (see **Fig. 5**). **Fig. 6** represents the number of entries into the central point of the EPM or number of tries into the open arm. From the above graph it is clear that test 1 and test 4 groups showed lesser entry into the centre of the plus maze.

While, test 3 group showed better responses when compared with test 4 or pipeline water treated animal group. From **Fig. 7**, it is clear that the other groups spent a few seconds (sec) trying to enter the open arms from the center platform when compared with the control group. The total duration of time used to try to enter the open arms of control group and other groups are expressed in mean ± SEM is 123.3 ± 19.29 sec, 30.8 ± 3.1 sec, 92.6 ± 15.95 sec, 86.2 ± 16.4 sec and 52 ± 18.83 sec respectively. **Fig. 8** shows light microscopic (magnification 400 X) pictures of pancreatic tissue (H & E staining). Pancreatic islets (IL) and acinar cells (AC) in different groups.

a. Control group showing normal islets (lightly stained cells) with acinar cells (surrounding with darkly stained cells).

b. Test 1 group showing ruptured islets portion with normal acinar cells.

c. Test 2 group showing normal islets with surrounding acinar cells.

d. Test 3 group showing cellular atrophy in islets with normal acinar cells.

e. Test 4 group showing ruptured islets with normal acinar cells.

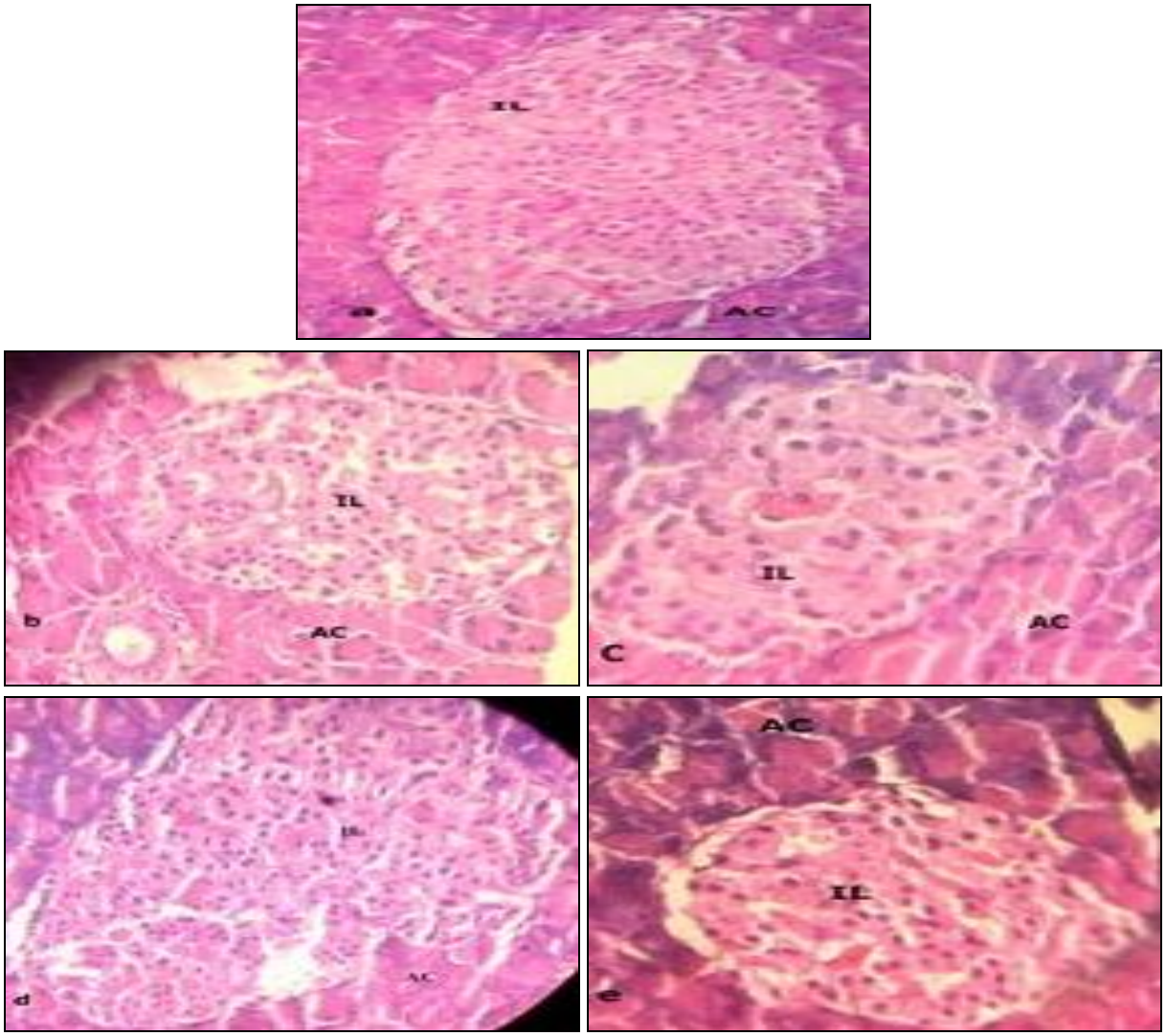

FIG. 8: RAT PANCREAS HISTOLOGY. Pancreatic islets (IL) and acinar cells (AC) in different groups. (a) Control group showing normal islets (lightly stained) with acinar cells (surrounding with darkly stained). (b) Test 1 group showing ruptured islets portion with normal acinar cells. (c) Test 2 group showing normal islets with surrounding acinar cells. (d) Test 3 group showing cellular atrophy in islets with normal acinar cells. (e) Test 4 group showing ruptured islets with normal acinar cells.

DISCUSSION: At present, many health-related issues arise from environmental challenges that include the availability of drinking water supply, sewage and solid waste management. As per the report of the world health organization (WHO), the drinking water of hundreds of millions of people is seriously polluted, and by 2025 half of the world's population will be living in water-stressed areas [22]. Water pollution is a substantial change of water bodies due to anthropogenic activities. Drinking water may contain various impurities that are of physical, biological and chemical nature. The most dangerous impurity is of biological nature, which causes human health problems [23]. The main issue regarding drinking water sources in urban areas is due to lack of natural groundwater sources, appropriate management of industrial and agricultural waste, lack of appropriate systems that controlled for water supply by means of stable interruptions in the systems due to technical problems. Another challenge in drinking water supply in both the rural and urban areas is collection of drinking water from open sources, nature or design of water storage tank, construction, decontamination procedures and management of water supply [24]. This worsened quality of the supplied water ultimately causes health issues. In our study, we evaluated the effect of daily used drinking water and its relation to blood glucose level and anxiety-related behavioural responses using an elevated plus maze (EPM) in experimental rats. Water is a universal solvent and an important constituent of the body and has a vital role in body functions. Drinking water helps to

maintain body fluids and homeostasis. Human body is exposed to various chemical reactions that may be happening by contaminated air, water or food. Many studies have reported on the aspects of the toxicity of metals or ions in drinking water. These studies report that some metals like lead, mercury, cadmium and metalloid arsenic which has a negative effect on physiology and develop an increased risk of incidence of diabetes and related metabolic syndromes [25-27]. Recent study findings suggested that low or moderate arsenic levels in drinking water or food slowly affect our system and is associated with the incidence of diabetes [28]. But no report has yet proved the aspects of anxiety behaviour and its correlation in daily used drinking water. This study focuses on the relation between anxiety and blood glucose levels during the consumption of drinking water.

Drinking water and its quality are associated with several factors, for example, the area of the drinking water source, depth of well, distance from the septic tank and storage procedure of water. Drinking water from natural sources is underground water, which allow their physicochemical composition and organoleptic properties to remain constant and also protect from the risk of contamination [29]. The quality of water is determined by three major parameters such as physical, chemical and microbiological properties. However, the quality of water depends on the permissible limits of ions present in it, pH level, TDS level, absence of bacteria and so on. But sometimes, heavy metals appear in water based on industrial wastes.

It reaches our body and accumulates in our system. Once it is absorbed, our body cannot get clear it off. Even though there are no immediate problems, heavy metals keep on accumulating in our bodies. Later, it develops heavy metal poisoning, including damage to all organs, disrupts the function of red blood cells, the central nervous system, physiological and behavioural problems. Severe toxicity from these metals may cause cancers [30]. Another review discussed that using metal-contaminated water leads to hair loss, liver cirrhosis, renal failure and neural disorders [31]. A high level of certain toxic metals in the water that causes acidity of drinking water has been associated with the incidence of type 1 diabetes [32].

Drinking water has a laxative effect due to a higher concentration of magnesium and sulphate ions [33], but in our study diarrhoea was not reported in animals due to the presence of permissible limit of magnesium and sulphate ions as per the guidelines followed. In this study, we assessed all the parameters of the drinking water. Both well and pipeline water sources were included in the study and the quality of drinking water was analyzed. In our result, the pH of the water sample was under permissible limits except in sample 2, which was collected from the rural area. From **Table 1,** it is clearly seen that water sample 2 was more acidic than other drinking water samples. The variation of pH is due to the effect of the water source. Moreover, from **Table 2** *Klebsiella* sp. was identified only from the test 1 group which was treated with water sample 2.

Klebsiella sp. is a gram-negative bacteria, belonging to the family Enterobacteriaceae, which was not observed in the other groups after treatment or in the same group before the treatment. So, it is assumed that the presence of *Klebsiella* sp. in the faecal sample may be due to the acidic nature of the water sample 2 when compared with the other samples. In this observation, we suggest that this is not only the effect of drinking water, but also there are disturbances observed during acidic water treatment. Simultaneously, all identified bacteria are under normal flora and do not cause any pathogenic effect. Environmental changes may be a significant factor in the growth of bacteria. *Staphylococcus* sp. is gram-positive bacteria that is not a normal gut flora [34].

In this study, the growth of bacteria. *Staphylococcus* sp. is a gram-positive bacteria and part of normal gut flora of both humans and animals [34]. However, the number may increase during gut infections. In this study, the growth of *Staphylococcus* was observed in faecal samples of animal groups when treated with drinking water collected from rural area 1 (test 1), rural area 2 (test 2) and urban area 2 (test 4) samples. The increased number of Staphylococcus in test 1, test 2 and test 4 does not suggest the development of a gut infection in animals. However, some factors in the water enhanced the growth of *Staphylococcus*.

This work studied the effect of drinking water on the total number of faecal flora, which is proportional to the number of gut flora. However, the activity and effects of gut flora during drinking water treatment was not studied here. Gut microbiota contributes a significant host glycaemic regulation and insulin sensitivity as per the report of Gérard and Vidal [35]. Gut bacteria also influence the nervous system, including the stress-associated hypothalamic-pituitary-adrenal (HPA) axis [36]. Appropriate environmental factors, endogenous or exogenous, influenced the growth of gut microbiota, which may be altering the secretion of neurochemicals and modulate the gut-brain axis [37]. Hence, further studies are needed in the area to specify the role of the gut biome.

Generally, faecal indicator bacteria are *Escherichia coli*, faecal coliforms, and faecal *Streptococci* typically used to measure the quality of water [38] for various purposes, including drinking water supply purposes. They are natural inhabitants of the gastrointestinal tracts of humans and other warm-blooded animals. These bacteria, in general, cause no harm. Well water, generally a source of groundwater that is conventionally considered to be the water source least susceptible to contaminants like indicator bacteria. This is due to the nature of groundwater from deep or limited aquifers. Our study assessed total coliforms in the drinking water that might be distinguished in each of the samples.

All the samples were assessed by indicator bacteria having measurable numbers, but it was not specified in our study. Our study sample named sample 1 was not disturbed with any other indicator bacteria, but others are having a measurable number of faecal coliforms. This is explained by several reasons that may be due to the effect of nearby connection with a contaminated source such as leakage from wastewater or contaminated surface water or subsurface sources such as a septic tank, a broken or leaking drain line or improperly designed well. Other findings from our study of chlorinated pipeline water sample 1 treated group showed a smaller number of coliforms compared with the other groups. Chlorination is the universal method for disinfection [39] of drinking water and reduces epidemic diseases [40]. Seasonal variations is also a significant factor in the growth of coliforms in water [41].

Stress can be defined as anything that tends to change the control of our body and emotions. The stress response is a series of physiological and behavioural changes involving the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system that helps the organism to manage these tasks [42]. Stress mediating responses results in hypersecretion of cortisol leading to obesity, insulin resistance, dyslipidemia, hypertension and glucose intolerance and plays a major role in the development of metabolic syndrome [43]. Mukherjee and Banerjee's study reported that metabolic syndrome animals spontaneously developed anxiety-like response [44]. The EPM is a widely accepted test in the study of anxiety in rodents and other animal models [45, 46].

The present study also evaluated the drinking water effect and anxiety behaviour in experimental groups with the EPM. In this study, behavioural responses with EPM is an effective tool to examine anxiety behaviour. However, the study of stress level was not proved based on hormonal status and EPM co-relation. A large cross-sectional study says that drinking plain water decreased the risk of depression and anxiety in adults [14,] but simultaneously, another study showed the point that there is a bidirectional link between mental health and metabolic status [47].

Few clinical studies had been done on the association of anxiety disorder with metabolic syndrome based on triglyceride level and blood pressure [48]. Uncontrolled secretion of cortisol has been implicated in the development of diabetes and obesity; this highlights the role of psychological stress on both conditions [49]. Water is one of the constituents of our body composition, and its deficiency leads to body imbalances. Although, the availability of natural sources of water or getting unpolluted water is a rarity nowadays. Association of hydrogen-rich water and improved mood, anxiety, and autonomic nerve function, a study by Mizuno *et al.,* showed that the hydrogen-rich water administration is an effective method to reinforce the quality of life and maintain good health [50]. An interventional study conducted in school children showed that promoting drinking habits that reduced the risk of overweight among them [51]. Our study concluded that animals treated with drinking water had a significant weight increase as the age.

There was no weight loss observed in animal groups. Another finding in our study shows that the animal group treated with drinking water having acidic pH and a detectable amount of lead was significantly reduced when correlated with the changes in the body weight. The focussed area of the study was to assess the anxiety behaviour of the experimental rats and their blood glucose level correlation during the period of drinking water treatment. In general, when the body is under stress, the adrenal glands start the release of glucose stored in various organs, which often leads to elevated levels of glucose in the bloodstream. Altered blood glucose can damage neuronal function. The EPM values showed increased anxiety in diabetic rats when compared with non-diabetic rats, which was evident in the decreased number of open arm entries and less time spent in the open arm as explained in Rajashree *et al*., study but they reported no gross locomotor activity changes in diabetic groups [52]. In the present study, it is observed that there was an alteration in fasting blood glucose levels in various groups at various weeks. From **Fig. 1**, it reflects in the rat groups treated with sample 1 and sample 4.

But the present result was not statistically significant on the 80th day of the experiment. In animal model to induce type 1 diabetes, there was anxiolytic-like effect in the EPM behavioural test. The test result showed a significantly higher frequency of entries in open arms, lower entries in close arms and a higher percentage of entries in open arms in the treatment group than in the diabetes untreated group [53]. Our results showed that there was variation in the fasting blood glucose levels when compared with the animals' initial blood glucose levels. Although, it is a short period of study and showing an elevated glucose level, we assume that if the treatment is extended for a longer period, it may develop in to a diabetic state. Naturally and electrochemically reduced water has a significant therapeutic role in oxidative stress. A study reported that naturally reduced water decreases anxiety-related behaviours and prevent oxidative stress in rats [54]. The present study does not explain the oxidative stress due to the factors associated with drinking water and related elevation in blood glucose levels. According to the present study we observed that animals treated with drinking water sample 2 from well water from the rural area and sample 5 pipeline water from an urban area and their response in the EPM test's in closed arm entries showed that longer duration than the other groups that spent in the closed arms. According to an earlier study, there is increased time spent in open arms indicating a lower degree of anxiety in the experimental animals [55]. It indicates that there is a suspected factor of drinking water that may influence animal EPM behavioural responses. The light microscopic photograph of the pancreas section showed the destruction of pancreatic islets compared with surrounding acinar cells (see **Fig. 8**). It appeared as spaces or cellular necrosis in test 1, test 3 and test 4 groups after exposure to water treatment. This change reveals that unwanted substances in the water samples are responsible for the abnormalities. This finding agrees with the study of Ramesh *et al*., reporting that substances like lead and cadmium are potent endocrine disruptors that can damage the normal texture of the pancreas [56]. In the light of the present observation, we hereby suggest that the reason may be the factors of drinking water like acidity, indicator bacteria and other unknown factors in drinking water. Further studies can reveal the reason which was not traced in this study.

CONCLUSION: A slight elevation in the fasting blood glucose level was observed in animals after treatment with drinking water. The changes in fasting blood glucose level and the relation of anxiety behaviour was observed in the EPM test responses of non-stressed animals. These observations may be due to the effect of factors of drinking water. This study revealed that factors of daily used drinking water may develop metabolic abnormalities and anxiety-related disorders in the future. The gut microflora communicates with the brain through different neural and hormonal pathways called the gut-brain axis especially through neurotransmitters and signalling molecules. Further studies are to be done in the areas of factors of drinking water, the role of the gut microbiome and its relation to the status of blood glucose and related issues.

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