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Effect of polluted water on DNA integrity of *Labeo rohita* inhabited in river Ravi

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Abstract: Aquatic pollution is responding to DNA damage in several aquatic organisms including fishes. It can cause malignancies, reduced growth, abnormal development, and decreased survival of embryos, larvae, and adults. Genetic analysis of animal relies on high yields of pure DNA and consequent analysis relies on the quantity and quality of DNA. An organic method of DNA isolation was used to isolate the DNA from different organs tissues, fins and scales of fish *Labeo rohita.* In the current study, two different groups reared (obtained from Fish Seed Hatchery Manawan, Lahore) and natural (collected from the river Ravi of province Punjab, Pakistan) fish *Labeo rohita* have been taken and were evaluated for DNA quality and quantity, along with studied DNA damage through gel electrophoresis and comet assay. The quality and quantity of isolated DNA from different organs were observed as tissue>fins>scales of fish *Labeo rohita*. The quality of isolated DNA from both groups (reared and natural) of fish *Labeo rohita* in tissues, fins, scales, and total mean value of each sample is (1.890 & 1.328 µg/µl, 1.683 & 1.264 µg/µl, and 1.780 & 1.262 µg/µl) while the total mean of DNA quantity in each sample is (1998.75 & 1276 µg/µl, 1381.5 & 1152, and 1378.75 & 1231.25 µg/µl) respectively, and a significant difference was found on DNA quantity and quality in both groups. Comet assay was performed for the study of DNA damage and results were compared in a reared sample and natural samples. The total mean value in grams of reared and the natural group is calculated 22.44 ± 0.34g and 20.13 ±0. 483g, while the mean length of both groups reared and natural is l2.80 ± 0.12cm and l3.55 ± 0.09cm respectively. In reared group tail was not observed while in the natural group tail was observed which indicate the DNA damage. The current study finds polluted aquatic environment badly damage the DNA of fish *labeo rohita*.

Keywords: Aquatic pollution, Fish organs (tissue, fins and scales), *Labeo rohita*, toxicity, river Ravi, and *Comet assay*.

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I. INTRODUCTION

Ecogenotoxicology addressed the potential effects of environmental pollution and genotoxic agents on the ecosystem, also the effect of these pollutants on organisms. These deadly environmental factors (EFs) are assessed by adopting several methods of genetic toxicology [1]. These EFs cause genetic mutations, damage in DNA, and various kinds of cancers in living organisms, such as humans and other aquatic species [2]. Similarly, the aquatic environment (AE) also affected by EFs. Fish used as a well-known genetic model for the analysis of pollution effects on aquatic life [3-4]. Today's scientists focused on the study of potential threats of pollutants on the AE, and they have used fish as the best model organism to evaluate the severe effects of metals (teratogens, carcinogens, mutagen, and clastogens) on life [5] because AE serve as suitable repositories for man's technological and biological wastes [6].

Pollution outcomes consist of a level of biological organizations, from subcellular to molecular level, by way of community-level to an organism population, and as a result of socio-economic concerns. Some physical factors such as ultraviolet rays, excessive temperature, heavy metals and other ionizing radiation also responses to a level of biological organizations along with DNA damage. Moreover, the AE also becomes polluted by the addition of agricultural, industrial, and home effluents containing more than a few natural and inorganic pollutants into streams and small rivers without being properly handled and are the main sources of contaminants in AE [7-8]. Pollution effects are greatly determined by synergetic methods related to the

combination, chemical mixture and the kinds of contaminants. The kinds of responses also depend on the developmental stage of the fish and influenced by different EFs such as salinity, temperature, oxygen, and pH [9]. Among heavy steel ions, some have no direct harmful effect on DNA; however, they play an essential role in the oxidative damage of DNA [10]. Heavy metals and organic contaminants are some of the most important pollutants that enter the AE by anthropogenic things and affect the DNA quality of aquatic organisms [11-12]. So, to perform molecular research on aquatic life, the researchers are facing poor DNA quality which is a critical problem for most amplification-based analysis, such as low quality of DNA decreases the PCR reaction efficiency. Also, DNA harm can most likely occur due to long-time exposer to a hazardous AE. That is why it is planned to study the severe effect of aquatic pollution on fish genetics [13].

Various methods are available to evaluate the pollution impact on DNA integrity of fish. The current study described the effect of pollution on DNA quality and quantity that was isolated from different organs (tissue, fins & scales) of fish *Labeo rohita* samples (private fish hatchery Lahore and river Ravi) and the level of DNA damage was evaluated by two different methods; gel electrophoresis and Comet assay.

II. Results & Discussion

The Natural (polluted) and Reared (non-polluted) samples of *Labeo rohita* were used in this research work. Four samples of a natural group from four different sites of river Ravi and four samples of Reared group were collected from Fish Seed Hatchery Manawan, Lahore to assess the quality, quantity and DNA damage of *Labeo rohita*.

DNA damage was measured by adopting two different methods gel electrophoresis and comet assay. The organic method was used for DNA extraction and the comet assay alkaline comet assay method is used. The mean total weight of reared group was 22.44g while it was 20.l28g in the Natural group. The mean total length of the reared group was l3.55cm while l2.8cm in the natural group. The quality and quantity of isolated DNA from different organs were observed as tissue > fins > scales of fish *Labeo rohita* that explain in (Table 1).

DNA qualification

In the current study, we perform DNA isolation from tissue, fins and scales of two groups (natural and reared) of fish *Labeo rohita*, and focus on examining the effect of aquatic pollution on DNA quality and quantity in both groups of fish samples. Fishes use fins as locomotor organs that propel and used to turn the body of fish into the water. The quality and quantity are measured between the natural and reared. The quality and quantity of DNA samples isolated from fins; the total mean values of DNA quality and quantity in the natural group are (1.26 and 1152 µg/µl), while in samples of the reared group it is (1.68 and 1381.5 µg/µl) respectively. The current study defines higher quality and quantity of DNA in the reared group as compared to the natural group. The quality and quantity of DNA samples isolated scales of the reared group the total mean values are (1.78 and 1378.75 µg/µl), while in the natural group the total mean values of DNA quality and quantity are (1.26 and 1231.25 µg/µl), which defines the DNA quality and quantity is higher in the reared group as compared to the natural group. Furthermore, the quality and quantity of tissue samples in the reared group is (1.89 and 1998.75 µg/µl) while in the natural group it is 1.328 and 1276 µg/µl). These findings revealed the DNA isolated from reared group is of higher quality and quantity. The overall results of DNA quantity and quality analyses in the current study are tissues have more DNA as compared to the fins and scales of both samples as shown in Table 1.

Comet assay analysis

Genotoxicity representing DNA damage is caused due to aquatic toxicity. In the current study, we perform two methods comet assay and gel electrophoresis to check the level of DNA damage in both samples. For gel electrophoresis, only the DNA samples were taken from each group and run for evaluating the DNA damage and fragmentation in samples. We noted samples collected from river Ravi doesn't show proper bands of DNA as compared to samples of fish belonging to the reared group of *Libo Rohita* that collected from the different site of river Ravi. The % of DNA damage is the representation of the size of the tail of DNA is formed. The findings of comet assay in the current study of reared group and natural group revealed in the reared group no tail is formed that indicating no DNA damage, while in samples of the natural group (samples obtained from river Ravi) several tails of DNA damage were noted. It means DNA damage was present in the natural group of *Labeo rohita* and no DNA damage in the reared group. The Mean S.D (Mean ± S.D) of the Comet assay is defined in Table 2. The illustrations of DNA damage and fragmentation in gel electrophoresis and *Comet assay* of both reared and natural groups are presented in Figure 1.

Fish serves as a useful genetic model for the evaluation of pollutants level in the aquatic environment [3, 6]. *Labeo rohita* was chosen as a bio-indicator in this research work for evaluating the DNA quality and concentration in three organs of tissue, fins and scales respectively.

Table 1: Samples of fish *Labeo rohita* that obtained from different sites of each group reared and natural, their molecular weight and length, and quality and quantity of DNA that isolated from different organs tissue, fins and scales:

Samples	Locations	(Weight / length)		Tissue		Fins		Scales	
		Weight (g)	Total length (cm)	Quality (µg/µl)	Quantity (µg/µl)	Quality (µg/µl)	Quantity (µg/µl)	Quality (µg/µl)	Quantity (µg/µl)
Natural Group	N1	20.28	l3.30	l.423	1130	l.237	1209	l.28	1225
	N2	22.39	l2.90	l.342	1055	l.311	1007	l.24	1120
	N3	l8.18	l2.50	l.320	l565	l.241	1189	l.32	1240
	N4	l9.66	l2.50	l.230	l355	1.275	1133	l.2l	1340
	Total Mean	**20.13**	**l2.80**	**l.32**	**1276**	**1.26**	**1152**	**l.26**	**1231.25**
Reared Group	R1	24.00	l4.00	2.12l	1335	1.670	1210	l.892	l385
	R2	22.60	l3.50	1.957	1270	1.555	1253	l.934	l255
	R3	22.l9	l3.59	1.835	2485	1.857	1686	l.630	l4l0
	R4	20.99	l3.20	l.648	2905	1.652	1377	l.742	l465
	Total Mean	**22.44**	**l3.55**	**l.89**	**1998.75**	**1.68**	**1381.50**	**1.78**	**l378.75**

The present study designed to evaluate the effect of pollution on different organs of fish *Labeo Rohita* is an inhabitant of the river Ravi; and results showed that DNA is damaged in aquatic species by pollution as shown in (figure 1) of gel electrophoresis and comet assay. This DNA damage might be due to production of nascent reactive species such as hydroxyl ions (OH^-), superoxide ion of oxygen (O_2^-), hydrogen peroxide (H_2O_2) in cells by inductive oxidative stress caused by different pollution. These ions/radicles are produced in normal conditions in cells, which are readily neutralized by variety of enzymatic and scavenging process. Furthermore, cells has internally repairing system by expression of DNA repairing genes [14]. Previous studies also explained DNA fragmentation and infertility, oxidative stress, and genetic factors can occur in response to long term exposure to the polluted environment or industrial toxins [15], such as fishes in urban and industrials areas showed higher damage as compared to reared fishes [16]. Similarly, the effect of pesticides is also studied on lizard *Tupinambis merianae* showed same results of DNA damage [17] and another study of heavy metals (copper 30 µg L^{-1}) effect on coral *Montastraea franksi* showed that DNA was damaged statistically significant [14].

The water quality of effluent and surface water was monitored with bioassays in aquatic animals. Genotoxins are chemicals that responsible for DNA damage in a variety of aquatic organisms and fishes ultimately affecting the economy of fish production significantly. Genotoxicity not only reduces the "fitness" in wild fish populations but also pose risk to human health via the food chain [18]. In the current study DNA was isolated from different organs; tissue, scale and fins of fish *Labeo rohita* that collected from two different aquatic environments, the reared and the natural. Using a spectrophotometer, the quality of DNA in samples of both groups was checked at A260 / A280nm and concentrations of DNA in grams. The current study revealed high quality (tissues; 1.89, fins; 1.68, and scales; 1.78 µg/µl) and concentration (tissues; 1998.75, fins; 1381.50, and scales; 1378.75 µg/µl) of DNA in samples of the reared group of fish *Labeo rohita* as compared to the quality (tissues; 1.328, fins; 1.264, and scales; 1.262 µg/µl) and quantity (tissue; 1276, fins; 1155, and scales; 1231.25) of the natural group. The study found DNA isolated from tissue samples is of higher quality and had high concentration as compared to DNA extracted from fins and scales sample of *Labeo rohita*, these results are in line with previous reported study on fish Vega-Retter and his fellows reported the same kinds of results [19].

Table 2: Samples of fish *Labeo rohita* and *Comet tail* length (Mean ± S.D):

S. No	Subject	Mean Tail Length (µm) ± S. D
1	Control	Normal round cells
2	Site 1	18.2 ± 1.50
3	Site 2	l7.7±l.02
4	Site 3	17.2± l.70
5	Site 4	16.9±0.75

Furthermore, the concentration of DNA in scales samples of fish that get from the private fish form is higher than the fish DNA samples of the natural group that obtained from the river Ravi. The findings of the current study verified the results of the previous study of

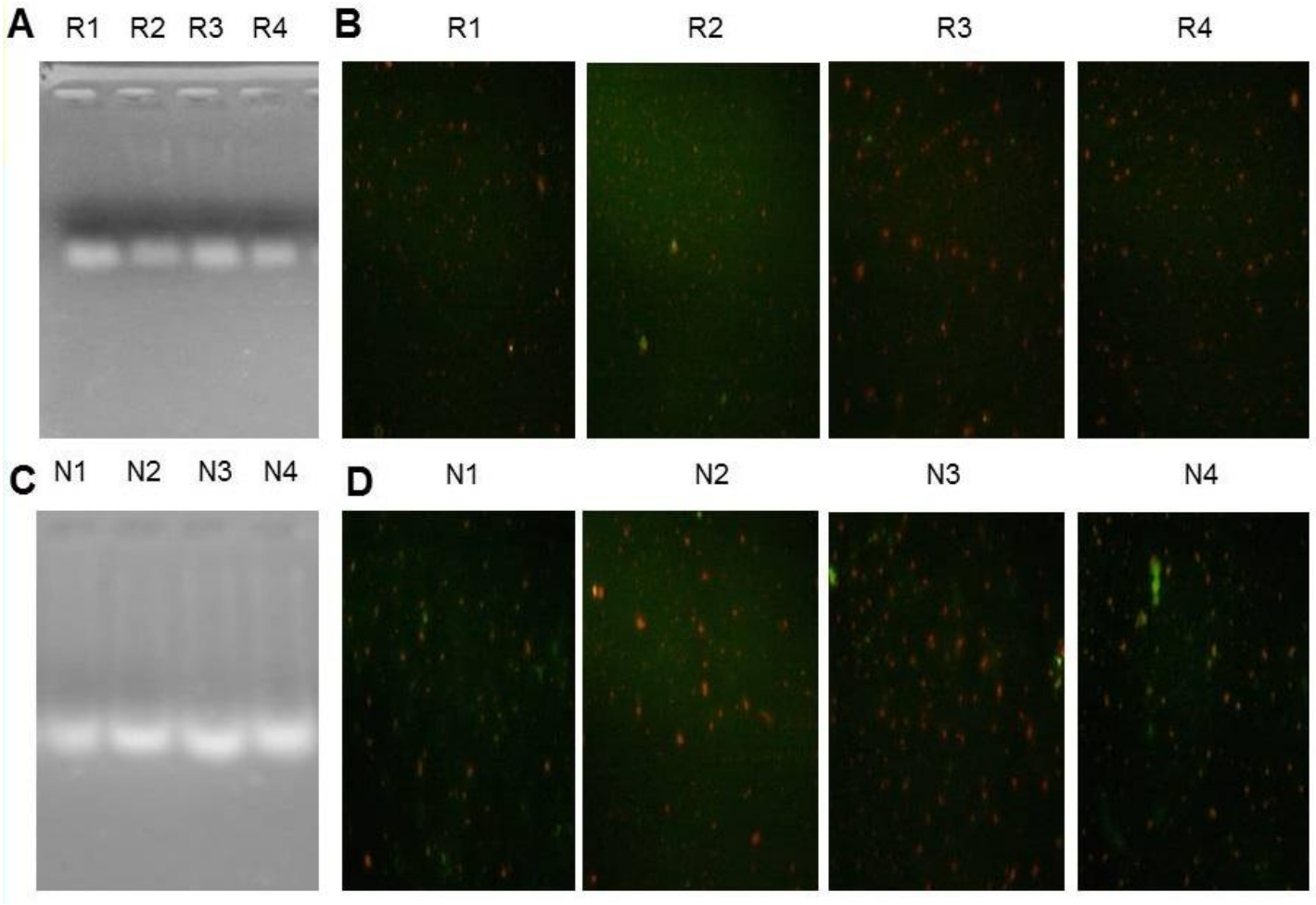

Figure 1: DNA damage findings of *Labeo rohita*: A) gel electrophoresis findings of *Labeo rohita* from reared samples. **B)** Comet Assay results of *Labeo rohita* of Reared populations. **C)** Gel electrophoresis findings of *Labeo rohita* from Ravi River. **D)** Comet assay results of fish *Labeo rohita* samples obtained from river Ravi.

Mehboob and his co-researcher published in 20l5 they reported DNA quality and quantity badly effects due to pollution, he studied the fish species of *Cirrhinus mrigal* that also get from river Ravi [20]. In addition, the current study also revealed the fish samples of *Labeo rehita* that collected from the Ravi river which having high weight and big length give a low concentration of DNA in (tissues; 1055,, fins; 1007, and scales; 1120 µg/µl) and also bad quality (tissues; 1.342, fins; 1.311, and scales; 1.24 µg/µl) DNA respectively. It is concluded that the DNA concentration of reared group is higher than the natural group noted, and findings of the current study become more coherent, compared to the findings of Tripathi and his fellows, who reported that the nucleic acid quantity is decreases in polluted samples [20].

Damage was also checked by comet assay and results were compared between samples of the reared group and natural group. In reared group tail was not formed while in the natural group tail was formed which indicate DNA damage in samples of the natural group of *Labeo rohita*. When the quality of samples was observed, there is no fragmentation in reared samples were noted, while all-natural samples of fish *Labeo rohita* showed fragmentation and this fragmentation increased with the increasing of fish weight. The findings of the current study are very close to the findings of Flammarion and his lab mates that published in 2002 on title level of DNA damage in cells [21]. Furthermore, the current study also supported by the findings of Czene and his co-researcher online in 2002, he also reported that the DNA fragmentation caused most likely due to pollution in fishes [22]. Our results supported the theory that the genetic analysis of aquatic species will be used as a promising marker for the identification of contaminant effects on aquatic species. As we observed more fragmentation in those fish samples get longer exposer of polluted water. It means long term exposer to pollutants caused greater DNA fragmentation which results in greater DNA damage.

III. Conclusion

Pollution causes severe genetic changes and is a big threat to aquatic life including fishes. In the current study we study the fish samples for the identification of the severe effect of pollution on DNA of fish Labeo rohita for quality and concentration of DNA, and also we study the DNA damage due to pollution. The current study reported that in non-polluted (reared fish) samples DNA quality and concentration is pure and high compare to the fish sample of river Ravi which we get poor quality and low concentration of DNA. Furthermore, at present is also noted that the DNA obtained from tissue samples is pure and of high concentration as compared to scales and fins. The order of DNA quality and quantity in a different organ of fish are like this (tissue ≥ scales ≥ fins). Moreover, the findings of comet assay in the present study describe the fish sample obtained from river Ravi results severely DNA damage due to the polluted aquatic environment. Our study highlights the importance of clean water reserves that are going to polluted day by day due to the increase in industries and population. Furthermore, our study described, pollution as a big challenge for aquatic life.

IV. Materials & Methods

Sample collection

This study was conducted on fish collected from the Ravi river. Ravi is located along the India Pakistan border and meanders substantially along the alluvial plains of the Amritsar and Gurdaspur districts of Punjab before entering Lahore, Pakistan [23]. Four samples of the natural group were collected from different sites of the river Ravi and reared group is collected from Fish Seed Hatchery Manawa, Lahore Pakistan. The samples were persevered in an ice box and then tissue, fins and scales are separated for DNA isolation (Figure 2).

DNA extraction and quantification

DNA was isolated from the fish samples of tissue, fins and scales by a Wasko *et al* method with some modification [24]. To enhancing the DNA quantity and quality following modifications were made in the standard protocol; 50 mg sample was taken from each tissue, fins and scales samples, the lysis buffer 350µl was added. For scales, 30 µl of urea was added in a lysing solution. Proteinase-K is added 20µl for tissue samples, 30µl for fins and 50µl for scales respectively. The tubes holding samples were incubated at 56°C, 60°C and 70°C for tissue, fins and scales samples overnight. 400µl phenol was added and centrifuged at l3000 rpm for ten minutes and repeat this step two time. 500µl of pure isopropanol with l0µl sodium acetate was added and samples were placed for 1-2 hours in the refrigerator. The supernatant was removed and 250µl of 70% ethanol was added and centrifuged for 5 min at 8000rpm to purify the DNA. The supernatant was discarded and place the pallet for air dry at room temperature overnight. The dried DNA pellet was dissolved in 50µl of TE buffer and saved at 4°C for future use. The quality and DNA concentration were measured by spectrophotometer at 280, 260, and 230 wavelengths. The quality and quantity are measured by applying the following formulas.

DNA Quality = Absorbance at 260/280nm
DNA Quantity = OD at 260nm x dilution fold x 50/l000

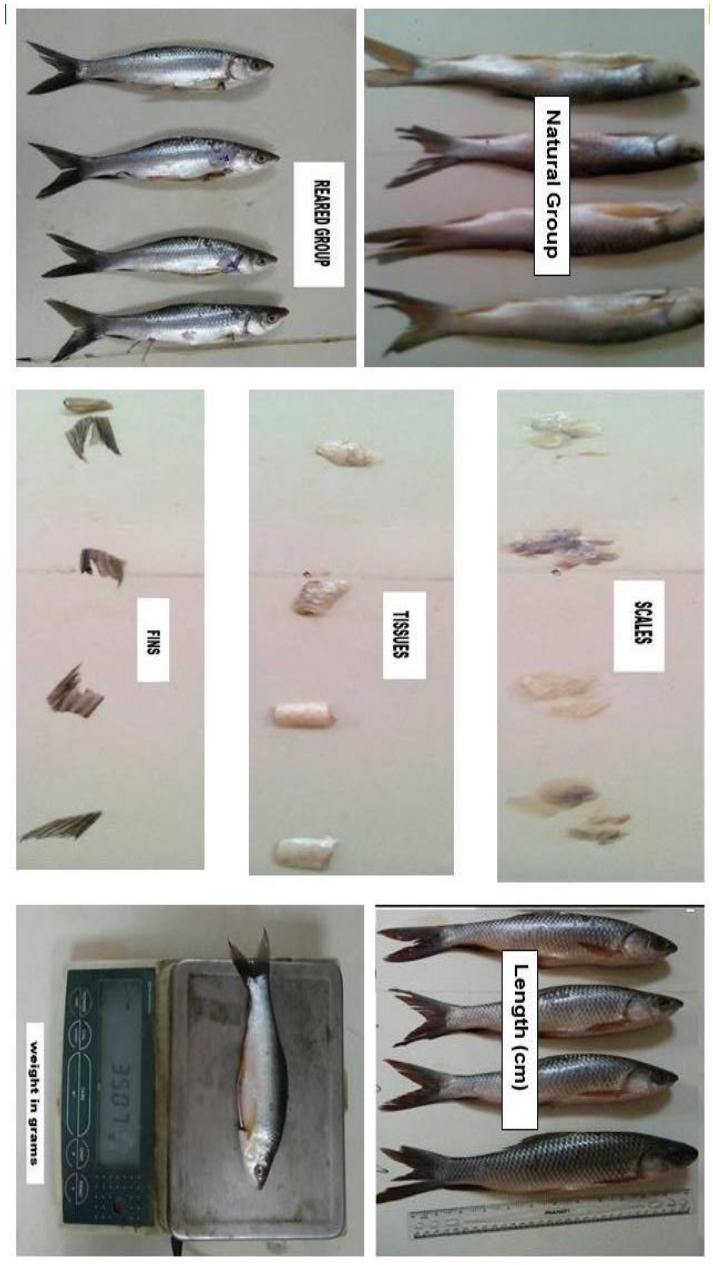

Figure 2: Samples of fish *Labeo rohita* that collected from different sites of river Ravi (Natural group) and private fish hatcheries (Reared group), and weight and length of each sample was calculated.

Comet assay procedure

The comet assay was performed according to the previously described protocol [25], under a fluorescence microscope at different magnification. 50 cells were scored from each slide. Cells having DNA damage had a comet-like appearance with a definite tail that defines the level of DNA damage. Cells without head were excluded from the calculation. While the cells having a definite round shape, appearance shows no DNA damage. By using CASP software the level of DNA damage was analyzed.

Authors' contribution: Design and supervision of the experiments: M.Noman and S.Ali; Sample collection: M. Tayyab and M.U. Farooq; Experiments perform: M. Tayyab and M.U. Farooq; DNA isolation and electrophoresis analysis: M. Tayyab, and S. Ali; Comet assay analysis: M. Tahir, M. Tayyab, and M.F.Tahir; manuscript writeup: M.Noman, M.Tayyab, and M.Tahir; critical analysis: M.Noman and M.F.Tahir. Funding for this study was supported by M.Tahir and S.Ali.

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