EVALUATION OF GENOTOXIC POTENTIAL OF GROUNDWATER FROM INDUSTRIAL AND FARMING SITES ON COLON CANCER CELL LINES

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DECLARATION

I declare that the dissertation entitled **"EVALUATION OF GENOTOXIC POTENTIAL OF GROUNDWATER FROM INDUSTRIAL AND FARMING SITES ON COLON CANCER CELL LINES"** has been prepared by me under guidance of Dr. Sanjeev K. Thakur, Assistant Professor, Center for Biosciences, School of Basic and Applied Sciences, and Dr. Sandeep Singh, Assistant Professor, Center for Genetic diseases and Molecular medicines, School of Emerging Life Sciences Technologies, Central University of Punjab. No part of this dissertation has formed the basis for the award of any degree or fellowship previously.

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CERTIFICATE

It is to certify that **RIMPLEJEET KAUR** has prepared her dissertation entitled **"EVALUATION OF GENOTOXIC POTENTIAL OF GROUNDWATER FROM INDUSTRIAL AND FARMING SITES ON COLON CANCER CELL LINES",** for the award of M.Sc. degree of the Central University of Punjab. She has carried out this work at the Center for Biosciences, School for Basic and Applied Sciences, Central University of Punjab.

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ABSTARCT

"Evaluation of Genotoxic Potential of Groundwater from Industrial and Farming sites on Colon Cancer Cell Lines"

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Water is critical component on earth which is essential for life. There are number of reservoirs of water like surface water, groundwater etc. used for drinking, domestic as well as industrial purposes. Numerous studies have shown that water is contaminated by many pollutants like chemicals including pesticides, fertilizers, and heavy metals including arsenic, uranium, and exposure to this contaminated water may cause number of health problems including cancer. It has been found that in Punjab, specifically Malwa region's groundwater is highly contaminated by pesticides and heavy metals which may correlate to several health problems in this region. In this context, mechanisms of genotoxic potential activity of the groundwater, from thermal plant and villages Deon and Joganand, was estimated in the present study. The groundwater caused reactive oxygen species production, mitochondrial membrane depolarization, which ultimately caused cell death. Groundwater also affected the antioxidant system enzymes, so all these factors may be the cause of health problems in this region.

Signature of student Sign of supervisor Signature of Co-supervisor

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LIST OF ABBREVIATION

Sr. No.	Full Forms	Abbreviations
1.	Deoxyribonucleic acid	DNA
2.	Ultraviolet	UV
3.	3-(4,5 – dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide	MTT
4.	Dimethylsulphoxide	DMSO
5.	Nicotinamide adenine dinucleotide phosphate	NADPH
6.	Reactive Oxygen Species	ROS
7.	Dulbecco's Modified Eagle's medium	DMEM
8.	Fetal Bovine Serum	FBS
9.	Phosphate Buffer Saline	PBS
10.	Sodium Hydroxide	NaOH
11.	Dihydrodichlorofluroscien diacetate	H_2DCFDA
12.	5, 5′, 6, 6′-tetrachloro-1, 1′, 3, 3′-tetraethylbenzimi- dazolylcarbocyanine iodide	JC-1
13.	Hydrochloric acid	HCl
14.	Total Dissolved Solids	TDS
15.	Superoxide Dismutase	SOD
16.	Magnesium chloride	$MgCl_2$
17.	World Health Organization	WHO
18.	Ethylenediaminetetraacetic acid	EDTA
19.	Carbon dioxide	CO_2
20.	Milligram	mg
21.	Milliliter	ml
22.	Microliter	µl

Chapter 1
Introduction

Water, required invariably by all living things to survive, has been known as the elixir of life. Surface water, ground water and underground water make up the main reservoirs of water, consumed for domestic as well as industrial purposes. Mostly, it is the ground water that is utilized for multiple purposes, present just below the earth's surface (Gupta and Agrawal, 2011). For drinking purpose, there are number of water supply sources like hand-pumps, tube-wells, dug-wells and submersible pumps. Increase in population size has taken its toll on the water sources, decreasing these resources in order to meet up the increasing demands (Dhembare, 2007). For all domestic, drinking as well as industrial purposes, people depend upon these sources. Drinking water, of late has been found to be contaminated with different types of pollutants (Gupta and Agrawal, 2011), gradually resulting in number of health problems.

Different studies have revealed that there are number of places where the drinking water has been polluted by different contaminants such as heavy metals, chemicals including pesticides and fertilizers. Heavy metals, such as cobalt, copper, manganese, molybdenum, vanadium, strontium and zinc, are the higher atomic weight elements possessing metallic substance like characters at room temperature. Although, these are essential for carrying out various physiological functions, but excessive amount of these heavy metals have proven to produce toxic effects on organisms. Other heavy metals like mercury, cadmium and lead are not beneficial for organisms and their accumulation can cause serious illness in organisms (Singh *et al.,* 2011). Water is polluted by many types of heavy metals which come from industrial as well as agricultural wastes (Aulakh *et al.,* 2009).

World population is increasing in stochastic manner, so to fulfill the ever increasing food requirements, farmers use pesticides and fertilizers to increase the yield (Mahajan and Tuteja, 2005). Thus, agriculture is becoming one of the main sources of water toxicity owing to number of chemicals used by the farmers which make the water toxic. These pesticides enter into the ground water through the soil. By this

way, we can say that pesticides and fertilizers are main sources for water toxicity. Drinking water is also contaminated by disinfection byproducts like chlorination by products and others like arsenic, nitrates etc. (International Agency for Research on Cancer, 1991). Numerous studies conducted to determine the underlying problem have failed to yield an answer, thus, demanding further studies in this direction. Increase in population size, industrialization, urbanization and agriculture are some of the problems upheld as the prime reasons for water toxicity (Aulakh *et al.,* 2009).

This research work has been carried out with an aim to evaluate the effects of water on human cells. For this purpose, water samples were collected at different time points of Malwa region and tested on cancer cell lines.

1.1 Hypothesis

Past research support the evidence of contaminated water in Malwa region of Punjab. The data suggests that pesticides and heavy metals are main reasons behind contamination of water. As compared to other regions of Punjab, utilization of pesticides is more in Malwa region because this area is mainly cotton growing area. Hence the contaminated water may result in genotoxicity which may gradually culminate in various health problems including cancer.

1.2 Rationale behind current research

The water in Malwa region observed to be contaminated by chemicals and heavy metals, which may cause toxicity to cells through ROS production, DNA damage or mitochondrial instability which cause apoptosis.

1.3 Objectives

To meet the needs of research aim following objectives has been formulated;

1. Physicochemical characterization of water sample from different regions of Bathinda.
2. To assess the genotoxic potential of water samples on human cell lines.
3. To measure the oxidative stress and antioxidant activity in response to water treatment on human cell lines.

Chapter 2

Review of literature

Water is used for different purposes like washing, agriculture, drinking. Drinking water sources are classified as surface water and ground water. Water is contaminated by different types of contaminants like manmade contaminants and natural contaminants (Chaudhary *et al.,* 2002; Gupta and Agrawal, 2011). Although, ground water is less contaminated than surface water, ground water has been found to be contaminated by different types of heavy metals which may enter in water by different sources like mining, agriculture, industries etc. (Figure 2.1) (Chaudhary *et al.,* 2002). Due to growing population and industrialization, the demand of water has also increased in the recent times. Therefore, ground water and surface water is supplied to urban cities which are contaminated by heavy metals (Dixit *et al.,* 2003).

Figure 2.1 Contaminants in ground water of India (Chaudhary *et al.,* 2002)

Contaminated water is held responsible for occurrence of numerous diseases, thus, resulting in higher mortality. In India, there are number of fresh water sources, but in comparison to urban areas, rural areas have shortage of safe drinking water of good quality (Sankararamakrishnan *et al.,* 2005).

2.1 Contaminants present in water

Water is contaminated by different types of substances, collectively called contaminants of water, which mainly encompass heavy metals, chemicals including pesticides and fertilizers, other salts, microbial contamination, etc. Heavy metals, which are higher atomic weight elements, are main contaminants of water. Heavy metals has a metallic substance like character at room temperature. Heavy metals like cobalt, copper, manganese, molybdenum, vanadium, strontium and zinc required in only trace amounts and excessive amount having the propensity to cause numerous deleterious effects on organisms. Other heavy metals like mercury, cadmium and lead have proven to be non-beneficial for organisms, their accumulation causes serious illness in organisms (Singh *et al.,* 2011).

Arsenic is the main heavy metal found in drinking water causing harmful effects. In Bangladesh, 52 districts have been recorded to have arsenic contamination in ground water which has been found to be more than 50µg/l which is higher than the prescribed range. Arsenic causes skin lesions in people even at low concentrations of 0.005–0.01 mg/l in drinking water (Yoshida *et al.,* 2004). It also causes hyperkeratosis with many cracks in the skin. With an aim to provide safe water to the people, International agencies have drilled up deep wells. Surface water is free from arsenic but contaminated with infectious organisms. In West Bengal, arsenic was found in the water. When urine and hair samples were used for clinical examination it showed arsenic toxicities in the people, about 37.2% arsenic neuropathy was noticed in the people (Chowdhury *et al.,* 2000). The capital city of India, Delhi has also been found to have heavy metals like cadmium, selenium, manganese and copper in water, resulting in a number of infectious diseases (Dixit *et al.,* 2003). Heavy metals enter in the water through different sources. Cadmium is used as an anticorrosion agent and used in Ni-Cd Batteries. Cadmium causes many health effects such as tubular

dysfunction resulting in kidney damage (Jarup *et al.,* 2000). Mercury is also a dangerous heavy metal found as a water contaminant. Mercury is used in thermometer, barometers, cosmetics, dental surgeries etc. Because it is an allergen, it's exposure causes lung damage, eczema (Jarup, 2003). When the heavy metal's limit crosses the permissible limit, they cause bad effects (Table 2.1) (Singh *et al.,* 2011). Heavy metals enter into the human body through different sources like soil, water etc. causing diseases like Indian childhood cirrhosis, a liver disease occurs due to copper toxicity.

Table 2.1 The main heavy metals and their health effects

Sr. No.	Heavy metals	Permissible limit (mg/l)	Sources	Health effects
1	Arsenic	0.02	Pesticides, fungicides and metal smelters	Bronchitis, dermatitis, poisoning
2	Cadmium	0.06	Welding, electroplating, pesticides, fertilizers, Cd, Ni batteries, nuclear fission plant	Renal dysfunction, lung diseases, lung cancer, bone defects like Osteomalacia, Osteoporosis, increased blood pressure, kidney damage, bronchitis, gastrointestinal disorder, bone marrow cancer
3	Lead	0.1	Paint, pesticides, smoking, mining, automobile	Mental retardation, in children, development delay, fatal infant encephalopathy, congenital paralysis,

			emission, burning of coal	sensor neural deafness, damage to nervous system, liver, kidney, stomach
4	Manganese	0.26	Welding, fuel addition, ferromanganese production	Inhalation or contact causes damage to central nervous system
5	Mercury	0.01	Pesticides, batteries, paper industries	Tremors, gingivitis, minor psychological changes, spontaneous abortion, nervous system damage, protoplasm poisoning
6	Zinc	15	Refineries, brass manufacture, metal plating, plumbing	Corrosive effects on skin, cause damage to nervous membrane
7	Chromium	0.05	Mines, mineral sources	Damage to nervous system, fatigue, irritability
8	Copper	0.1	Mining, pesticides production, chemical industry, metal piping	Anemia, liver and kidney damage, stomach and intestinal irritation

Source: Adopted from Singh *et al.,* 2011.

Various studies have shown that the liver of the infected infant contains excess amount of copper binding protein (Nayak *et al.,* 1975; Portmann *et al.,* 1978), Copper initiates liver injury and cause alterations in liver functions (Nayak and Chitale, 2013; Walker, 1999). Chromium is one of the 14 heavy metals that causes pollution as well as different health problems (Paustenbach *et al.,* 2003) and has been rightly declared as carcinogenic by different agencies (Beaumont *et al.,* 2008; Linos *et al.,* 2011). In leather tanneries and chrome plating industries, chromium is being used in high amount which also contributes to water contamination (Schaffner *et al.,* 2010). Because the remaining toxic waste is not properly cleaned by industries before release into environment, so ultimately, it causes gastrointestinal, dermatological abnormalities and abnormal hematological functions and it was highlighted that, Kanpur is main city, the groundwater of which is contaminated by chromium due to high number of leather tanneries industries (Sharma *et al.,* 2012). In North India, the heavy metals like chromium, lead, arsenic and zinc are reported in gallbladder cancer patients (Chhabra *et al.,* 2012). Lead is used in building materials, pots and sweetens port wine. Lead cause brain damage in children more because the blood brain barrier not properly developed, so it passes to brain and causes damage. Lead causes lead encephalopathy and neurotoxicity in adults (Lidsky and Schneider, 2003). Long time exposure causes kidney damage (Mortada *et al.*, 2001). It is a carcinogen, it causes stomach cancer, lung cancer etc. (Steenlend and Boffetta*,* 2000). Lead is cytotoxic agent at very low concentration, causes hemolysis by toxic effects on red blood corpuscles (Mrugesh *et al.,* 2011).

To increase the crop yield, fertilizers and pesticides are used in fields which enter in the soil and water and cause their contamination. Many types of pesticides are used like Organochlorines, Organophosphates etc. In Haryana, the farmers of Hisar district use DDT, Endosulfan and Cypermethrin frequently (Kumari *et al.,* 2008). In Kanpur, the Malathion concentration is higher than other pesticides, but pesticides like DDE, DDT, Aldrin, Ethion, Methyl parathion and Endosulfan were not detected in both the surface and ground water samples (Sankararamakrishnan *et al.,* 2005). According to WHO, the range of fluorides set as 1.5mg/l, but in drinking water there is high amount of fluorides are present which cause Fluorosis problems in the people (Suthar et al.,

2008). In Rajasthan, different sites of Sambhar Lake have more than 400mg/l nitrates due to which people are suffering from gastric cancer and dental diseases (Joshi and Seth, 2009). Hanumangarh has high amount of fluorides in the range of 3-4mg/l, in drinking water and excessive intake of fluorides in water cause skeletal and dental fluorosis (Suthar et al., 2008). International studies have been found that, In Woburn, Massachusetts has contaminated water due to presence of chlorinated products. These chlorinated products made the drinking water harmful for people (Lagakos *et al.,* 1986).

Various other contaminants like Uranium, radon and radium are present in drinking water; Uranium is naturally occurring radioactive material which is present in rocks and soils and released in environment by different ways like mining, combustion from coal and phosphate fertilizers. The World Health Organization has set recommended level for Uranium is <15µg/l (WHO, 2011). Water is also contaminated by industrial, agricultural wastes like Trichloroethylene, Tetrachloroethylene and 1, 1-dichloroethane, asbestos which cause cancer. (Andersen *et al.,* 1993).

2.2 Water contaminants and human health

Different types of heavy metals are found in the water which directly as well as indirectly causes bad effect on human beings, plants and animals. These metals enter in our body and accumulate in the body tissue, process known as Bioaccumulation. In this way they affect the food chain and cause serious health defects (ul Islam *et al.,* 2007). Leafy-vegetables have high Bio-accumulation factor for heavy metals than non-leafy vegetables (Zhuang *et al.,* 2009).

According to world health organization, recommended level of arsenic is 10µg/l. Due to presence of arsenic in drinking water; internal cancers cases are observed (World Health Organization, 2011). Because skin is quite sensitive to arsenic, skin lesions are some of the most common and earliest nonmalignant effects related to chronic Arsenic exposure (Guha, 2005). Arsenic exposure causes number of other defects also like neurological effects, hypertension, peripheral vascular disease, cardiovascular disease, respiratory disease, diabetes mellitus, and malignancies including skin cancer, lung cancer (Ferriccio *et al.,* 2000) because skin, lung, bladder,

kidney, liver, and uterus are considered most affected sites of As-induced malignancies (Yoshida *et al.,* 2004). A study in West Bengal explains that the use of arsenic contaminated water cause hepatotoxicity, hepatomegaly and Non cirrhotic portal fibrosis that are characterized by spreading out of portal zones with streaky fibrosis (Guha, 2005). The association between heavy metal stress and altered sperm function as well as seminal enzyme inhibition (Sengupta *et al*., 2013) was noticed in Southern Assam. Due to heavy metals like cadmium and arsenic, the alterations are found in the sperm function parameters like hypo-osmotic swelling, acrosome reaction, and nuclear chromatin de-condensation in men in the affected persons. Due to low dose of cadmium skeletal damage occurs and it also causes kidney damage (Alfven *et al.,* 2000; Nordberg *et al.,* 2002). Cadmium causes many health effects. Cadmium is an allergen, its exposure causes lung damage, eczema and also causes tubular dysfunction which ultimately cause kidney damage (Jarup *et al.,* 2000). Lead is a carcinogenic in nature therefore it causes stomach cancer, lung cancer and gliomas (Steenlend *et al.,* 2000). It also causes lead encephalopathy and neurotoxicity in adults (Lidsky and Schneider, 2003). Long time exposure of lead causes kidney damage (Mortada *et al.,* 2001).

Surface as well as groundwater is contaminated by organophosphates and Organochlorines pesticides. These are chemicals, used by farmers to increase the productivity, which gradually accumulate in the crops. When these crops are eaten, these chemicals enter in our body and get accumulated in the body tissue (Bioaccumulation) which ultimately disturbs the food chain which causing deleterious health defects (ul Islam *et al.,* 2007). Pesticides like Organochlorines have been related to breast cancer in post-menopausal women (Garcia, 2003). In Thriuvallur district, ground water is main source of drinking water. But this ground water is contaminated by pesticides which cause headache, jaundice, memory loss, bradycardia, respiratory diseases etc. in the people (Jayshree and Vasudevan, 2007). Normally in drinking water, the nitrate level is less than 2mg/l, but farmers use fertilizers in the fields to fulfill the nitrogen requirements to increase the productivity, as a result the nitrates level increase very rapidly (Sankararamakrishnan *et al.,* 2005).

2.3 Water contamination and birth defects

The effects of drinking water on birth defects are evaluated on National as well as International level. An International survey in an area of northern New Jersey indicates the presence of Trihalomethane, Dichloroethylene, Carbon tetrachloride, Benzene etc. in drinking water which cause birth defects. Trihalomethane causes CNS defects; oral cleft defects, and cardiac defects, level more than 100ppb reduces the birth weight by 70.4gm, same way presence of Carbon tetrachloride and Trichloroethylene in water lowers birth weight and central nervous system defects, neural tube defects were also observed. Benzene also causes neural tube defects and cardiac defects (Bove *et al.,* 1995). In city of Las Vegas (Nevada), perchlorate is detected in drinking water; a chemical which was known to affect the thyroid function in newborns. But there was no difference, when thyroxin level was measured in water containing perchlorate and without perchlorate (Li *et al.,* 2000). On the other hand, the chlorinated organic has been observed to cause childhood leukemia and increased prenatal deaths also (Lagakos *et al.,* 1986).

When carelessly disposed materials (chemicals, byproducts) enter into water, they cause contamination and cause serious health defects including birth defects (Kurzel and Cetrulo, 1981). Numerous studies are observed in the relation of heavy metals as well as pesticides with birth defects. Pesticides cause central nervous system and musculoskeletal birth defects (Marshall *et al.,* 1997). High concentration of Arsenic in water causes birth defects, In Bangladesh, Arsenic cause spontaneous abortions and neonatal deaths (Milton *et al.,* 2005). Mercury is also a dangerous heavy metal which causes brain damage. Arsenic also causes methaemoglobinemia in children (Craun *et al.,* 1981) which is formed when nitrite converts the ferrous iron of hemoglobin (Hb) into the ferric form (Fewtrell *et al.,* 2004) methaemoglobin cannot bind to oxygen. Same way the presence of copper in water causes hepatic cirrhosis in infants (Nayak and Chitale, 2013; Baker *et al.,* 1995) which was considered endemic in Indian infants but in past few years it is also found in non-Indian infants as well (Sriramachari and Nayak, 2008). It has been found that women who are exposed to increased level of lead and chlorinated products have increased stillbirth cases (Aschengrau *et al.,* 1993).

2.4 Water in Malwa region of Punjab

Due to success in the agricultural green revolution, Punjab is one of the India's most prosperous states. Punjab is leading grain producing state. Seventy percent of the population is directly or indirectly associated with agriculture (Singh, 2008). Number of pesticides and fertilizers (Table 2.2) are used to increase the yield which causes contamination of drinking water.

Table 2.2 Consumption of fertilizers in Punjab (000, nutrient tones)

Year	Nitrogenous	Phopsphatic	Potassic	Total	Consumption per hectare (KG)
2007-08	1317	341	37	1695	213
2008-09	1332	379	55	1766	223
2009-10	1348	383	56	1787	226
2010-11	1403	435	33	1911	243
2011-12	1409	455	72	1936	246

Source: Agriculture at a glance, Department of Agriculture, Government of Punjab, Chandigarh 2012, (Blaurock-Busch *et al.,* 2014)

In Muktsar and Patiala, groundwater is not suitable for drinking and irrigation purpose because it is contaminated by different types of anions and cations (Kumar *et al.,* 2007). It was estimated that, drinking water is toxic due to presence of fertilizers, pesticides and fluorides, these pesticides are known to be carcinogenic and the presence of these chemicals in blood of cancer patients indicates harmful impact (Thakur *et al.,* 2008). There are different types of pesticides like Heptachlor, Endosulphan, Alderin which are found in groundwater. Pesticides are also held responsible for spontaneous abortion and premature births. In the affected areas, people have been observed to have blue line on the gums and also gastrointestinal morbidities (Thakur *et al.,* 2010). In cotton-growing area, Bathinda has toxic drinking water due to presence of pesticides. In Talwandi Sabo, high amount of pesticides like Endosulfan, Monocill, Ethion are used as compared to Chamkor Sahib (Thakur *et al.,* 2008). Heavy metals like arsenic, cadmium also present in the water of Malwa region

(Blaurock-Busch *et al.,* 2014). Studies show that most of deaths in the Malwa region of Punjab are only due to presence of heavy metals in drinking water (Sharma, 2012).

Table 2.3 Report on cancer incidences and prevalence (2011)

District	Current Cancer cases detected	Death cases due to Cancer	Cancer Incidences (per lakh)	Cancer prevalence (per lakh)	Cancer suspects (per lakh)
Barnala	588	780	98.7	229.7	183.2
Bathinda	1627	2058	125.8	284.9	272.2
Fatehgarh Sahib	588	924	106.3	273.3	251.6
Ferozpur	2136	2461	113.9	245.2	387.8
Moga	840	1674	88.4	264.7	565.1
Mansa	1053	1212	134.8	290.0	338.7
Muktsar	1177	1791	136.3	343.7	466.0
Patiala	1513	1498	86.8	172.7	397.8
Sangrur	1483	2284	93.4	237.3	487.9
Malwa region	11005	14682	107.4	250.8	390.4

Source: Adopted from State Health Systems Resource Centre, Punjab

Due to toxic drinking water, people are affected by number of health problems. A data collected by State Health Systems Resource Centre, Punjab (Table 2.3) highlighted that due to intake of highly contaminated water, Malwa region has high number of cancer cases (Sharma, 2012). In Malwa region mainly four districts Mansa, Faridkot, Bathinda and Ferozpur have poor quality drinking water and this water is thought to be the reason behind high rate of cancer. Mortality rate is high due to cancer which depends upon gender and farming (Indian council of Medical Research, 2001). These regions are mostly affected by different diseases due to water toxicity.

In Malwa region, water is contaminated by higher percentage of uranium which causes health problems to the people of this region. World health organization has set the recommended level for uranium is <15 µg/l (WHO, 2011) but results of surveys

shows that there is high concentration of uranium in drinking water than permissible limit (Singh, 2008) which is originated from industries like thermal plants, fertilizer factories, chemical factories and cement factories. It causes renal and lung defects (Parihar *et al.,* 2013) and increased risk of fertility problems, reproductive cancers (Singh, 2008).

Geochemical studies show that the water of Malwa region has high salinity and total dissolved solids which make the water toxic (Blaurock-Busch *et al.,* 2014). Villagers attributed higher occurrence of cancer due to unhygienic living conditions and poor quality of drinking water due to presence of many toxic metals like aluminum, manganese, lead and uranium which has carcinogenic or mutagenic potential, the combined effects of these toxins enhances carcinogenicity and mutagenicity (Singh *et al.,* 2009). In Talwandi Sabo, there is high amount of heavy metals like cadmium, arsenic, selenium, chromium etc. as compared to Chamkor Sahib (Thakur *et al.,* 2008). In Malwa region, three districts like Moga, Bathinda and Faridkot have arsenic metal and pesticides in the drinking water which causes brain cancer and skin lesions (Lalwani *et al.,* 2004; Sidhu *et al.,* 2014). Studies by different institutions shows maximum TDS of more than 10,000ppm was found in the samples taken from the hand pumps situated in villages viz. Arianwala, Tehna, Chehna, Jaito of Faridkot district and few sites of Jawaharwala village of district Sangrur. This quality of water causes cancer in the people of these areas and intake of this toxic water by most people in Malwa belt is the main cause of mortality (Sharma, 2012; Sharma *et al.,* 2013). Fluorides cause joint and bone problems in people of Malwa regions (Singh and Sharma, 2014).

Chapter 3

Materials and methods

3.1Materials

3.1.1 Chemicals

All materials used for cell culture like DMEM (Dulbecco's Modified Eagle Media), FBS (Foetal Bovine Serum), trypsin, antibiotic solution etc. were purchased from Invitrogen. Other chemicals used in research work like DMSO (Dimethylsulphoxide), MTT (3-(4, 5-dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide), Ethedium bromide, Sodium chloride (NaCl), Sodium hydroxide (NaOH), Ethylenediaminetetraacetic acid (EDTA), Tris buffer, etc. were purchased from Loba and Invitrogen.

3.1.2 Instruments

During research work different types of Instruments were used which have been listed below in the table.

Table 3.1 List of instruments with their manufacturing company

Sr. No.	Name of Instrument	Manufacturing Company
1	Scientific Balance Machine	Mettler Toledo
2	Autoclave (vertical) NSW-227	Calton
3	CO_2 Incubator	New Brunbwick, UK
4	Electrophoresis Unit	Tarsons
5	Mutiplate reader	Syatronics
6	Inverted microscope	Olympus Magnus
7	Mini Centrifuge	Spinwin
8	pH Meter	Mettler Toledo
9	Refrigerated Centrifuge 5430R	Eppendorf, Germany
10	Water Bath	Julabo
11	UV-VIS double beam 2202 Spectrophotometer	Systronics

12	Gel documentation machine	BIO-RAD
13	Laminar air flow	Calton

3.1.3 Cell lines under study

For the research work, two cell lines HCT116 wild type and HCT116 p53 mutant type were obtained as a kind of gift from Prof. Tapas Mukh Upadhyay, Former Director, National Center for Human Genome Studies and Research (NCHGSR), Punjab University, Chandigarh.

HCT116 cell lines are human colon epithelial cells. The main function of these cells is to extract water, salts and nutrients from partially digested food and then push the residue in rectum and anus for expulsion.

Table 3.2 Characteristics of HCT116 p53 wild type and HCT116 p53 mutant type cell lines

Sr. No	**Property**	**HCT116 p53 wild type cell lines**	**HCT116 p53 mutant type cell lines**
1	Source organism	*Homo sapiens*	*Homo sapiens*
2	Tissue	Colon	Colon
3	Morphology	Epithelial	Epithelial
4	Culture properties	Adherent	Adherent
5	Genes expressed	carcinoembryonic antigen (CEA)	carcinoembryonic antigen (CEA)
6	Biosafety level	1	1
7	Disease	Colorectal carcinoma	Colorectal carcinoma
8	Population Doubling time	Approximately 21 hours.	Approximately 21 hours.
9	Special feature	Normal p53 genes	Mutated p53 genes

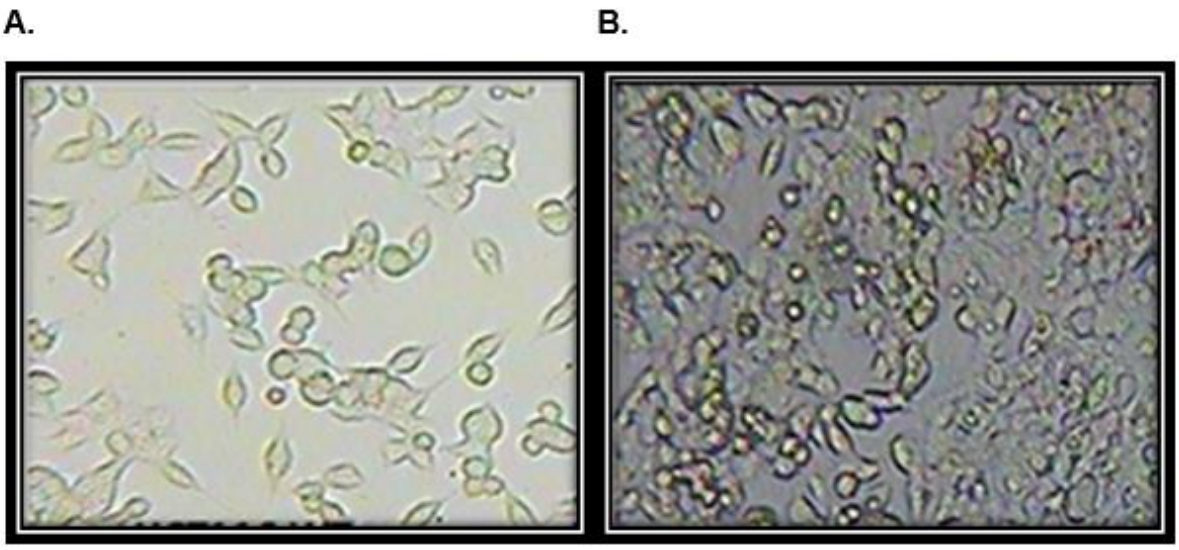

Figure 3.1 Microscopic views of A. HCT116 wild type and B. HCT116 p53 mutant cell lines

3.1.3a Why using these cell lines for study?

These cell lines are derived from colon cells, which extract water from digested food. To analyze the water toxicity on human health, these cell lines were selected. Toxic water shows its bad effect on colon cells, because during digestion water expose to these cells more. Toxic water cause different types of bad effects on colon cells and due to this reason number of diseases occur. For this study, I have taken HCT116 wild type cells in which p53 protein is active and other is HCT116 p53 mutant, in which p53 protein is mutant. This research work has been put forward with an aim to determine the effect of water, obtained from the different villages of Malwa region, on person suffering from colorectal cancer in the process determining the role played by p53.

Methods

3.2 Experimental design and protocols used in whole experimentation

The brief plan of work has been given below:

Experiment 1: Survey of villages

Experiment 2: Physicochemical analysis of water

Experiment 3: Estimation of oxidative stress

Experiment 3.1: Estimation of free radicals production by H_2DCFDA assay

Experiment 3.2: Estimation of mitochondrial membrane potential by JC-1 assay

Experiment 4: Estimation of cell viability after water treatment using MTT assay

Experiment 5: Estimation of antioxidant enzyme activity ~~analysis~~

3.2.1 Routine assay in cell culture lab

3.2.1.1 Cell Culturing

After 70% confluency, the cells were sub-cultured. Otherwise in case of less confluence, media was changed by removing old media with fresh media which has FBS, penicillin/streptomycin, ciprofloxin. For passaging, cells were detached from surface by using trypsin and then harvested by centrifuging the cells at 1200rpm for 5 minutes. Then after removing supernatant, pellets were taken and re-suspended in 2ml media. Then that was transferred in 25cm^2 culture flasks and incubated in incubator.

3.2.1.2 Maintenance of cell lines

For proper growth of cell lines, the cell lines were maintained in 25cm^{2}and 75cm^2 culture flasks by adding DMEM supplemented with 10% FBS, penicillin/streptomycin and ciprofloxin which prevent cell lines from any contamination then cell lines were incubated at 37^{o}C with 5% CO_2 and 95% humidity.

3.2.1.3 Sub-culturing of cell lines

After 70% confluency, the cell lines were sub-cultured in 25cm^{2}culture flasks. Before sub-culturing all the materials which were used for sub-culturing, were kept in water bath after removing from refrigerator for came down the temperature to 37^{o}C to prevent any cold stress. Then, the cells were trypsinized means detached from surface by adding 1ml trypsin, after 5 minutes the trypsin was inactivated by adding 1ml media containing FBS. Then, the cells were harvested by centrifuging at 1200rpm for 5 minutes at 4^{o}C temperature. The supernatant was discarded and pellet was re-suspended in 2ml media and transferred into 75cm^2 flask which has 8ml media. Then, the cells were incubated in incubator. Media was changed after every 2 days. For different assays the cells were taken.

3.2.1.4 Cryopreservation and thawing of cell lines

For future use, the cell lines were preserved in freezing media with 10% DMSO in the cryovials and kept at -80ºC. To culturing the dry preserved cell lines, revival of the cell lines was done by thawing at 37ºC and then re-suspended in 5ml media and transferred in 15ml falcon tube. Then to remove the DMSO, the cells were centrifuged at 1200rpm for 5 minutes. Then pellet was re-suspended in 3ml media and reseeded in culture flask containing 2 ml media and incubated at 37ºC. Media was changed after each day.

3.3 Survey of villages

Survey was done in 34 villages of two districts, Mansa and Bathinda, of Malwa region of Punjab. These selected regions are “high risk” zone of Punjab which has reported higher incidences of cancer mortality. Survey involved three aspects; obtaining information on cropping patterns, name of pesticides which are used in fields and samples collection. Punjab follows two crop cycle, in one cycle, rice and cotton are two major crops which mature during summer and harvested during autumn, in second cycle, wheat is a major crop which matures during winter and harvested during spring.

3.3.1 Sample collection

After survey of villages, samples were collected two times from 5 sites of Bathinda region (Table 3.3) after cultivation of *rabi* crops.

Table 3.3 Sampling sites and abbreviated form of samples used in the following section

Sr. No.	Sample	Sample site	Source
1	R1	Opposite Thermal Plant/ National Fertilizers Limited	Hand-pump
2	R2	Inside Thermal Plant/ National Fertilizers Limited	Motor

3	R3	Village Deon	Hand-pump
4	R4	Village Deon	Tube-well
5	R5	Village Joganand	Hand-pump

3.4 Physicochemical water analysis

3.4.1 Total dissolved solids (TDS) (Fatima *et al.,* 2007)

Total dissolved solids are the suspended organic and inorganic substances like metals, chemicals, salts etc. in the water. Water is classified depending upon the TDS value per liter volume. TDS is measured. Low TDS value indicates good water quality whereas high TDS value means low water quality**.**

Materials

Beakers, Oven, Analytic weighing balance, water samples

Procedure

To measure the TDS of water samples, first of all beakers were cleaned and dried properly. Then, dried beakers were weighed on weighing machine and the weight noted down. Then, each water sample was poured in previously marked beakers up to 100ml. Then, they were kept in oven at 105^{o}C overnight to evaporate the whole water until dry mass was remained. Next day the beakers were removed from oven and again these dry beakers were weighed. Then, TDS was calculated by using the readings.

3.4.2 pH of water samples (Fatima *et al.,* 2007)

Materials

pH meter, water samples (filtered and unfiltered)

Procedure

In order to analyze pH, water samples were filtered to remove the innumerable suspended particles. The water samples were filtered by using filter paper with 0.25µ pore size. To check the pH of the water samples, the water samples were filtered and then the pH was noted by using pH meter.

3.4.3 *N*-bromosuccinamide assay (Pandey *et al.,* 2014)

In water, numbers of pesticides are also present which contaminate the water. To detect the pesticide concentration like Malathion concentration in water, *N*-bromosuccinamide assay was done. In the presence of *N*-bromosuccinamide, Malathion is oxidized and various oxidation products are formed. The reacted *N*-bromosuccinamide gives color with safranine dye. The unreacted Malathion concentration is estimated by decrease in the intensity of color of safranine.

Materials

Malathion pesticide, Hydrochloric acid (HCl), *N*-bromosuccinamide, Safranine dye

Procedure

For detecting the Malathion concentration in water samples, the standards of different Malathion concentrations were prepared. Then in glass tubes, 1ml of each water sample was added, and then 2M HCl was added, after that 50mM *N*-bromosuccinamide was added. Then these solutions were shaken continuously for 10 minutes. After this, safranine dye was added and color intensity was estimated at 530nm on UV- Spectrophotometer.

3.5 Estimation of oxidative stress

3.5.1 H_2DCFDA (2′, 7′-dichlorodihydrofluorescein diacetate) assay (Baviskar *et al.,* 2013)

It is very sensitive ROS detection assay. In stressed conditions, ROS production increases which damage the cells and also cause DNA damage. H_2DCFDA is a fluorescent probe which reacts with several ROS including hydrogen peroxide, hydroxyl radicals and peroxynitrite. The H_2DCFDA passively diffuses into cells and is retained in the intracellular level after cleavage by intracellular esterase. Upon oxidation by ROS, the non-fluorescent H_2DCFDA is converted to the highly fluorescent 2', 7'-dichlorofluorescein (DCF). Dead cells produce ROS. So the fluorescence is related to the number of dead cells.

Materials

H2DCFDA (2′, 7′-dichlorodihydrofluorescein diacetate), phosphate buffer solution

Procedure

Two 96 wells were seeded with 100µl of $6x10^4$ cells/ml of HCT116 p53 wild type and HCT116 p53 mutant cell lines in Laminar air flow and incubated at 37ºC in incubator for 24 hours. Then, on next day, the cells were treated with water samples as per experimental design. Then treated cells were again incubated for 24 hours in incubator. Then, 50µl of H2DCFDA and phosphate buffer solution was added in each well after 24 hours and then kept for 20 minutes in dark. After 20 minutes, the media was removed and 50µl of PBS buffer was added in each well. Then, fluorescence production was measured at excitation 485 and emission 530.

3.5.2 Estimation of DNA damage by DNA laddering assay (Chen *et al.,* 2004)

Different toxic substances cause bad effect on cells either by ROS production, which cause DNA damage or by other reasons. To see the DNA damage by toxic substances, DNA was isolated from the treated cells and it was then seen by running on agarose gel.

Materials

Lysis buffer, Phenol: chloroform: isoamyl alcohol (PCI), Ethanol, 5M Sodium chloride solution, Agrose, TAE buffer

Procedure

Two 96 wells were seeded with 100µl of $6x10^4$ cells/ml of HCT116 p53 wild type and HCT116 p53 mutant cell lines in the Laminar air flow and incubated at 37ºC in incubator for 24 hrs. Then, on next day, the cells were treated with water samples as per experimental design. Then treated cells were again incubated for 24 hrs. in incubator. Then, the cells were harvested by centrifuging at 1200rpm at 4^0C for 5 minutes after 24 hrs. 3nd DNA was isolated from treated cells.

DNA isolation from treated cell lines

The cell pellets were resuspended in Lysis buffer solution and incubated at 60ºC for 15 minutes. Then, added Phenol: chloroform: isoamyl alcohol (PCI) after 15 minutes and mixed until emulsion was formed. After that, they were centrifuged at 10,000rpm at room temperature for 10 minutes. Then, upper clear layer was taken and 5M NaCl and chilled 70% ethanol was added, mixed and again centrifuged at 10,000rpm at room temperature for 10 minutes. Then, pellet was taken and added chilled 70%

ethanol and again centrifuged at 10,000rpm at room temperature for 10 minutes. Then pellets were dried and resuspended in TE buffer. Then, isolated DNA was run at 0.8% agrose gel at 90 voltages, 150 amperes.

3.5.3 Estimation of Mitochondrial membrane potential

3.5.3.1 JC-1 (5, 5′, 6, 6′-tetrachloro-1, 1′, 3, 3′-tetraethylbenzimi-dazolylcarbocyanine iodide) assay (Wong *et al.,* 2002)

Mitochondrial membrane potential is an important parameter of mitochondrial function which is used as an indicator of cell health. For this parameter JC-1 assay is used. JC-1 assay is a very sensitive assay for mitochondrial membrane potential detection. For this assay JC-1 fluorescent probe is used. JC-1 is lipophilic fluorescent molecule with the different emission wavelength i.e. green and red fluorescence and detector of mitochondrial membrane potential. This cationic dye can selectively enter into mitochondria and reversibly change color from green to red as the membrane potential increases. In healthy cells with high mitochondrial membrane potential, JC-1 spontaneously forms complexes known as J-aggregates with intense red fluorescence. On the other hand, in apoptotic or unhealthy cells with low mitochondrial membrane potential, JC-1 remains in the monomeric form, which shows only green fluorescence.

Materials

JC-1 (5, 5′, 6, 6′-tetrachloro-1, 1′, 3, 3′-tetraethylbenzimi-dazolylcarbocyanine iodide) dye.

Procedure

Two 96 wells were seeded with 100µl of $6x10^4$ cells/ml of HCT116 wild type and HCT116 p53 mutant cell lines in Laminar air flow and incubated at 37^oC in incubator for 24 hours. Then, on next day, the cells were treated with water samples as per experimental design. Then, treated cells were again incubated for 24 hours in incubator. Then, next day, 40µl of JC-1 dye was added in each well and kept for 20 minutes at 37^0C. Then fluorescence was noted down Excitation at 490nm and Emission at 527, 590nm.

3.6 Estimation of survival rate

3.6.1 MTT (3-(4, 5-dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) assay (Baviskar *et al.,* 2013)

MTT assay is used to check the growth inducing or inhibitory effect of various substances on the cell lines. MTT is a dye when it enters into the cells; the mitochondrial enzyme succinate dehydrogenase reduces the MTT dye into the insoluble dark purple colored product that is called formazan. The formazan product is soluble in DMSO and then its concentration can be measured by using spectrophotometer. Reduction of MTT dye is related to number of viable cells.

Materials

MTT (3-(4, 5-dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) dye (5mg/ml), Phosphate buffer, DMSO (Dimethyl sulphoxide)

Procedure

Two 96 wells plates were seeded with 100µl of $6x10^4$ cells/ml of HCT116 wild type and HCT116 p53 mutant cell lines in the Laminar air flow and incubated at 37^oC in incubator for 24 hours. On next day, the cells were treated with water samples as per experimental design. Treated cells were again incubated for 24 hours. Next day, the media of cells was removed and cells were washed with phosphate buffer solution. Then 100µl of 0.5mg/ml MTT dye was added in each well. Then plates were incubated at 37^oC for 4 hours in dark place. After 4 hours, the MTT dye was removed from cells and purple colored formazan precipitates were dissolved in DMSO. The cells were kept for 20 minutes to dissolve the precipitates. Then after 20 minutes, absorbance of treated cells was taken at 570nm.

3.7 Estimation of antioxidant enzyme activity

During pathological conditions like cancer, diabetes etc. oxidative stress is the main factor which is determined. Antioxidant enzymes protect our body from any dangerous effects of oxidative stress. To analyze the Antioxidant enzyme activity of HCT116 cell lines which are previously treated with water samples, different analysis assays were done. For this analysis, cell lysates were prepared after destroying the cell membrane, a solution which contains proteins and enzymes, is come out from cell. So enzyme and protein activities are analyzed by this process.

3.7.1 Superoxide Dismutase (SOD) assay (Marklund and Marklund, 1974)

In stress conditions, the large number of free radicals are produced which cause toxic effects on the living organisms. To protect the body from these toxic effects, antioxidant enzymes like superoxide dismutase, catalase etc. are produced. SOD provides first line of defense against toxic effects of elevated ROS production. SODs convert superoxide to hydrogen peroxides which is further catalyzed by catalase enzyme.

Materials required

6mM EDTA, 6mM Pyragallol, 0.1M Tris Hcl buffer

Preparation of cell lysates

Five 90mm culture dishes were seeded with HCT16 cell lines in Laminar air flow and incubated at 37ºC in incubator for 24 hours. Then next day, the cells were treated with water samples as per experimental design. The treated cells were again incubated for 24 hours in incubator. Then for preparing the cell lysate, cells were harvested and then Triton X -100 was added. Then the cells were freeze at -4ºC. After freezing, cells were thawed and freeze repeatedly. After this cells were centrifuged at 14000 rpm for 5 minutes and supernatant was stored at -80ºC.

Procedure

During this assay, in 100µl sample, 1.5ml Tris HCL buffer, 0.5ml 6mM EDTA and 1ml of 6mM pyragallol was added and then mixed them properly and absorbance was taken at 420 nm on UV-Spectrophotometer.

Calculation

Pyrogallol autoxidizes rapidly in alkaline aqueous solution, this reaction is inhibited by superoxide dismutase depending upon presence of the superoxide anion radicals, ($O_2^{\cdot -}$) in the reaction. The autoxidation was studied essentially during the first step(s) and the rate was taken from the linear increase in absorbance at 420 nm which is seen for a number of minutes after an induction period of some 10 s. the relationship between absorbance at 420nm and oxygen consumption was observed by this assay.

% inhibition = Absorbance Control- AbsorbanceTreatmentx100/Control = *X*

50% inhibition = *X*/50 = *Y* U/ml

3.7.2 Catalase assay (Chance and Maehly, 1955)

Catalase is a cellular antioxidant enzyme which is present in peroxisomes of cells. It protects the body from toxic effect of high concentration of hydrogen peroxide. It catalyzes the hydrogen peroxide into molecular oxygen and water, without the production of free radicals. So measurement of Catalase activity is very important in pathological conditions.

Material required

Potassium Phosphate buffer, Hydrogen peroxide, Cell lysate

Procedure

To estimate the Catalase enzyme activity, Hydrogen peroxide and Phosphate buffer was added, which was taken as positive control and absorbance was noted down at 240nm. Then in this combination cell lysate was added and again reading was taken. In this way absorbance value of each sample was noted down.

Calculation

Catalase enzyme changes hydrogen peroxide into water and oxygen and protects the body from toxic effects of hydrogen peroxide. The Catalase activity was measured by following formula:

Units/ml = [(A/min (Blank) – A/min (Sample)]. d .1)/ V X 0.0436

Where, A/min = Change in Absorbance per min

d = dilution factor

V = sample volume

0.0436 = e^{mM} (Extinction coefficient) for hydrogen peroxide

1 = reaction volume in ml

3.7.3 Glutathione reductase assay (Sedlak and Lindsay, 1968)

Glutathione reductase is flavoprotein that catalyzes the reduction of oxidized glutathione to reduced glutathione using NADPH as hydrogen donor, so indirectly it acts as antioxidant. Glutathione reductase plays an important role in preventing the oxidative damage by maintaining the glutathione level in the cells. With the help of glutathione peroxidase enzyme glutathione is responsible for minimizing the hydrogen peroxide level.

Materials required

0.1M Potassium phosphate buffer of pH 7.5, 0.2mM EDTA, 1.5mM $Mgcl_2$, 0.5mM NADPH, 2mM Glutathione oxidized

Procedure

To analyze the enzyme activity, 0.2ml cell lysate was taken, in this 0.2M Potassium buffer, 0.2mM EDTA, 1.5mM $MgCl_2$, 0.5mM NADPH and 2mM Glutathione oxidized added, then mixed properly and absorbance was taken at 340nm on UV-Spectrophotometer.

Calculation

Glutathione reductase is an indicator of oxidative stress. Its activity is measured by reduction of glutathione disulphide to glutathione by reduction of NADPH.

Glutathione disulphide + NADPH $\longrightarrow$ 2 moles Glutathione + $NADP^+$

Units/ml = $A/min/6.22x\ 10^{-3}.d$

Where, A/min = Change in Absorbance per min

d = dilution factor

$6.22x\ 10^{-3} = e^{mM}$ (Extinction coefficient) for NADPH

Chapter 4

Results and Discussion

The results of entire research work have been divided into following sections depending upon the type of parameters.

a. **Survey of villages of Bathinda and Mansa Districts of Punjab**
b. **Physicochemical water analysis**
c. **Determination of water genotoxicity in human cells**
d. **Estimation of Antioxidant enzymes activity**

Section a.

Survey of villages of Bathinda and Mansa Districts of Punjab

Total 34 villages of two districts, Mansa and Bathinda which are “high risk” zone of Malwa region of Punjab, were surveyed. These selected regions are reported to have higher incidences of cancer mortality. The survey had three aspects; obtaining information on cropping patterns, Sample collection and collecting information about the pesticides used in fields (Table 4.1). It was observed that there are three main crops- rice, cotton and wheat which are cultivated in these regions and mainly two pesticides are used by farmers - Malathion and Monocrotophos, to protect their crop from pest infestation and to subsequently increase yield of crops.

Table 4.1 Surveyed villages of Bathinda and Mansa districts

Sr.no.	Name of villages	Pesticides used	Crop cycle	District
1.	Begalehra	Malathion, Monocrotophos, Alpha Endosulfan Aldrin, Aldicarb, Chloropyriphos, Acephate Alphametharinetc.	**Major crops** - Wheat, Mustard, Cotton and Paddy. **Minor crops** - Bajra, Maize, Barley, Sugarcane, Pulses etc.	Bathinda
2.	Lehramohabbat			
3.	Nathana			
4.	Gill patti			
5.	Ghudda			
6.	Akliaklan			
7.	Bhuchokhurd			
8.	Deon			
9.	Jodhpur romana			
10.	Kotshmeer			
11.	Pathrala			
12.	Phoosmandi			
13.	Jodhpur pakhar			
14.	Siwian			
15.	Bhagivander			
16.	Gatwali			
17.	Korieana			
18.	Kutiwalkalan			
19.	Singo			
20.	Tungwali			
21.	Deon			
22.	Joganand			
23.	Mansa khurd			Mansa
24.	Khara			
25.	Barnala			
26.	Chkerian			
27.	Jogga			
28.	Bhupal			
29.	Ralla			
30.	Burjharike			
31.	Burjjhanbran			
32.	Jhuneer			
33.	Sardoolgarh			
34.	Bhudladha			

Section b.

Physicochemical water analysis

Water is an important resource for living things, maintaining different types of function in our body, and up to 3-4 liters of fluids per a day is needed. One can survive without food for several weeks due to reserve fat but without water survival becomes difficult for more than 3 days owing to inability of our body to reserve water. About 2/3 part of human body is water. Water is contaminated by different types of contaminants like pesticides, heavy metals, which cause different types of health problems (Nordberg *et al*., 2002; Jarup *et al* 2000; Nayakand Chitale, 2013).

So, the physical parameters of water samples were estimated after filtration process. To check the different water parameters, the water samples were filtered with 0.2µm filter paper.

4.1 pH of water samples

pH is the negative logarithm of hydrogen ion for measuring acidity and basicity on a scale from 0-14. If, water has more free Hydrogen ion, then it is said to be acidic (pH <7) and alkaline in nature if more hydroxyl ion (pH >7). pH is one of the most important parameter in water chemistry because any change in pH directly related to biological and chemical properties of water. Normally, the pH of normal water is 7 which is good for drinking purpose. But, water pH can be decreased or increased depending upon the type of contaminants in water (Fatima *et al.,* 2007) due to which water may be of bitter in taste which can affect the mucous membrane (Sharma, 2012).

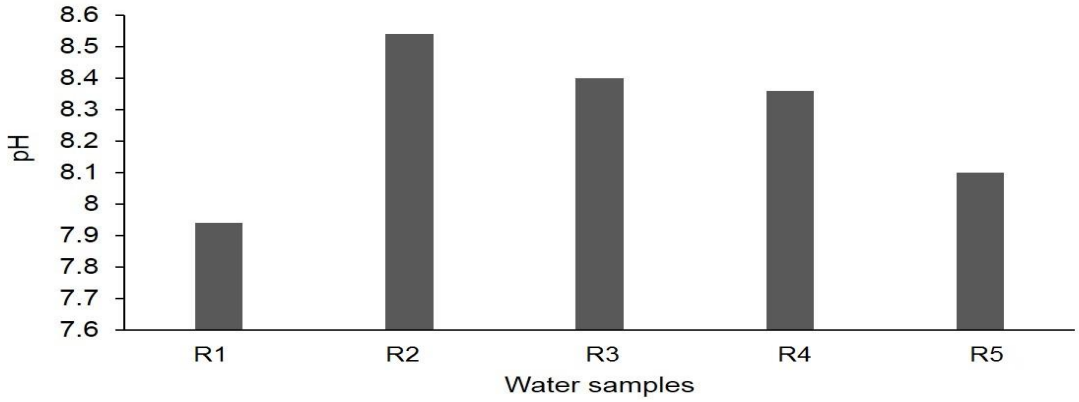

Figure 4.1 pH of water samples after filteration with 0.2µm filter paper

To estimate the pH of the different water samples, firstly water samples were filtered using a 0.2µm pore sized filter paper. Then, pH of filtered was measured with the help of pH meter. It was observed that pH values of water samples are ranging from 7.2 to 8.4, which are within the maximum permissible limit as prescribed by WHO, showing slightly alkaline nature of water samples which may cause skin dryness (Sharma, 2012).

Among the water samples tested, R2 and R3 showed a significant alkaline nature. It indicates that the water samples may contains particles which are basic in nature.

4.2 Estimation of Total dissolved solids in the different water samples

Water is contaminated with different types of minerals and impurities which may be either suspended or dissolved. In order to determine the total dissolved minerals and impurities in water samples, the water sample was filtered and TDS of the filtered

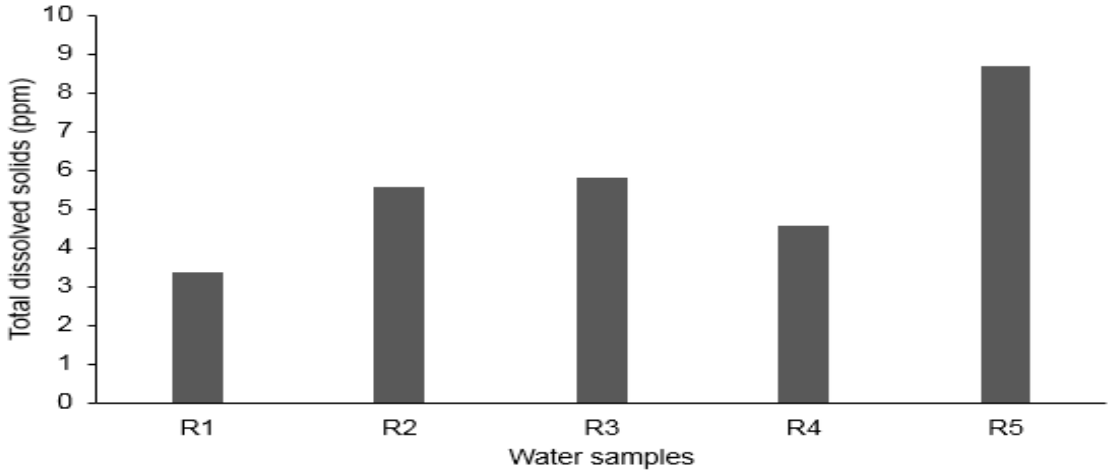

Figure 4.2 Total dissolved solids of water samples

samples were measured using the TDS meter. The permissible limit for TDS has been stated to be in the range of 500ppm. Any change in the TDS value is not good for health, making it a crucial parameter for evaluating the quality of water.

It was observed that except from R1, TDS value of all water samples was more than the permissible value that is 500ppm. Sample R5 was observed to have highest TDS value indicates the presence of high amount of minerals and other particles in the

dissolved form which increase the TDS value of this water. Similarly, TDS content for sample R2, R3 and R4 was also high underlining high content for minerals and other solids in the water which may cause gastrointestinal irritation and corrosion (Sharma, 2012)

4.3 Estimation of Malathion concentration in water samples by NBS assay

For the purpose of high yield production, farmers use number of pesticides like Organophosphorus or Organochlorine. Since our survey showed Malathion was one of the most commonly used pesticides and there is an assay available, we decided to look ~~of~~ for its presence in all the water samples. In order to estimate the Malathion concentration in water samples, *N*-bromosuccinamide assay was performed. Standards of different Malathion concentration were prepared and absorbance was taken at 530nm. Then, Malathion concentration in water samples was estimated with the help of standards absorbance. The Malathion gets oxidized in the presence of *N*-bromosuccinamide. The reacted *N*-bromosuccinamide gives color with safranine dye. The un-reacted Malathion concentration is estimated by decrease in the color of safranine.

Table 4.2 Malathion concentration in water samples

Sr. No.	Sample	Malation Concentration (mg/l)
1	R1	92
2	R2	72
3	R3	132
4	R4	12
5	R5	192

It was observed that all samples have Malathion pesticide, out of all samples, field's water like sample R5 (Table 4.2) showed higher Malathion concentration as compared to other field's samples like sample R3 and R4. The order of decrease in Malathion concentration in samples, was R5>R3>R4. On the other hand, sample R4 which was tube well water sample, has least amount of Malathion concentration, it

can be said that Malathion is not present in deep ground level of farming site, same as farming sites, industrial water samples have also Malathion pesticide.

According to FAO (Food and Agricultural Organization), 2000, the permissible concentration of Malathion is set at 0.4mg/L. Let's take an example, suppose in water sample R5 192mg/L Malathion concentration was found, if a person drink 3 liters water per day, then daily consumption of Malathion will be 576mg per day which is too much high concentration and may cause toxicity to the human cells. So, to evaluate the genotoxic effects of Malathion on human colon cancer cells, biological assays were performed.

Section C

Genotoxicity analysis

Studies have found that pesticides and heavy metals cause oxidative stress in the cells by producing the reactive oxygen species (Valko *et al.,* 2007) which are further responsible for number of health problems (Andollahi *et al.,* 2004). So, after analyzing the physicochemical characteristics, reactive oxygen species were estimated.

4.4 Estimation of oxidative stress

4.4.1 Estimation of Reactive oxygen species production

ROS was measured in cells treated with water samples for 24 hours using H2DCFDA stain. H2DCFDA is a fluorescent dye which reacts with oxygen free radicals to produce fluorescent signal indicating higher ROS will lead to high intensity signal of H2DCFDA.

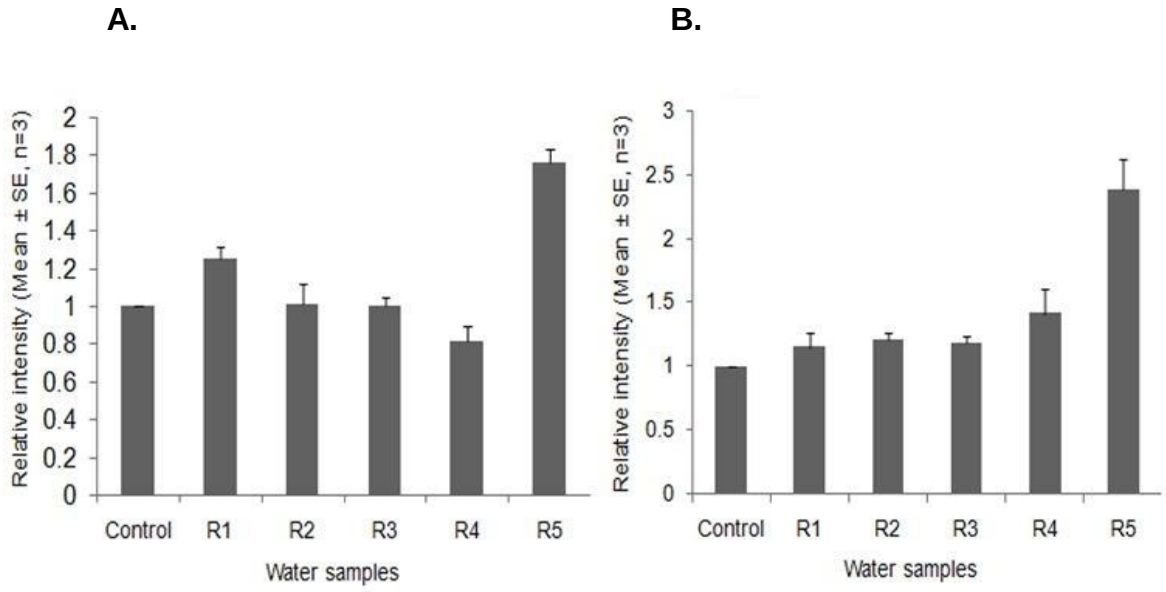

Figure 4.3 Formation of Reactive oxygen species in A. HCT116 wild type B. HCT116 p53 mutant cells in response to different water samples treatment. Data is expressed as mean value ± S.E. (n=3).

Results indicated that, sample R5 caused significant amount of ROS production in both HCT116 wild type and p53 mutant cell lines, as indicated by high fluorescence, which may have the potential to cause DNA damage, gradually culminating in cell

death, R5 followed by R1 which also produced more reactive oxygen species in comparison to control but sample R4 produced less fluorescence with respect to other samples, thus indicating less production of ROS.

4.4.2 Estimation of DNA damage by DNA laddering assay

To investigate the DNA damage due to pesticides containing water samples, the cell lines were treated with water samples. ROS production is related to DNA damage. DNA damage was observed only in sample R1, R4 and R5, in which R1 and R5 reported to more amount of ROS producing water and R4 was least amount of ROS producing water. DNA was isolated from the cells after water treatment and run on the gel. No significant results were observed. In figure 4.4, we can see that in Control,in which cells were treated with distilled water, have much stable DNA as compared to treated cells. But on other hand, relative to R1, R4 and R5 caused more DNA damage in the cells (Figure 4.4). Our result indicate towards genotoxic nature of water which may be attributed due to presence of pesticides, heavy metals and other contaminants in it. These contaminants may cause direct DNA damage as well as through production of free radicals.

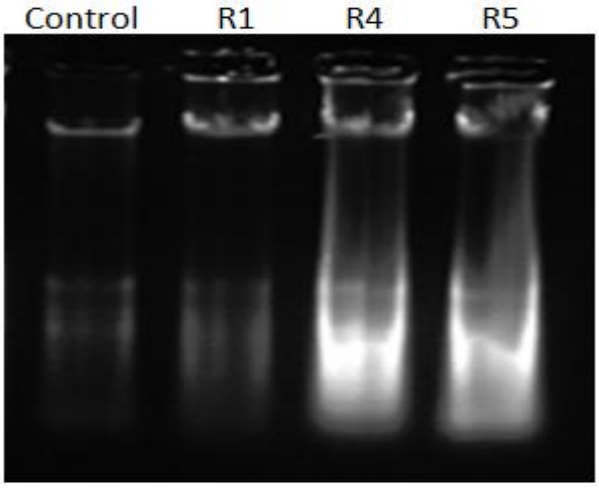

Figure 4.4 Appearance of DNA fragmentation in HCT116 wild type cell lines in response to water samples R1, R4 and R5

4.4.3 Estimation of Mitochondrial membrane potential by JC-1 (5, 5′, 6, 6′-tetrachloro-1, 1′, 3, 3′-tetraethylbenzimi- dazolylcarbocyanine iodide) assay

Mitochondrial membrane potential is an important parameter which is used as an

indicator of cell health. For estimation of the mitochondrial membrane potential, JC-1 assay was done (Wong *et al.,* 2002). JC-1 is a fluorescent probe, after entering the mitochondria; it produces red and green fluorescence depending upon the mitochondrial potential. In the case of healthy cells with high mitochondrial membrane potential, JC-1 spontaneously forms complexes known as J-aggregates with intense red fluorescence. On the other hand, in apoptotic or unhealthy cells with low mitochondrial membrane potential, JC-1 remains in the monomeric form, which shows only green fluorescence.

A. B.

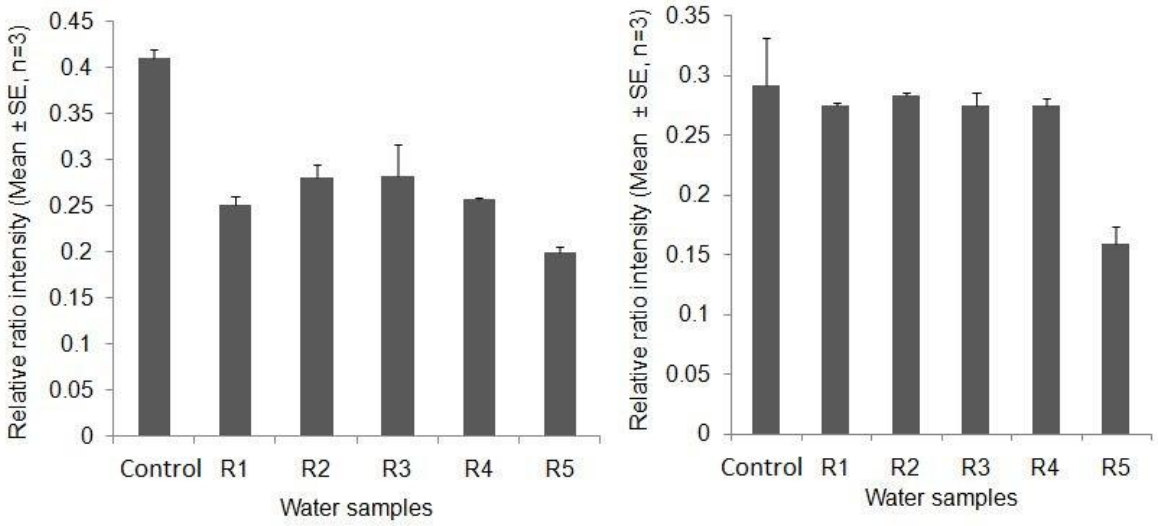

Figure 4.5 Mitochondrial membrane polarizationof A. HCT116 wild type and B. p53 mutant cells in response to water samples. Data is expressed as mean values ± S.E. (n=3).

Further, HCT116 wild type and mutant type cells were treated with water samples and then JC-1 dye was applied according to the protocol. Results showed that there was a significant decrease in the mitochondrial membrane polarization in HCT116 wild type.

Specifically, sample R1, R4 and R5 showed significant decrease in membrane polarization which highlighted that mitochondrial membrane is depolarized. Sample

R5 showed less polarized membrane among all water samples which may cause mitochondrial dependent cell death.

After applying same method on mutant cells, it was observed that, there was no significant difference in the membrane potential of Control cells and treated cells except sample R5.

4.5 Estimation of cell survival rate in response to water exposure

For estimation of survival percentage of cells after water treatment, the cells were treated with water samples and subsequently, with the help of MTT dye, their survival percentage was estimated. It was observed that the cell survival rate was seen to decrease to about 60% in treated cells with respect to control. Moreover, it was seen that 40% cell viability was reduced after the water treatment.

Specifically, sample R5 showed a survival rate of 50%. It was thus observed that among all water samples evaluated, R5 was found to be more toxic for cells. This sample showed a value of 8 gm per litre TDS content, which was higher than the permissible range of 0.5-1 gm per litre. This can, thus, be co-related with the fact that high amount of dissolved solids along with pesticide contamination in water samples can prove to be detrimental to the cells survival, leading to the cell death gradually. In comparison to wild type, cell survival rate of p53 mutant cells was observed to be more (except in case of R5). Studies reported that in the presence of p53 gene, during stress conditions, damaged cells undergoes apoptosis (Chipuk *et al.,* 2004) and so cell survival rate in HCT116 wild type cells was less, on the other hand in p53 mutant cells, survival rate was increased because mutation in p53 gene leads to activation of various cell signaling pathways which may resulted in preventing the damaged cells to undergo apoptosis and proliferate independently.

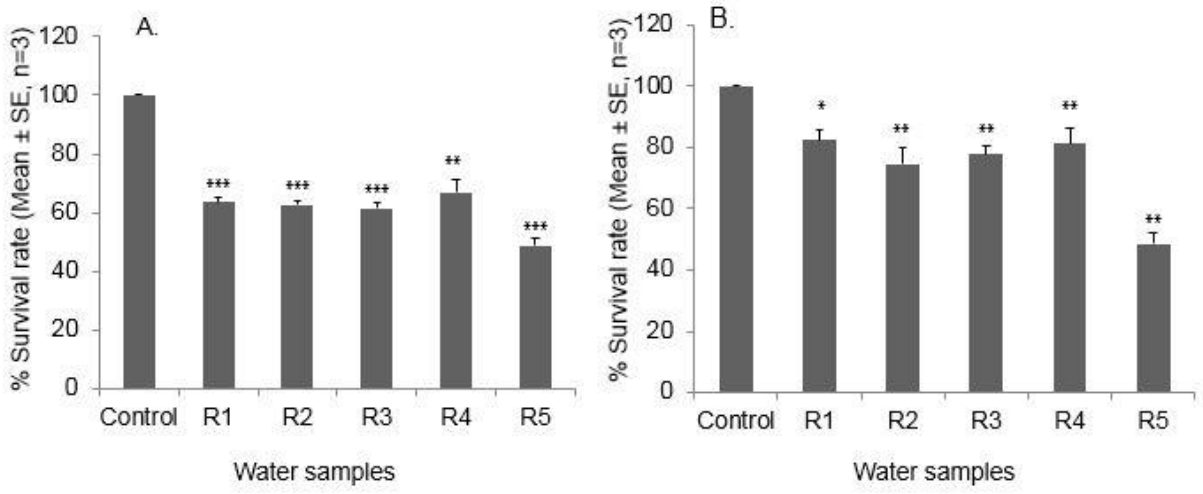

Figure 4.6 Percent Survival rate of A. HCT 116 wild type and B. HCT116 p53 mutant type cells in response to water samples. Data is expressed as mean values ± S.E. (n=3) and had been analyzed by student-t test and compared with respect to control. Statistical significant results were indicated by stars (*).

4.5.1 Estimation of survival rate of pretreated cells

In order to determine the survival rate of cells which were previously subjected to stress conditions. The stress used in present study was short time exposure (2hours) to water sample R1 or R5 followed by culture of cells in normal media for 24hours. Afterwards the earlier mentioned treatment was given to the pretreated cells for 48 hours followed by MTT assay.

Results suggested that, cells in Samples R1, R2 and R4 which are without any pretreatment, have shown high survival rate as comparison to R3 and R5 (Figure 4.7A.). Hence, Sample R5 was estimated to be more toxic to the cells as compared to other samples, on other hand R1 was less toxic. So, to evaluate the defense system activity and resistance on the repeated exposure of water on cells, cells were pretreated with water R1 and R5, and the subsequent survival rate was observed. Results depicted that in comparison to other samples, on repeated exposure to water, there is significant increase in survival rate as seen in Sample R3 and R5 treated cells which were pretreated with R1 water sample. On other hand, when cells were pretreated with water sample R5, all samples showed increased survival rate.

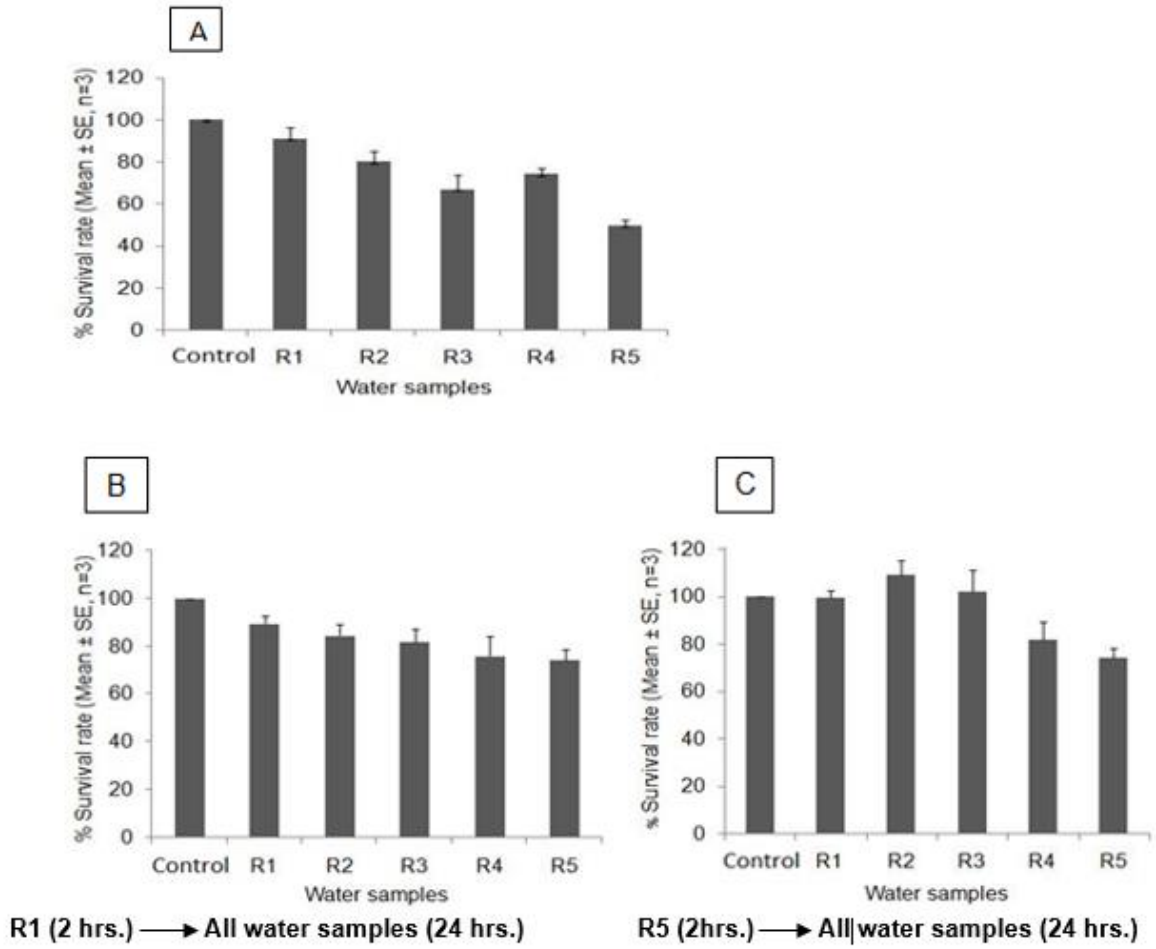

Figure 4.7 Percent survival rate of HCT116 wild type cells in response to A. without pretreatment of water samples B. R1 pretreatment and C. R5 pretreatment. Data is expressed as mean values ± S.E. (n=3).

In sample R1, R2 and R3, proliferation rate was observed and survival rate of samples R4 and R5 was also increased in comparison to without pretreated cells. So, it was revealed that short time exposure activated the stress mechanisms which protected the cells from harmful effects of water.

4.5.2 Investigation of cell growth after Long term culture

To investigate the change in cell's structure and proliferation rate after exposing the cells for long time to stress conditions, the cells were treated with all water samples and incubated for 10 days in incubator, after every 3 days fresh media was added and treatment was given. After 10 days, cells were observed under microscope.

There was more dead cells containing floating masses were observed in water sample R4 and R5 as compared to control cells. Water sample R1, R2 and R3 cells were also not normal; their morphology was changed due to stress conditions. Same results were seen in HCT116 p53 mutant cells, in R4 water treated cells; there were dead cells which were floated in media as mass. On the other hand, Cells treated with R2 and R5 caused significant change in cell's morphology as comparison to cells treated with other water samples. In the end, it was observed that there was not enough live cells to obtain any significant result in MTT assay while the control cells showed normal growth rate.

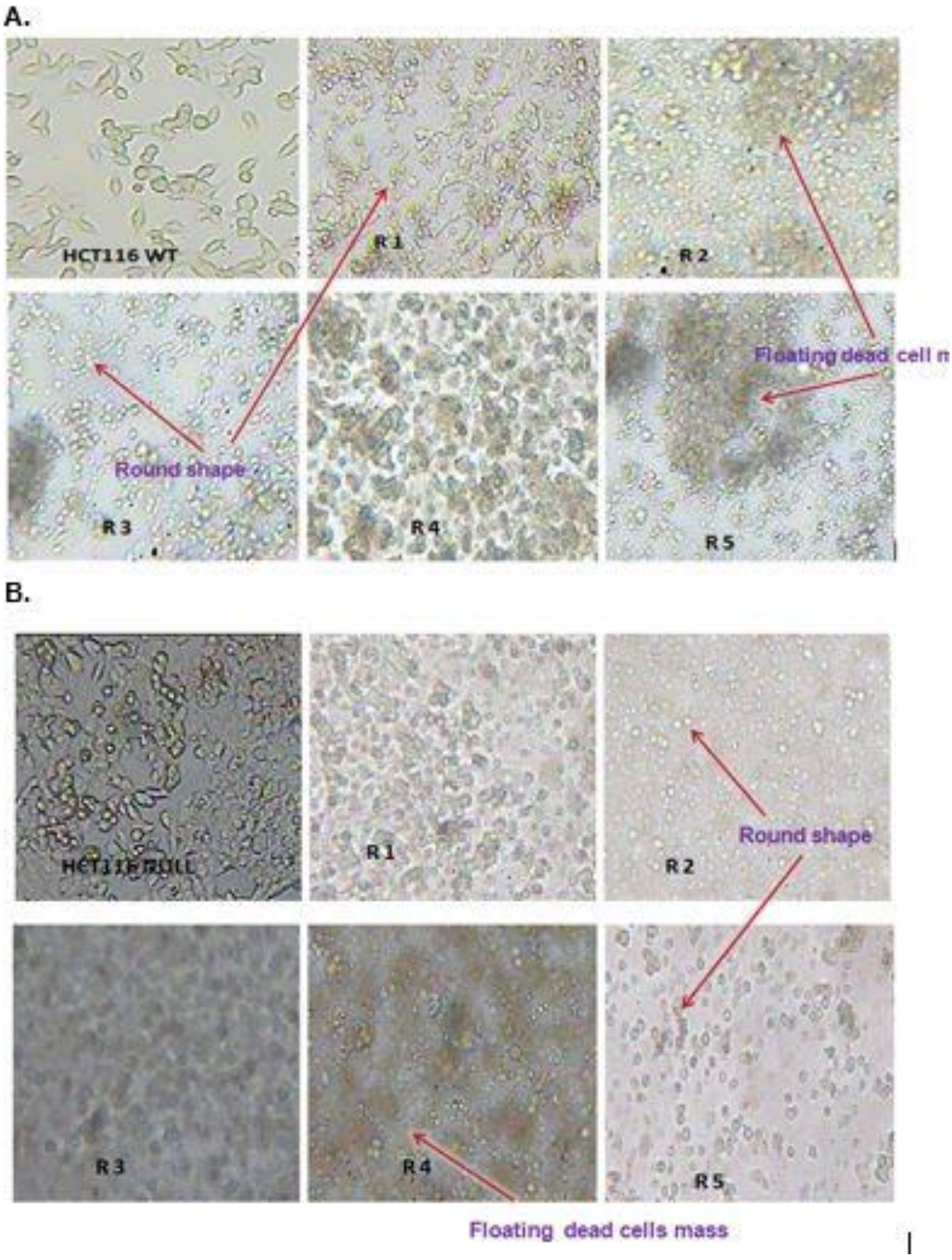

Figure 4.8 Observation of cell morphology and death rate in response to water samples after exposed the A. HCT116 wild type and B. HCT116 p53 mutant type cells for long time (10 days).

Section d

Antioxidant enzyme activity analysis

4.6 Estimation of antioxidant enzyme activity

Antioxidant enzymes are the enzymes which prevent or decrease the free radicals production in cells and thus prevent the cells from damage, like Superoxide dismutase (SOD), Catalase, Glutathione reductase etc. (Michiels *et al.,* 1994). During stress conditions, antioxidant system is also affected.

4.6.1 Estimation of Superoxide dismutase enzyme activity

It is first line defense against free radicals because it converts the super oxides into hydrogen peroxide which is further processed by next antioxidant enzyme named Catalase. To analyze enzyme activity, cell lysates were taken and after making the reaction mixture, activity was observed by taking the absorbance at 420 nm wavelength on spectrophotometer. It was observed that, all water samples treated cells showed less Superoxide dismutase enzyme activity as comparison to control. Sample R5 showed significant decrease in enzyme activity, which may be the reason of high number of reactive oxygen species in R5 treated cells, which I have already analyzed. On the other hand, R4 treated cells showed significant increase in SOD activity in related to other samples. Overall decrease in SOD activity indicates that antioxidant defense of cells weakens upon exposure to the water.

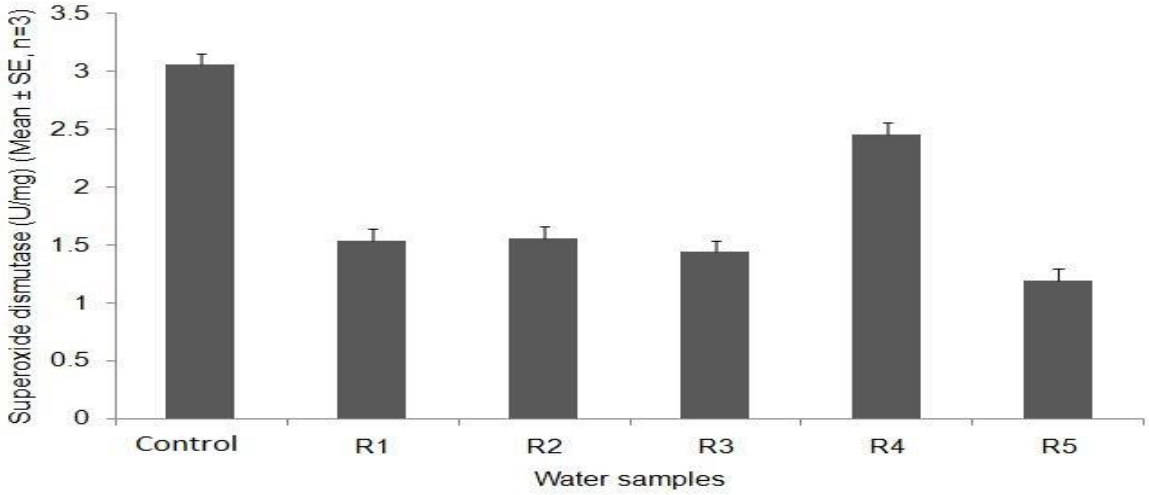

Figure 4.9 Superoxide dismutase activity in HCT116 wild type cells in response to water samples. Data is expressed as mean values ± S.E. (n=3).

4.6.2 Estimation of Catalase enzyme activity

Catalase is an antioxidant enzyme which converts the hydrogen peroxide in oxygen and water without production of free radicals. Enzyme activity was observed at 240nm. After this analysis, it was observed that Catalase activity was decreased in water samples R2, R3 and R5 treated cells as compared to control. On other hand, the activity was significantly increased in R4 treated cells. Also, it is compared with the survival rate results, in which survival rate of R4 treated cells was high as compared to other samples, so it estimated that in R4 treated cells, this enzyme played role to protect the cells from free radicals which produced during stress conditions.

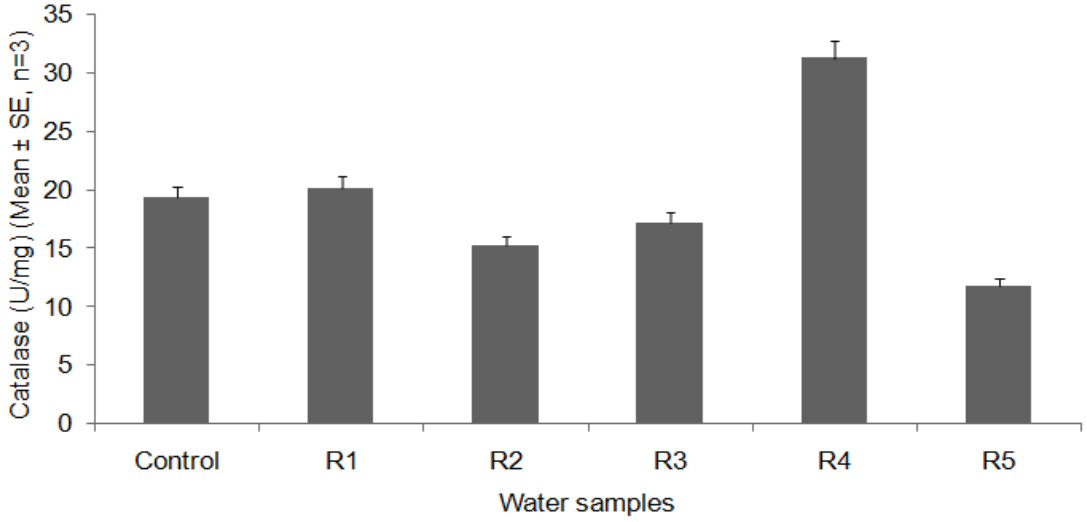

Figure 4.10 Catalase activity in HCT116 wild type cells in response to water samples. Data is expressed as mean values ± S.E. (n=3).

4.6.3 Estimation of Glutathione reductase enzyme activity

Glutathione reductase is in directing antioxidant enzyme which decreases the hydrogen peroxide level with the help of NADPH which donate the hydrogen when oxidized glutathione changes into reduced glutathione. To analyze the activity, cells were treated with water samples and as per protocol absorbance was taken at 340nm. It was observed that all samples showed significant increase in the glutathione reductase activity, especially R4 treated cells as comparison to Control, but except R5 treated cells which showed less glutathione reductase activity which may be the reason of less survival rate.

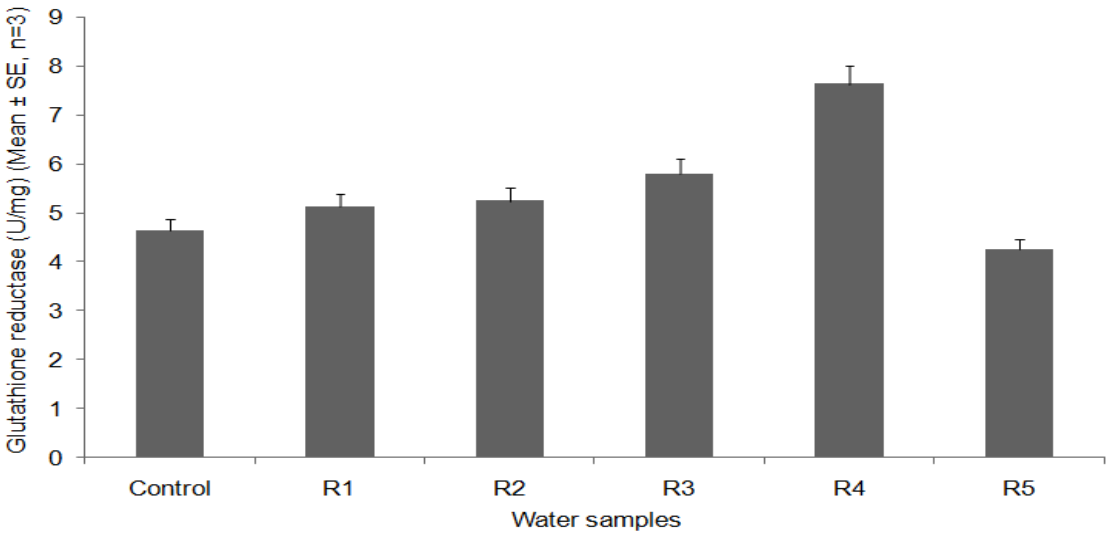

Figure 4.11 Glutathione reductase activity in HCT116 wild type cells in response to water samples. Data is expressed as mean values ± S.E. (n=3).

Chapter 5

Discussion

Concern to genotoxicity of water such as, from industrial and farming sites of Malwa region, is increasing due to increasing reports of water contamination in the recent years. Water contamination is significant in health concerns because for numbers of purposes we depend upon water for domestic, drinking as well as for industrial purposes (Chaudhary *et al.,* 2002). There are number of studies suggesting the water contaminants like heavy metals (Singh *et al.,* 2011; Sharma, 2012), pesticides (Thakur *et al.,* 2008; Thakur *et al.,* 2010) etc. are the main reasons of different health problems including Cancer in Malwa region (Parihar *et al.,* 2013; Koccher *et al.,* 2012; Blaurock-Busch *et al.,* 2014).

To evaluate the water genotoxicity of industrial and farming sites of Malwa region, a survey was done to know the pesticides which are used by farmers and crops which are cultivated in the fields. After this survey, 5 samples were collected from Thermal and villages Deon and Joganand, to study the water genotoxicity effects on human cells. Prior to evaluating the water genotoxicity in human cells, physicochemical characterization was done. The results suggested that all water samples show pH in permissible limit which is 6.5-8.5 (WHO, 2011) indicating that water samples may contain particles which may be alkaline in nature. It was observed that, all samples showed high TDS value than the permissible value (500ppm/l) prescribed by WHO. Out of all water samples, R5 showed significant increased TDS value which may be due to heavy contaminants.

Numerous studies reported that pesticides are present in water of Malwa region. (Sharma, 2012; Singh 2008; Thakur *et al.,* 2008). After analyzing the TDS and pH of water samples, Malathion concentration was estimated. It was observed that water sample R1 (92 µg/ml), R3 (132 µg/ml) and R5 (192 µg/ml) showed significant Malathion concentration but, water sample of village Deon (Tube-well) showed least amount of Malathion concentration(12µg/ml), it was revealed that pesticides did not reach in deep level.

There are very less *in vitro* studies which suggested the water genotoxic effects. In present investigation, attempts were made to evaluate the effects of water on

mitochondrial membrane polarization due to generation of oxidative stress. During stress conditions, free radicals cause DNA damage and mitochondrial membrane depolarization, consequently cell death occurs (Behl *et al.,* 1995). Out of all samples, R5 sample showed significant increase in free radicals production in both HCT116 wild and HCT116 p53 mutant cells. During stress conditions, mitochondrial membrane becomes depolarized; cytochrome c and other pro-apoptotic proteins release and initiate the mitochondria dependent cell death (Ott *et al.,* 2007). The mitochondrial membrane polarization was analyzed by JC-1 dye (Wong *et al.,* 2002). The results suggest that as all water samples caused depolarization of mitochondrial membrane, but R5 showed significant increase in depolarization in both HCT116 wild type p53 mutant cell lines.

It has been found that due to oxidative stress, cell survival rate is also affected (Ott *et al.,* 2007; Martindale and Holbrook, 2002), hence the cell survival rate was estimated in response to water treatments after oxidative stress analysis. The results suggests that all water samples treated cells showed 60% survival rate but R5 treated cells showed significant decrease in cell survival rate (50%). In HCT116 p53 mutant cells, the survival rate was up to 80% in all water samples except R5 which showed 50% cell survival rate. It can be said that in HCT116 wild type cells, p53 cause cell apoptosis due to which the cell number was decreased and on other side, in p53 mutant cells, damaged cells do not undergoes apoptosis and cell number was increased (Chipuk *et al.,* 2004). The stress used in present study was short time exposure (2hours) to water sample R1 or R5 followed by culture of cells in normal media for 24hours. Afterwards the earlier mentioned treatment was given to the pretreated cells for 48 hours followed by MTT assay. It was observed that as comparison to without pretreated cells, the cell survival rate was increased in R1 and R5 pretreated cells, which shows that short time exposure may activate the stress mechanism which protects the cells from harmful effects of water. Then, the long term effect of water on both, HCT116 wild type and p53 mutant type cells, was observed. To analyze this, the cells were exposed to water samples for 10 days, the results suggest that cell shape was changed during stress conditions and also high death rate was occurred due to which there were not enough cells to do the MTT assay to

check the survival rate.

Stress conditions also results in the imbalance of the antioxidant system, in which the antioxidant enzymes are present which scavenge the free radicals in cells (Apel and Hirt, 2004; Valko *et al.,* 2007; Amin and Hashem, 2012). To analyze the antioxidant system activity in response to water treatment, antioxidant enzymes activity was evaluated. The results shows that superoxide dismutase activity and Catalase activity was decreased by water samples, as comparison to other samples, activity was significantly high in R4 and less in R5 treated cells. The Glutathione reductase activity was high in all water samples treated cells except R5. There was significant higher activity was observed in R4 treated cells. So, it may be revealed that good activity of antioxidant enzymes may be the reason of low death rate in R4 and less antioxidant enzyme may be the reason of high death rate of R5 treated cells.

So ultimately we can say that water of Bathinda region is not good for health, it may cause genotoxicity in people of the region which may lead to health problems.

Conclusions and Future Perspectives

In the present study, physicochemical, genotoxic potential and antioxidant system of water treated cells, has been investigated. The results of this study suggest the following conclusions;

- In water samples, acidic macromolecules may be present which may be filtered out with filter paper and so after filtration, pH was increased.
- Malathion pesticide was found in all water samples, water from village Deon and Joganand (Hand-pump) have high amount of Malathion pesticides like 132µg/ml and 192µg/ml respectively. On the other hand village Deon (Tube-well) showed very low amount of Malathion concentration, it means Malathion did not reach into deep level water.
- Increased free radicals production, mitochondrial depolarization and DNA damage was seen in water sample treated cells but R5 showed more free radical production and DNA damage due to which survival rate was 50%. The results of survival rate after pretreatment suggests that first exposure of R5 sample was activated the defense system, so survival rate of cells was increased during repeated exposure to stress conditions. Long time exposure to water samples, R2 and R3 cause morphological changes in cells and R4 and R5 cause cell death.
- Antioxidant system was also affected by R5 sample, which may be the reason of death rate of cells and in R4 treated cells antioxidant system is less affected as comparison to other samples, due to which there was less cell death in R4 treated cells.

Further studies are required to evaluate the genotoxic effects of water from farming and industrial sites with large number of samples from Bathinda region. This study should be done on primary cells. Studies are required to analyze heavy metals and pesticides interaction, whether heavy metals may quench the impact of pesticides or not.

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