GENOTOXICITY OF DISINFECTION BY-PRODUCTS USING COMET ASSAY

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(2016)

In the name of Allah,
The most Gracious, The most merciful.

GENOTOXICITY OF DISINFECTION BY-PRODUCTS USING COMET ASSAY

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF MASTER OF SCIENCE

IN

ENVIRONMENTAL SCIENCE

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CERTIFICATE

It is hereby certified that the contents of this thesis entitled

GENOTOXICITY OF DISINFECTION BY-PRODUCTS USING COMET ASSAY

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Dedicated to my beloved parents and grandparents.

Their continuous efforts, support and endless prayers have

made me what I am today.

ACKNOWLEDGEMENTS

I am thankful to ***Allah*** Almighty for being with me all the way and for giving me more than I expected.

Completing this work would not have been easy if it were not for the support and encouragement provided by my teachers. Clay P. Bedford said that a student can be taught a lesson for a day but if one can teach him to learn by creating curiosity then he will carry on the course of learning as long as he is alive, and that is exactly what my supervisor ***Dr. Imran Hashmi*** has done throughout this whole experience. I am deeply thankful to him for sharing his knowledge and always showing deep concern, care, patience and encouragement. He came up with the wisest and quickest solutions to every problem I ever had during my research.

I must express my gratitude to ***Dr. Muhammad Arshad*** and ***Dr. Habib Nasir*** for responding to my questions and queries promptly.

I would also like to acknowledge the lab demonstrators and seniors of this institute, to whom I am indebted for their help. My special thanks to my senior ***Romana Khan*** for assisting me time to time whenever I needed her guidance.

And last but not the least, my ***family*** and ***friends***, for providing me much needed escape from my studies whenever I needed it and for helping me keep things in perspective.

Asna Lodhi

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LIST OF ABBREVIATIONS

$CHBr_3$	Bromoform
$CHCl_3$	Chloroform
ClO_2	Chlorine Dioxide
DBPs	Disinfection By-products
DNA	Deoxyribonucleic acid
EU	European Union
GC	Gas Chromatography
HAAs	Haloacetic Acids
HANs	Haloacetonitriles
LMPA	Low Melting Point Agarose
MCLs	Maximum Contaminant Levels
mg/L	Milligrams per Litre
NaClO	Sodium Hypochlorite
NMPA	Normal Melting Point Agarose
NOMs	Natural Organic Matters
OTM	Olive Tail Movement
PAA	Peracetic Acid

PBS	Phosphate Buffer Saline
RBC	Red Blood Cells
SCGE	Single Cell Gel Electrophoresis
SSB	Single Strand Break
THMs	Trihalomethanes
TL	Tail Length
USEPA	United States Environmental Protection Agency
WBCs	White Blood Cells
WHO	World Health Organization
µg/L	Micrograms per Litre
µL	Microlitre

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ABSTRACT

Bromoform and Chloroform are trihalomethanes and are among the most frequently detected drinking water disinfection by-products. They have been associated with various adverse effects on human haematological parameters, which are an important indicators of well-being. The prime objective of current study was to conduct a dose-response assessment to investigate the genotoxic and haematological effects of trihalomethanes on humans. Blood samples of healthy subjects were exposed to different concentrations (10, 30 and 50 µg/mL) of chloroform and bromoform *in vitro* to analyse how these compounds affected the haematological count with increasing dose concentrations. Headspace gas chromatography analysis was also conducted on samples to assess the difference between measured and spiked values of doses. Comet assay results showed that mean tail lengths values for bromoform (13.6, 23.0, 46.6 µm) were higher than chloroform (13.4, 22.4, 36.8 µm) at the three observed doses, depicting that bromoform has a higher genotoxic potential. Other indexes included were %DNA in Tail (%) and Olive Tail Moment (OTM) which showed the same trend. The haematology results indicated that the target compounds affected some haematological parameters in a dose dependant way. Haemoglobin (HGB) and mean corpuscular haemoglobin concentration (MCHC) levels lowered as they were significantly affected ($P<0.05$) by bromoform at all administered doses whereas the platelet (PLT) and red blood cell (RBC)count lowered significantly ($P<0.05$) only at at doses 30 µg/mL and 50 µg/mL. Conversely the damage caused by chloroform was minor ($P>0.05$). Both the compounds had no significant effect ($P>0.05$) on hematocrit (HCT) and white blood cell levels (WBC) as compared to the control.

Chapter 1

INTRODUCTION

Access to safe drinking water is not only basic human right but also vital for maintenance of human health. Just like development and health issues, access to clean drinking-water is not only an issue of local and regional but national level as well. It has been observed that improvement of drinking-water quality and making it easily accessible favors poor people the most irrespective the area is urban or rural (WHO, 2004).

Unfortunately, a major portion of the world's population has limited or no access to sufficient and safe water supplies, which is why both developed and developing countries encounter burden of waterborne diseases and fatality (Sobsey *et al*., 2003). Approximately 1.3 billion people residing in low-income countries are deprived from access to safe drinking-water. About 2.2 million deaths occurred in 1998, owing to diarrhea, most exclusively in these low-income countries, where there is no availability of drinking-water (Luby *et al*., 2001).

There are huge repercussions for human health environment as a result of contamination of water resources (Khan *et al*., 2013). That is why investigation of water contamination has become the primary focus of environmental scientists in recent years (Muhammad *et al*., 2011).

The water that is for the sole purpose of human consumption ought to be free from chemical materials and micro-organisms in concentrations that would prove to be a

health hazard. Drinking-water supplies must not only be free and safe from dangers to health, but it must also be aesthetically attractive as well (WHO, 1958).

Water and Sanitation sector is highly uncared for in Pakistan. A good number of households in Pakistan lack access to safe drinking water. As of 2005, around 38.5 million people of the total population lacked access to clean and safe drinking water sources in Pakistan. According to World Health Organization (WHO), every year about 7 1.8 million people die from diarrheal diseases in developing countries and 88 percent of these diarrheal diseases are accredited to unsafe water supply and poor sanitation (Khan *et al*., 2007).

Disinfection is crucial to avert the transmission of waterborne diseases and to guarantee water quality from the water distribution networks to the consumer's tap (Legay *et al*., 2011). Disinfection treatment methods include chlorination, chlorine dioxide, chloramines, ozone, and ultraviolet light. Among all the commonly used water disinfectants, chlorine by far is the most predominant disinfectant being used worldwide (NRC, 1987). It is strong oxidizing and disinfecting agent that has such wide acceptance pertaining to its economic availability and effective disinfection properties. Its capability to destroy bacteria and viruses helps eradicate water borne diseases such as cholera, typhoid and dysentery. Chlorine-based disinfectants are highly popular owing to their high oxidizing potential and permanence, which provides a bare minimum level of residual chlorine all the way through the distribution system and provides protection against microbial recontamination. For this reason it is highly prioritized as a cost-effective disinfection approach especially in developing countries like Pakistan (Muntaha, 2014).

The process of chlorination has been used successfully since 1908 (USA) for drinking water disinfection and it still continues to be broadly used for the purpose but unfortunately just like other disinfectants chlorine also leads to the formation of unwanted disinfection by-products (Siddique *et al*., 2012).

In raw water, chlorine has the tendency to react with natural organic matter (NOM) resulting in the formation of a variety of DBPs such as trihalomethanes (THMs) and haloacetic acids (HAAs). The formation of DBPs is highly dependent on water source quality, like concentration and properties of natural organic matter (organic precursors) and bromide levels (inorganic precursors). Their formation may also vary with change in factors like chlorine dose, contact time water and chlorine, reaction solution , pH and temperatures (Garrido & Fonseca, 2009).

Identification of DBPs and alarm over their likely adverse health effects promoted extensive research activity in USA as well as Europe in order to curtail cancer risks. The World Health Organization (WHO), United States Environmental Protection Agency (USEPA), and the European Union (EU) introduced regulations for trihalomethanes in drinking water. In 1979, 100 µg/L was established as the maximum concentration limit of trihalomethanes (THMs) in drinking water by USEPA. In 1998, Stage 1 Disinfectants (D)/DBP Rule was issued by USEPA that lowered the acceptable levels of THMs to 80 µg/L and for the first time regulated haloacetic acids, bromates and chlorite to 60 µg/L, 10 µg/L and 1000 µg/L respectively. In 2009, the European Union amended the predetermined limits for maximum trihalomethanes concentration from 150 to 100 µg/L (Tello *et al*., 2013).

Ever since the first publication in 1974 about the toxic by-products produced when chlorine reacts with organic matter, a lot of epidemiologic studies have evaluated the genotoxic effects associated with exposure to these toxins. A number of researchers have linked exposure to DBPs in drinking water to an increased risk of genetic damage and cancer (Villanueva *et al*., 2004).

Exposure to DBPs has been repeatedly found to be carcinogenic and mutagenic to humans. Not only this but adverse reproductive and fetal developmental effects, like low newborn weight, growth retardation and abortion have also been proven (Newbold & Eyles, 2005).

Chlorine and chloramines are the major disinfectants that produces significant amount of THMs as by-products. Trihalomethanes have detrimental side effects on humans. These types of compounds have been exposed to originate DNA damage, obstruct with the immune system and impair cell growth. A higher risk of eroding teeth enamel, asthma and eczema has been reported when exposed to THMs in water. They are also shown to cause a higher rate of miscarriage and birth defects. This class of compound neither degrades nor is digested by the body in fact it gets stored it in the fat tissues and secrete through different body fluids (breast milk, blood and semen) (Khan, 2015).

1.1 Hypothesis

Consumption of water containing trihalomethanes has genotoxic effects on humans.

1.2 Study Objectives

At present no considerable research is being carried in Pakistan on the genotoxicity of THMs. The study aimed to investigate the genotoxicity of disinfection by-products by integrating in vitro toxicology with focused genotoxicity analysis of two prevalent DBPs, chloroform and bromoform using comet assay.

The objectives are mentioned below:

1) Dose-response genotoxicity assessment using comet assay.
2) Analyse the comparative genotoxicity of THMs.
3) Examine whether the exposure affects haematological parameters.

1.3 Expected Benefits

Currently chlorine has wide applicability in water disinfection process and it has just as wider health concerns associated with it. This research aimed to acquire knowledge regarding these associated health issues and provide scientific support and rationale to highlight the negative impacts of THMs.

Following benefits were expected from this study:

1.3.1. Genotoxicity Assessment

This study would help evaluate the benefits and probable risks of disinfectants in drinking and would provide useful evidence about the genotoxic effects associated with trihalomethanes. It shall help implicate single strand DNA breaks as a feature of chloroform and bromoform exposure.

1.3.2. Level of Toxicity

This research would not only help determine whether the benefits brought from chlorination are more or less than the toxicity risks associated with it but would also help demonstrate the genotoxic response at different concentration levels.

1.3.3. Suitable Applications

This methodical, comparative, *in vitro* toxicological data would provide the water supply community with highly useful information to and would make then rethink before using chlorine as a disinfectant. Additionally, this research data would help in prioritizing DBPs and their associated compounds for upcoming in vivo toxicological researches and risk evaluation. Moreover these results would contribute to the ongoing and future researches on drinking water and leading cancer causes in developing countries especially.

Chapter 2

LITERATURE REVIEW

Water disinfection is a worldwide practice used in the treatment processes for drinking water to decrease the risk of infections associated with pathogens.

2.1. Disinfectants and Disinfection By-products

Regardless of the fundamental importance of water disinfection, disinfection by-products represent a significant matter of public health as various classes of DBPs have been identified in treated drinking water. Some DBP's are not only mutagenic, carcinogenic but genotoxic as well and many epidemiologic studies associate them with reproductive and developmental troubles.

The US Environmental Protection Agency in 1998 and 2005 set stage 1 and stage 2 rules respectively, for disinfectants and DBPs. According to stage 1 rule exposure to DBPs must be reduced for the customers of community water supply systems as well as non-transient non-community water supply systems. It also included those supply systems that served less than 10,000 customers and that added any disinfectant to the drinking water during course of the treatment process (EPA, 2001). The stage 2 rule was built upon former rules which stated the use of disinfection byproducts to improve your drinking water quality and provide additional public health protection from disinfection byproducts.

Monarca *et al*. (2004) conducted research on two commonly used disinfectants NaClO, ClO_2 and a relatively new one PAA, and evaluated how they led to the formation

of various toxic and genotoxic compounds in drinking water. They set up a pilot plant and added these disinfectants continuously to the pre-filtered lake water that was flowing into three basins. Plants, fish and molluscs were used to perform short-term *in vivo* tests while the *in vitro* tests were conducted using bacteria, yeast and human cells to study how genotoxic DBPs formed. The DBPs were identified using gas chromatography during the different treatments. The microbiological analyses were conducted on disinfectants to test their biocidal activity while the water quality was evaluated through chemical analyses. The drinking water plant was useful in examining the toxicity and genotoxicity of DBPs. The results explained the setting up of the pilot plant and effectively reported microbiological and chemical analyses.

2.2. Tihalomethanes (THMs)

More than 600 DBPs have been recognized to date now in drinking therefore making it somewhat impossible for all of them to be measured. Among these trihalomethanes and haloacetic acids are the most prevalent ones in chlorinated water (Weinberg & Krasner, 2002).

Currently, only 11 disinfection by-products are regulated and they mainly originate in chlorinated drinking water. Chloroform, bromoform, bromodichloromethane and chlorodibromomethane are among the four trihalomethanes regulated by the U.S. EPA in drinking water as a group and are known as total trihalomethanes. EPA has summed up the concentrations of these individual DBPs at 80 μg/L (EPA, 2006).

Table 1.3.3 : Levels for Total THMs EPA

Total THMs	MCLG	MCL
Bromoform	Zero	0.080 mg/L or 80 ppb (Sum of concentrations of total THMs) as an annual average
Chloroform	0.07 mg/L or 70 ppb	
Bromodichloromethane	Zero	
Dibromochloromethane	0.06 mg/L or 60 ppb	

Their maximum contaminant levels (MCLs) are based on the cancer data that has been collected over the past few decades through vigorous research.

Table 1.3.3 : IARC classification of THMs

THMs	Humans	Classification
Chloroform	Inadequate evidence for human carcinogenicity	Possible human carcinogen (Group 2B)
Bromodichloromethane	Inadequate evidence for human carcinogenicity	Possible human carcinogen (Group 2B)
Dibromochloromethane	Inadequate evidence for human carcinogenicity	Not classifiable as to it's carcinogenicity in humans (Group 3)
Bromoform	Inadequate evidence for human carcinogenicity	Not classifiable as to it's carcinogenicity in humans (Group 3)

2.2.1. Factors Affecting THMs formation

The formation trihalomethanes during the course of disinfection process is quite complex and is driven by several factors (pH, temperature, NOM, chlorine dose etc) that involve interactions. Many factorial analysis researches and strategies have been performed on different scales to identify and understand the most relevant and significant factors that influence the production of trihalomethanes.

Rodriguesa *et al*. (2007) used tentative data analysis method to identify the significant factors that effected the formation of trihalomethanes during water disinfection using chlorine. They setup two laboratory scale experimental prototype designed for studying multiple factors (FA concentration, chlorine dose, temperature, pH and bromide ion concentration). The results revealed that increase in FA concentration increase the concentration of less brominated THMs. Temperature which is very common environmental factor apparently speeds up the production of THMs. However, the solubility of most of the volatile THMs decreases. The formation of trihalomethanes is directly proportional to the amount of chlorine used to disinfect that is why the amount of chlorine must be reduced to minimum. High amount of bromide ions in raw water yields higher amounts of bromated THMs.

Liang & Singer, (2003) conducted a research on nine HAAs and four THMs. They evaluated several water treatment and quality parameters under controlled chlorination setting to verify their effect on the formation and distribution of the selected DBPs in drinking water. Raw samples were coagulated using alum after sampling from five water utilities and XAD-8 resin was then used to fraction them. The raw and coagulated water fractions raw along with the hydrophobic and hydrophilic water extracts

were chlorinated at pH 6 and 8 and then held at 20°C. The results revealed that the formation of trihalomethanes increased as the pH increased from 6 to 8 whereas the formation of HAAs decreased. However the formation of dihaloacetic acid was not affected much. At pH 8 the amount of THMs formed was more than HAAs, whereas at pH 6 the case was reverse. The hydrophobic fractions as compared to hydrophilic fractions always resulted in higher HAAs and THM formation, but during DBP formation in low humic content hydrophilic carbon played an important role. The hydrophilic fractions as compared to the subsequent hydrophobic fraction were relatively more reactive with bromine. More HAA precursors were generally removed by coagulation than THM precursors. Waters with high UV absorbance values were relatively more amenable to removal of OM through coagulation than the waters with low UV absorbance values. The results suggested that HAA precursors had a higher aromatic content than THM precursors.

2.2.2. Exposure Routes of Trihalomethanes

There are various pathways through which one may be exposed to trihalomethanes. The exposure may be dermal, through ingestion or inhalation. Dermal absorption accounts for 80% of the exposure to trihalomethanes. Swimming is a great culprit in this case as the pool water is disinfected and abundant in THMs.

Villanueva *et al*. (2006) made it the objective of their study to assess the lifespan exposure to THMs through ingestion, dermal absorption and inhalation. For this they designed a case-control study for bladder cancer in a hospital environment that was conducted form year 1998 to 2001 in five different regions of Spain. The people living in the catchment area adjacent to the hospital were taken as the research subjects. In order to

obtain information about their water related routine special interviews were conducted. The level of trihalomethanes, history of water supply source, and the year when water was chlorinated in the study area were confirmed by analyzing the water samples and handing out questionnaires to the authorities. Trihalomethane estimate levels covered about 79% of the total study subjects that were being exposed. The residential THM level was found to be 32.2 mg/L on average. Through ingestion the average exposure was 23.7 mg/day and this value was correlated with the THM exposure level of ingestion. Among 21% and 45% of the subjects from control group, that were not exposed to trihalomethanes through ingestion, were evaluated as somewhat little or highly exposed during showering or bathing and 5–10% of the total were exposed by way of swimming. The significance of different route assessment was underscored by the results from experimental studies that showed considerable differences in the trihalomethanes uptake and inner distribution via any of the studied routes.

Basu *et al*. (2011) investigated the hazard index and the lifetime cancer risk THMs along with their concentration through various routes like inhalation, oral ingestion and dermal absorption in the collected water samples from a treatment plant. Bromoform was found to be in higher concentration than chloroform. Among the studied pathways, 80–90% of the total risk was contributed by inhalation whereas oral exposure was lower in percentage as compared to it and dermal contact the lowest. Chloroform and Bromoform were found to be main THMs with high cancer risk. Chloroform has the highest risk in gaseous form and whereas bromoform through oral ingestion. Total THMs had hazard index higher than unity through oral route indicating high non-carcinogenicity. It was thought that the inconsistency among these three exposure

pathways may possibly be due to different concentration and speciation of trihalomethanes in the water. The sensitivity analysis by tornado diagram confirmed the Chloroform's high positive impact of chloroform to cause cancer was confirmed using tornado diagram which indirectly, established inhalation to be the major exposure pathway. This research recommended the adjustment of the regulatory issues associated to THMs based upon the related health risks of each THM and its exposure pathway.

Uyak, (2006) estimated the risk of cancer throughout a person's lifetime along with the hazard index THMs through different exposure routes (oral, dermal absorption and inhalation) via water taps in different districts of Istanbul. Chloroform, was found to be the most dominant THM in Istanbul tap water, followed by bromodichloromethane and dibromochloromethane. The results revealed that among the three studied exposure pathways oral ingestion posed the highest risk for cancer than the other two. The lifetime risks of cancer for chloroform and bromodichloromethane among all these districts was more than 106, which is negligible as defined by USEPA. Overall the results revealed that every year around 5 of the 8 million residents of Istanbul may perhaps get cancer from daily intake of such tap water.

2.2.3. Human Health Effects of Trihalomethanes

The consequences of trihalomethanes on public health have stirred apprehension ever since 1974 when chloroform was discovered and later in 1976 when it was classified as a potential carcinogen (Pressman *et al*., 2010).

The cancer potency factors of total trihalomethanes published by the USEPA have been used in various studies to calculate the risk of cancer posed by trihalomethanes.

Table 2.2.3 : Cancer Potency Factors

Compound	Cancer Potency Factor
Chloroform	0.0061 mg/kg/day
Bromodichloromethane	0.062 mg/kg/day
Dibromochloromethane	0 mg/kg/day (not cancerous)
Bromoform	0.0079 mg/kg/day

Shafiee & Taghavi, (2012) wrote an article review about the researches that highlighted the health impacts of trihalomethanes and stated that they have significant negative impact on child birth such as the child might be born under-weight, may suffer growth retardation or may be delivered premature. Many evidences also suggest that these DBPS are not only mutagenic but genotoxic as well and they have also been highlighted as a cause of bladder cancer. However, there are still some inconsistencies in this regard as different results have been reported by different studies.

2.3. Haematological Test

Enforceable standards for chlorite have only been publicized by very few countries up till now and unfortunately even less for chlorate (WHO, 2005). Many toxicological researches have been performed on animals and the results have clearly indicated haematological damage (anaemia etc) primarily due to exposure to chlorite. Impaired neurological development has also been observed along with delayed female sexual development, abnormalities of soft tissue and altered function thyroid function (UNEP, 2000). For humans haematological count is an important indicator of well-being.

Sen *et al*. (2011) performed a study to find out the effects of Dichloroacetic acid (DCA) on blood parameters in albino rats. The rats were fed with oral dose (125mg/kg/body weight) of target compound for 30-days, 60-days, 90-days. Standard methods were used collect and analyse the blood samples. A significant decrease was observed in glucose level, cholesterol level and haemoglobin level ($P<0.05$). The results showed that DCA is very toxic and its intake by humans must be minimum.

Toussaint *et al*. (2001) expose Japanese medaka (Oryzias latipes) in a diluter system for upto 9months to measure the concentration of bromodichloromethane (BDCM). They evaluated different parameters which included hepatocarcinogenicity, hepatocellular proliferation, haematology, and intra hepatic BDCM concentration. The results showed BDCM to be non-hepatocarcinogenic at the tested concentrations. Gallbladder lesions and bile duct abnormalities were evident in medaka treated with 1.424 mg/L concentration of BDCM, after a period of 6 and 9 months. After a period of 1, 4, and 20 days hepatocellular proliferation was observed. Even after 9 months the hematocrit, leukocrit, cell viability, and cell counts of treated fish showed a minor change as compared to control fish. At concentration 1.424 mg/L statistically significant gallbladder and bile duct abnormalities occurred.

Foster *et al*. (1997) investigated if any variations occurred in haematological malignancies with change in geographical areas of water supplies in South West peninsula of the UK. Haematological data was taken from the Leukemia Research Fund's Data Collection Study, and was mapped into 46 geographical areas of differing water supply. The results indicated significant heterogeneity in the incidence among water supply areas for two groups of disease-acute leukemias and myeloproliferative disorders.

During the study period considerable change was observed in pH, nitrate concentration and aluminium concentration Significant correlations was noticed between the standardized incidence ratios of five disease categories and some water quality indicators, especially aluminium and trihalomethane concentrations. The standardized occurrence of some haematological malignancies differed between geographical areas of water supply in South West England, plus the evidence suggested that deviation might be related with variation in water quality indicators.

2.4. Gas Chromatograph (GC) Analysis

Blount *et al*. (2006) performed a research to strengthen the studies exploring relation between VOC exposure and their adverse health effects, and to do so they developed an automated analytical method using SPME, capillary GC, and quadrupole MS. This SPME–GC–MS method produced excellent results and offered significant improvements as compared to the ones that were previously used and was promising enough to help expand biomonitoring efforts to assess non occupational VOC exposure.

Silva *et al*. (2006) developed a reliable analytical method to quantify trace levels of two ido-trihalomethanes namely dichloroiodomethane (IDCM) and bromochloroiodomethane (IBCM) in human blood. These analytes in the blood samples were extracted using SPME and then were then desorbed and separated by capillary-GC and then were analysed using mass. The accuracy of method for IDCM and IBCM ranged between 6 and 20% with detection limit of 2 ng/L for both IDCM and IBCM.

Bonin *et al*. (2005) developed a method to quantify trace levels of trihalomethanes and methyl tert-butyl ether (MTBE) in human blood. The SPME method

was preferred because of its high accuracy and low difficulty. SPME GC-MS method detection limits ranged from 0.3 to 2.4 ng/L. The method proved itself to be suitable for quantifying THMs and MTBE in blood samples tested from a diverse U.S. reference population.

2.5. Genotoxicity Assessment

Whether used in combination or alone disinfectants produce variety of DBPs that induce toxic and genotoxic activities which is why it has been more than quarter of a century since the genotoxicity and chemistry disinfection byproducts has been investigated (Plewa *et al*., 2004).

Chuang & Hu, (2004) used human and rat whole blood directly for their assessment using SCGE comet assay. It was found that 20µl of whole blood from both was plenty to perform comet assay, and that the comet images which were obtained from whole blood were similar to the isolated lymphocytes comet images. At 4°C the DNA remained undamaged for up to 4 hours and showed no apparent strand breakage at −80°C in 10% pre-cooled dimethyl sulfoxide when the whole blood samples were cryopreserved for upto 60 days. Rats were injected with Fe/NTA, a known carcinogen, to show that whole blood could be used for *in vivo* studies and DNA strand breakage was calculated in comparison with the lymphocytes. The DNA strand breakage in whole blood and isolated lymphocytes was the same which signified that for *in vivo* genotoxic studies whole-blood may be used. However, the only disadvantage of using the whole-blood technique was that RBCs cause interferences in *in vitro* studies. Nevertheless, RBC haemolysis and WBC isolation through centrifugation can overcome this setback. Otherwise the results

showed that the whole-blood technique was simple to using isolated lymphocytes whenever time is limiting factor.

2.5.1. Genotoxicity Assessment tools

Genotoxicity tests or tools are used for the evaluation of agents that have the potential cause alterations in DNA, as well as mutations. About 200 assays have been for the study of genetics, mutagenesis exploration and genotoxicity detection but they all differ from each other as they detect different genotoxic endpoints (Luttrell *et al*., 2008).

Table 2.5.1 : Some Common Assays for Detecting Genotoxicity

ASSAY NAME	ENDPOINT
***In vitro* assays**	
DNA abduct analysis	DNA adducts
Comet assay	DNA strand breakage
Alkaline elution assay	DNA strand breakage
Micronucleus assay in mammalian cells	Clastogenicity, aneugenicity
Unscheduled DNA synthesis in cells	DNA repair
Sister chromatid exchange in mammalian cells	DNA repair
Ames assay	Gene mutation
CHO H*prt* mutation assay	Gene mutation
Mouse lymphoma assay	Gene and chromosome mutation
Chromosomal aberration in mammalian cells	Clastogenicity, aneugenicity
***In vivo* assays**	
Rodent micronucleus assay	Clastogenicity; aneugenicity (somatic cells)
Sister chromatid exchange in rodents	DNA damage (somatic cells)

Unscheduled DNA synthesis in rodent liver	DNA repair (somatic cells)
H*prt* mutation assay	Gene mutation (somatic cells)
Tk mutation assay	Gene and chromosome mutation (somatic cells)
Transgenic rodent mutation assay	Gene mutation (somatic and germ cell)
Dominant lethal assay	Clastogenicity (germ cell)
Mouse heritable translocation test	Chromosome mutation (germ cell)
Mouse spot test	Gene mutation (germ cell)
Mouse specific locus test	Gene mutation (germ cell)

2.5.2. Comet Assay

Comet assay is a very useful technique to work with for genotoxicity assessment as compared to other assays. It is low cost, rapid, flexible, easily applicable, allows to work with small test substance and above all it can detect DNA damage of very low level. Human mutagens and carcinogens are being identified with the help of this assay (Tice *et al*., 2000).

This useful tool allows easy assessing of DNA damage and repair in individual eukaryotic as well as a few prokaryotic cells, and has is now applicable in diverse fields ranging from genetic toxicology to human epidemiology (Dhawan *et al*., 2009).

In this method the cells are embedded in agarose on a clean microscope glass slide and are then lysed with detergent and high salt to form nucleoids containing supercoiled loops of DNA linked to the nuclear matrix. Electrophoresis is performed at high pH which results in structures that resemble comets that are observed using fluorescence microscope. This assay is applicable for testing chemicals for genotoxicity, human biomonitoring and molecular epidemiology, monitoring environmental

contamination with genotoxins and performing elemental researches in DNA damage and repair (Collins, 2004).

The comet assay procedure developed by Singh *et al*. (1988) was slightly optimized to carry out this study. According to Dhawan *et al*. (2003) the method developed by N.P Singh for single cell gel electrophoresis (SCGE)/comet assay, combines biochemical techniques for detecting DNA single strand breaks, allows data collection at individual cell level, enabled to perform robust types of statistical analyses.

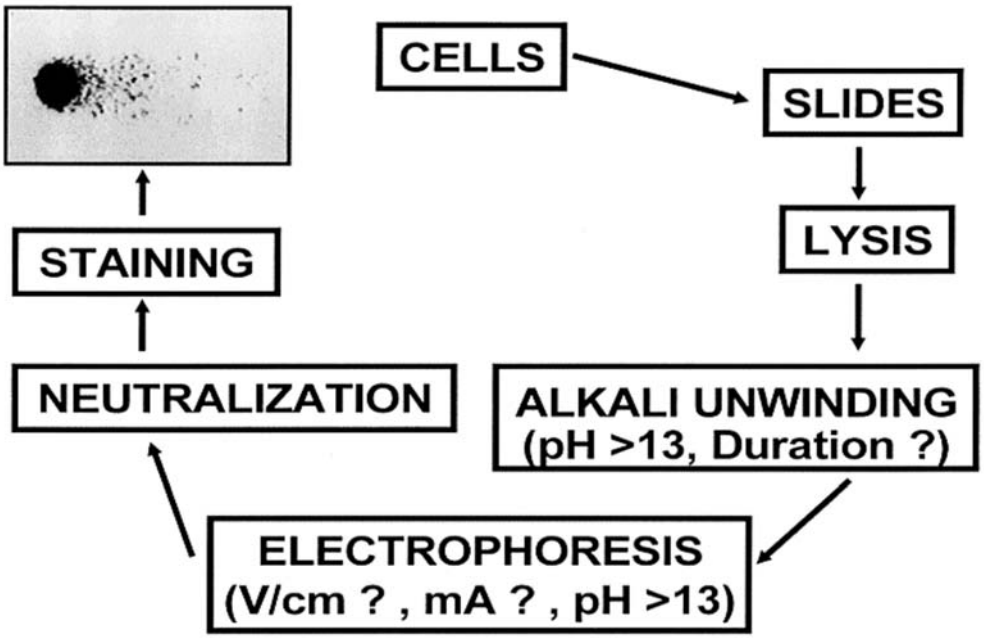

Figure 2.5 : Comet Assay Protocol

2.5.2.1. *In vivo* Assessments

As *in vivo* assays integrate the effect of biological assessment that is why for modeling purposes they are thought to be more suitable than *in vitro* assays (Burlinsona *et al*., 2007).

Narotsky *et al*. (2015) conducted multigenerational bioassay for reproductive toxicity using mixture of drinking water containing the four regulated trihalomethanes and the five regulated haloacetic acids as up till now their joint reproductive toxicity is unknown. To achieve their desired objective they exposed Sprague-Dawley rats (F1 and F2 generation from beginning till end of gestational period) to a mixture of trihalomethanes and haloacetic acids at concentrations that were 0, 500×, 1,000×, or 2,000× of the U.S. EPA's MCLs. The results revealed that maternal water uptake and body weight reduced at ≥ 1,000× and 2,000×. No observable effects were seen in pre-natal and post-natal survival. Unlike the F2 generation, at the time of birth F1 generation weights remained unchanged but after birth they reduced at the concentrations 2,000× and at ≥ 1,000× on PND6 and PND21 respectively. Male sperm motility was compromised at 2,000× and at 1,000× and 2,000× the puberty onset was also delayed. They drew the conclusion that a mixture of regulated disinfection by-products at a concentration up to 2,000× of the U.S EPAs MCLs did not have adverse effects on the fertility, retaining of pregnancy, pre and post-natal survival, or even the birth weights and that the observed delayed in puberty might have been secondary to decreased water intake.

Kogevinas *et al*. (2010) collected exhaled air, urine and blood samples from multiple non-smokers who swam in chlorinated pool waster, to evaluate if exposure to disinfection by-products is linked to biomarkers of genotoxicity. Sampling was done before and after swimming. They associated the concentrations of few selected THMs in exhaled air and the corresponding changes in micronuclei and DNA damage (comet assay) in lymphocytes, urine mutagenicity and micronuclei in urothelial cells. The

concentration of trihalomethanes, after swimming, in exhaled breath was seven times higher than the concentration before swimming. The results revealed that higher the concentration of THMs in exhaled air the more the micronucleated lymphocyte frequency changed. DNA damage as a result of swimming was not detectable by comet assay. There was significant increase in urine mutagenicity after swimming. However no considerable changes were associated to micronucleated urothelial cells. Overall the results supported possible genotoxic effects of being exposed to disinfection by-products from swimming pools.

2.5.2.2. *In vitro* Assessments

There are many advantages of *in vitro* genotoxicity tests and are widely accepted by scientific communities. They are inexpensive and the results can be achieved in relatively short period of time. However a drawback of using this type of assay is that it does not integrate DNA repair. *In vitro* assay may be performed on whole blood or lymphocytes (Fenech, 2013).

Guzzella *et al*. (2004) set up a series of short-term *in vitro* tests showing various genetic end-points, in order to examine genotoxicity of surface water after treatment with different biocides (NaClO, ClO_2, PAA) for disinfection. C-18 silica cartridges were used to concentrate the water sample prior to and after disinfection and for conducting bio-assays and chemical analysis the water concentrates that contained non-volatile organics were separated into different portions. The following in vitro tests were conducted on the water concentrates dissolved in DMSO: the Salmonella mutagenicity assay, SOS Chromotest, Microtox and Mutatox assay, gene conversion, point mutation and mitochondrial DNA mutability assays were conducted on the concentrated that were

dissolved in dimethyl sulfoxide (DMSO). The research outcomes explain that for the detection of genotoxicity SOS Chromotest and yeast assays were very. The extracts of surface-water were seldom toxic to majority of the considered test organisms, somewhat suppressing their possible mutagenic nature. The results also showed that among all of the tested biocides, water genotoxicity was increased by NaClO and ClO_2. On the other hand PAA somewhat decreased raw water activity.

Zhang *et al*. (2012) studied the impact on DNA damage of fifteen disinfection byproducts, in human HepG2 cells was using SCGE assay. Among the selected fifteen DBPs, four were THMs, six were HAAs, three were haloacetonitriles (HANs), and the remaining two were MX and CH. The results of showed that the minimal effective concentration at which considerable increase in OTM was observed, bromodichloromethane induced the highest DNA damage followed by dibromochloromethane, tribromomethane and trichloromethane in the same order among the four selected THMs. HAAs and HANs induced comparatively lower DNA damage. The toxicity of MX and CH was found to be similar to trichloroacetic acid and dibromochloromethane respectively. Among the six HAAs idoacetic acid was the most genotoxic DBP among the fifteen DBPs, followed by bromoacetic acid. The results showed that chlorinated DBPs were far less genotoxic compared to brominated DBPs and that using HepG2 cells for SCGE proved to be a sensitive tool for the evaluation of DBPs genotoxicity.

Liviac *et al*. (2009) carried out research on the genotoxic potential of Halonitromethanes. HNMs represent an up-and-coming class of DBPs that are quite alike halomethanes and are produced when is treated with chlorine and/or ozone.

Trichloronitromethane (TCNM) and bromonitromethane (BNM) were the two selected HNMs to conduct this study on human cells using comet assay and micronucleus assay. The results revealed that both compounds were significantly genotoxic in nature and induced high level of DNA damage, although the DNA damage repaired over time. Additionally, it was found that oxidized bases contributed 50–75% DNA-induced damage. Quite opposite to this, in the micronucleus frequency there were no positive effects were observed, neither in lymphocytes nor in TK6 cells.

Buschini *et al*. (2004) evaluated the genotoxicity of the commonly used disinfectants ClO_2 and NaClO in human WBC by using comet assay and strain D7 Saccharomyces cerevisiae. The effects of the disinfectants were compared to those of PAA, which they suggested as an alternative biocide. All the three turned out to be mildly genotoxic in WBC with the lowest effective dose being 0.2 ppm for ClO_2, 0.5 ppm for NaClO and PAA. The results obtained from Saccharomyces cerevisiae D7 test however showed considerably higher genotoxic affects the end-points at 5-10 folds higher doses than the concentration generally used for disinfection of water. The results revealed that all these disinfectants damage the DNA and that PAA shows effects similar to ClO_2 and NaClO.

Maffei *et al*. (2005) investigated the viability and possible effectiveness of an integrated methodology to analyse the genotoxicity of drinking water. This methodology used comet assay and micronucleus assays to estimate the damage imposed by water extracts on DNA and chromosomes blood cells of humans. During different seasons water samples were collected from Lago Trasimeno, Italy. Sodium hypochlorite and chlorine dioxide were then used to disinfect these collected samples. Human leukocytes

were incubated with the extracts of untreated and treated water. The results revealed that the genotoxicity of the collected water was increased by these disinfectants. Among the selected disinfectants, PAA damaged DNA the highest. None of the samples showed any detectable increase in micronucleus frequency. The multiple endpoint micronucleus assay indicated the collected water samples to be cytotoxic in nature, therefore concluding that the integrated methodology employed for the assessment of genotoxicity might be functional both for comparison of potential health risks of disinfected water and controlling quality of raw drinking water.

Comet assay was performed under highly alkaline conditions for this study. The next chapter gives a detailed step wise methodology of this research.

Chapter 3

MATERIALS AND METHODS

3.1. Test Materials and Instruments

Standard analytes chloroform and bromoform were purchased from Sigma Aldrich (USA) and Dr. Ehrenstorfer (Germany) respectively, with 99% purity. Methanol was acquired from Merck (Germany), whereas SPME (75 μm Car-PDMS) fiber was obtained from Supelco (USA). Fully automated instrument Sysmex XP-100 (Singapore) was used for haematology analysis. Gas Chromatograph used for analysis was Shimadzu GC-2010 (Japan). HS-SPME clear glass vials and EDTA tubes were supplied by Supelco (USA) and Improve Medical (Belgium) respectively.

For performing comet assay Tris-HCL molecular biology grade, low melting point agarose (LMPA) electrophoresis grade, normal melting point agarose (NMPA) electrophoresis grade were obtained from Scharlau (Spain) and Ethidium Bromide, sodium hydroxide pellets, Trizma base were supplied by Daejung (Korea).

3.2. Cleaning of Glassware

Throughout the research period all the glassware was first washed with soapy water, soaked overnight in chromic acid solution, rinsed with distilled water and then finally oven dried at 150°C.

3.3. Sample Preparation

3.3.1. Sample Collection

Over the course of this research 20 peripheral blood samples were collected from healthy humans, between age group 20 to 30 years, for gas chromatography (GC), genotoxicity and haematology analysis in EDTA vacutainers through venipuncture at ASAB (NUST) diagnostic laboratories. These sterile vaccutainer glass tubes contain EDTA, which prevents the blood from clotting. For headspace gas chromatography analysis, to avoid headspace loss, vacutainers were filled with blood completely. Immediately after the blood was drawn into the tube it was slightly shaken by hand to carefully dissolve the EDTA.

Figure 3.3 : Blood Withdrawal

3.3.2. Sample Storage

After the blood samples were withdrawn they were immediately delivered to IESE laboratory of Microbiology and Biotechnology. The samples were carefully

transferred in ice boxes to protect them from light and heat. Once the samples were brought to the lab, they were stored at 4°C until analysis. For comet assay analysis fresh blood samples were used and analysed within 4 hours of collection.

3.3.3. Cell Treatment

Prior to cell treatment, the blood samples were removed from the refrigerator and placed at room temperature for 20-30 minutes. Once the blood samples equilibrated with the room temperature 1mL aliquots of blood was withdrawn using a clean disposable air tight syringe and transferred into each of the four EDTA vacutainer. To administer the blood samples with different doses of chloroform and bromoform for cell treatment, both chloroform and bromoform were prepared in concentrations of 10, 30 and 50 µg/mL.

Aside from control remaining three samples were spiked with 10, 30 and 50 µg/mL doses of chloroform. The similar procedure was followed for bromoform sample preparation. Once the samples were spiked with doses of chloroform and bromoform they were placed in incubator at 37°C for 5 hours to allow cell treatment. Same procedure for followed for genotoxicity, haematological and HS-SPME analysis.

3.4. HS-SPME for Blood Sample Analysis

3.4.1. Standard Solutions

THM stock solution of 10,000 µg/mL was prepared in methanol following EPA Method 551.1. Working standard solutions of 1, 10, 20, 30 40, 50 µg/mL were prepared for chloroform and bromoform to obtain linear calibration curves.

3.4.2. HS-SPME technique

The blood samples were tested using gas chromatography to observe change in concentration of spiked dose, by means of headspace solid-phase micro extraction technique (HS-SPME). The extraction method used involved mixing 1mL of treated blood sample with equal volume of distilled water in SPME glass vial using a hot plate magnetic stirrer. Stirring was done at 40°C for 30 minutes. The SPME fiber was injected through the 1.5 mm thick PTFE/silicone septum of vial and sample was allowed to adsorb on the fiber. After 10-15 minutes the SPME fiber was retracted and sample was injected in GC. After calculating change in spiked dose concentrations the recovery efficiency (R) was also calculated using U.S EPA 555.1 method.

$$R = \frac{100\ (A-B)}{C}$$

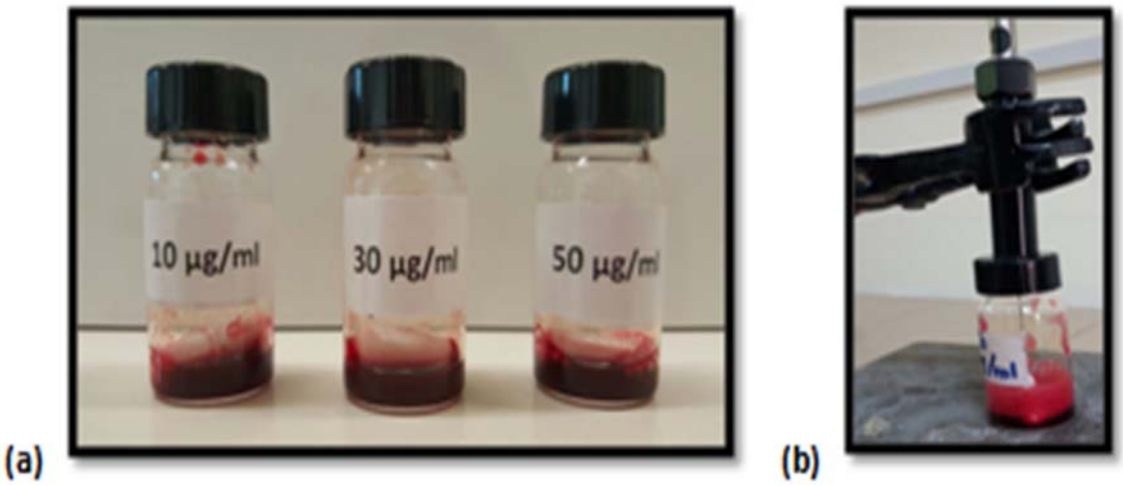

Figure 3.4 : SPME vials containing treated blood sample (a) SPME fiber injected into the vial (b)

3.4.3. Gas Chromatographic Conditions

The GC conditions were optimized as the injector and detector temperature are influenced by boiling point of analytes while the column temperature and carrier gas flow are critical factors for elution of analytes.

Table 3.4.3 : GC Conditions

Parameters	Values
1. Injector	
Pressure	48.2 Kpa
Total flow	126.9 mL/min
Temperature	220°C
Linear Velocity	24.4 cm/sec
2. Column	
Initial temperature	50°C
Final temperature	200°C
Temperature ramp	15°C/min
3. Detector	
Temperature	220°C
Current	0.03 nA
Gas Flow	4 mL/min

3.5. Comet Assay Protocol

3.5.1. Preparation of Reagents

The following reagents were prepared using autoclaved distilled water and glassware to avoid any contamination and factors that may interfere with results.

3.5.1.1. Phosphate Buffer Saline (PBS)

A tablet of PBS was dissolved in 100 mL distilled water and autoclaved for 15-20 minutes. After preparation PBS solution was stored at room temperature.

3.5.1.2. Agarose

For the preparation of slides two different agaroses of different percentages were prepared.

- **Normal Melting Point Agarose (NMPA)**

  For the preparation of 1% normal melting point agarose, 500 mg of NMPA was weighed and dissolved in 50 mL PBS buffer in a glass beaker. The beaker was placed in a water bath set at 90°C. The agarose was continuously stirred until it completely dissolved.

- **Low Melting Point Agarose (LMPA)**

  For the preparation of 2% normal melting point agarose, 1000 mg of LMPA was weighed and dissolved in 50 mL PBS buffer in a glass beaker. Just like NMPA preparation, the beaker was placed in a water bath set at 90°C and agarose was continuously stirred until it completely dissolved, to avoid formation of lumps.

3.5.1.3. Lysing Solution

For the preparation of 500 mL of lysing solution 2.5 N NaCl (73.05g), 100 mM EDTA (18.6g) and 10 mM Trizma Base (0.6g) were dissolved properly in 350 mL distilled water. Once the ingredients dissolved 4 g of NaOH pellets added to the mixture and dissolved by mixing vigorously for 20 minutes. After dissolving NaOH, its pH was adjusted to 10 using concentrated HCL or NaOH. The solution was then made 445 mL with distilled water using a measuring cylinder.

Finally at the time of use 50 mL of 1% Triton X-100 and 5 mL of 10% DMSO was added to the solution to make it 500 mL.

3.5.1.4. Alkaline solution

In order to prepare alkaline solution, two stock solutions were prepared.

10N NaOH

It was prepared by dissolving 200 g NaOH in 500 mL distilled water.

200 mM EDTA

It was prepared by dissolving 14.8 9 g EDTA in 200 mL distilled water.

To prepare 500 mL of working solution 15 mL of NaOH and 2.5 mL of EDTA stock solution was added to 482.5 mL distilled water. The pH of the solution was adjusted to >13.

3.5.1.5. TBE Electrophoresis Buffer

Tris Base (10.8g), Boric Acid (5.4g) and EDTA (1.86g) were dissolved in

1000 mL of distilled water, to make TBE electrophoresis buffer. The pH of the solution was adjusted to >13.

3.5.1.6. Neutralization Solution

Neutralization solution 0.4M Tris HCL was used. For 500 mL working solution 24.25 g of Tris HCL was dissolved in 500 mL distilled water.

3.5.1.7. Staining Solution

10x Stock solution

It was prepared by dissolving 5 mg in 25 mL distilled water.

1x Working solution

It was prepared by dissolving 1 mL of stock solution in 9 mL distilled water.

3.5.2. Procedure for Alkaline Comet Assay

The Singh *et al*. (1988) alkaline version comet assay protocol was followed as such with slight modifications (changes in gel strength, step durations). The slides were labeled properly before layering. During preparation of slides low humidity environments were preferred otherwise the gelling time of agarose was increased by 15 minutes to enhance the adherence of samples. The method was performed under dim light to prevent UV-induced DNA damage.

3.5.2.1. Slides Pre-coating

Typical three layer sandwich slides were made but before pre-coating the slides they were made grease-free and clean by dipping them in 70% ethanol and drying over blue flame. The NMPA agarose was maintained at a temperature of 37°C for pre-coating the slides. Half portion of the slide was dipped in LMPA to make smooth and uniform layer of agarose. Immediately after dipping, the lower part of the slide was wiped off to

remove the excess agarose and the agarose layer was allowed to solidify at room temperature. Pre-coating of slides provides enhanced anchorage for the subsequent layers of agarose.

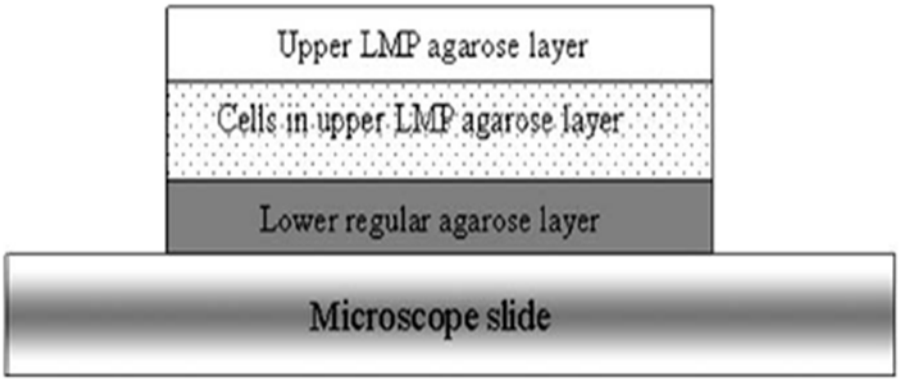

Figure 3.5 : Three Layer Sandwich Slide

3.5.2.2. Sample Pouring

Once the slides dried, LMPA-cell suspension was dropped onto the solidified NMPA layer. LMPA-cell suspension was prepared by mixing 75 µL of LMPA (37°C) with 10 µL of cell sample in a disposable micro centrifuge tube. Special care was observed to avoid formation of bubbles. The gel layer was allowed to solidify by placing the slides on ice pack for 10-15 minutes. After the second layer of agarose solidified , a third layer of agarose was made to occupy remaining pores in second layer and to ensure complete coverage of sample area on the slide by spreading 80 µL of NMPA over it. Immediately after layering cover slips were carefully placed on the slides. The gel was allowed to solidify for 10-15 minutes at 4°C.

3.5.3.3. Lysing

After the solidification of third agarose layer, the cover slips were removed carefully and were dipped in cold lysing solution that contains high concentration of salts and detergents. The slides were allowed to lyse in the solution at 4°C for minimum 1-2 hours.

Figure 3.5 : Slides Dipped in Lysing Solution

3.5.3.4. Alkali unwinding

After the lysing step excess buffer was drained from the slides after removing them from the lysing solution. Next the slides were carefully submerged in alkaline solution at room temperature for 20 minutes.

3.5.3.5. Electrophoresis

After alkali unwinding ice cold TBE buffer was poured into the electrophoresis tank and the slides were placed in the tank's slide tray. The tank was covered with lid and the slides were electrophoresed for 20-25 minutes at 24 V and 300 mA.

3.5.3.6. Neutralization

Next, the slides were lifted from the electrophoresis solution gently and placed on a flat tray. The slides were neutralized, using the neutralization solution, drop by drop for 5 minutes. This step was repeated 2-3 times and the excess solution was drained from the slides.

3.5.3.7. Staining

A few minutes after neutralization, the slides were stained using 80µL of 1x ethidium bromide staining solution. The slides were stained 2-3 times with the staining solution until a yellowish brown colour developed. Excess staining solution was drained using chilled distilled water

3.5.3.8. Drying

After staining, excess moisture was removed from the slides by drying them in the oven at 37°C for about 15 minutes.

3.5.3.9. Visual Analysis

Slides were then visualized to determine genetic damage by observing the stained using 1000x objective with Trinocular Fluorescent Microscope (Optika- B353FL). The microscope was equipped with a camera (AIPTEK: AHD-Z600), ocular micrometer of 10 µm and white LED/12V 20W illuminator. Images were taken and tail length was measured with ocular micrometer.

Figure 3.5 : Setup for Visual Analysis of Comets

3.5.3.10. Image Analysis

The comets images were analysed using CASP Lab software. It has been developed to work with coloured as well as or gray-scale images of fluorescence-stained comets. A measurement frame is drawn on the screen and its size adjusted. The adjustments are frozen to prevent accidental modification. The frame is moved onto a cell and measurement is activated. An intensity profile shows up on a "profile" window together with selected result values and the result can be saved. The software gives quantitative description of comets using different parameters like % DNA damage, comet tail length (µm) and levels of DNA damage. %DNA damage was determined by counting damaged cells having long tail like structure in each slide. Tail length was measured (Heepchantree *et al*., 2006) according to given formula:

Comet tail length (µm) = Maximum total length – Head diameter

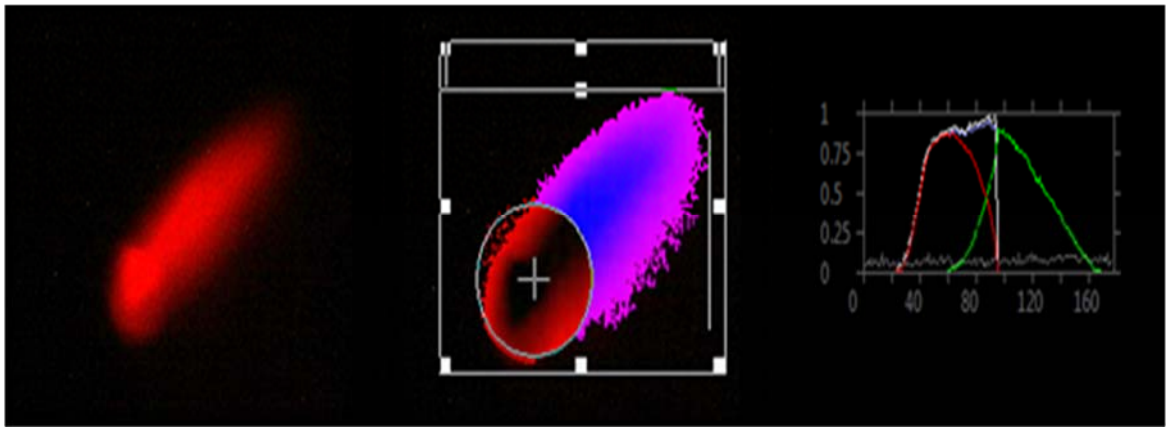

Figure 3.5 : Stepwise Demonstration of Image Analysis Using Casp Lab for Bromoform 50 µg/mL

Another parameter to evaluate the extent of DNA damage is to classify damage according to length of migrated DNA (tail length). In this research classification is no damage, low level damage (5 – 20%), medium level damage (20 – 60%) and high level damage (60 – 100%).

3.5.4. Haematological Analysis

After cell treatment the samples underwent complete blood count (CBC) test for analyzing the effect of target compounds on various haematological parameters. The equipment used to conduct the test was Sysmex XP-100 Haematology Analyser. The haematological parameters included in the CBC test were red blood cells (RBCs), white blood cells (WBC), platelets (PLT), hematocrit (Hct), mean corpuscular haemoglobin concentration (MCHC) and haemoglobin (Hgb).

Figure 3.5.4 : Sysmex XP-100

3.5.5. Statistical Analysis

Statistical analysis of all the data was carried out using ANOVA to estimate the significance of the differences found among the groups. $P<0.05$ was considered statistically significant. Correlation was also used to represent the association between concentration of target compounds and DNA damage.

RESULTS AND DISCUSSION

This chapter is based upon the results obtained from the series of experiments conducted to determine the genotoxicity of THMs and their effect on human haematological parameters. Phase wise discussion of results of this research is done below in detail.

4.1. GC Analysis

Calibration curves were plotted for both target THMs and an acceptable linear calibration curve with a regression coefficient (R^2) of 0.98 and 0.99 was obtained for chloroform and bromoform respectively (Figure 4.1).

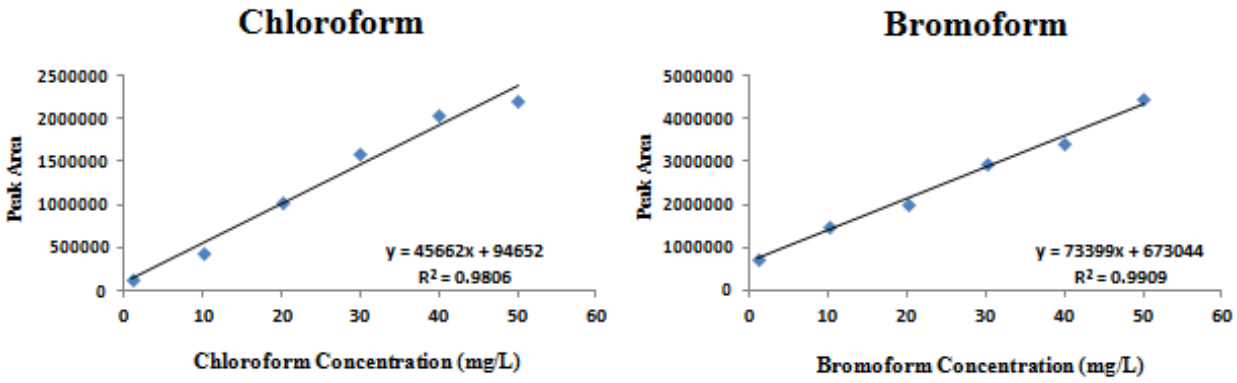

Figure 4.1 : Calibration Curve Showing Con. Vs Peak Response for Chloroform (a) & Bromoform (b)

Gas chromatography results revealed that 16.8-19.9% and 15.1–22.7% change in spiked dose concentration occurred after cell treatment in chloroform and bromoform respectively, indicating that the cell treatment was successful. This slight change of dose

concentration was probably due to headspace loss and volatilization occurring while transferring samples into SPME vials (Blount *et al*., 2006) .

Moreover, it is quite challenging to elucidate measured concentrations of THMs in blood samples and shall continue to pose challenges owed to their rapid metabolism and elimination (Aylward *et al*., 2008).

The respective chromatographic peaks for standards and samples of both THMs at dose 50 µg/mL may be observed in the Figure 4.2. The figure shows identifiable chromatographic peaks for chloroform and bromoform at retention time 3.95 and 7.74 minutes respectively.

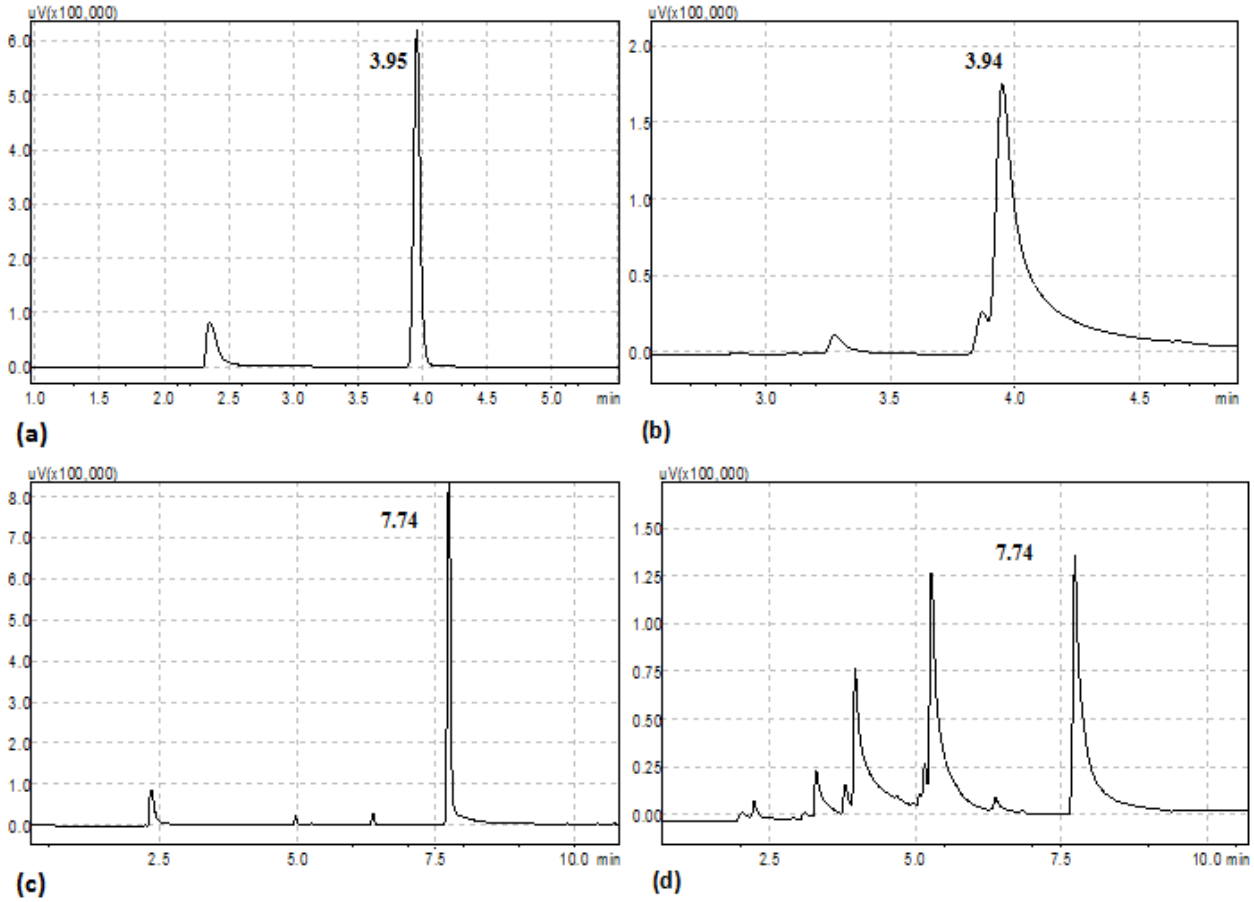

Figure 4.2 : Chromatographic Peaks for Standards and Samples of Chloroform (a,b) & Bromoform (c,d)

Recovery efficiency for HS-SPME was calculated to evaluate the method performance for THMs. According to USEPA percent recoveries must fall in the range of 70 to 120% for THMs. The HS-SPME helped to achieve acceptable recovery efficiency values ranging from 80.1 – 83.2% and 77.29 – 84.9% for chloroform and bromoform respectively, therefore establishing that HS-SPME technique is reproducible, fast and accurate.

4.2. Genotoxicity Analysis

The results obtained from comet assay were plotted in a series of graphs for the parameters tail length, tail DNA and olive tail moment (OTM). Disturbances were observed at all concentrations of chloroform and bromoform. For statistical analysis one-way ANOVA and Tukey's test were used to analyse data and make multiple comparisons.

4.2.1. Visual Inspection

There are four classes of DNA damage and the utilization of this system has been found to provide quantitative resolution which is sufficient for many purposes. The figure below puts the four classes (0, 1, 2, 3) of DNA damage to display.

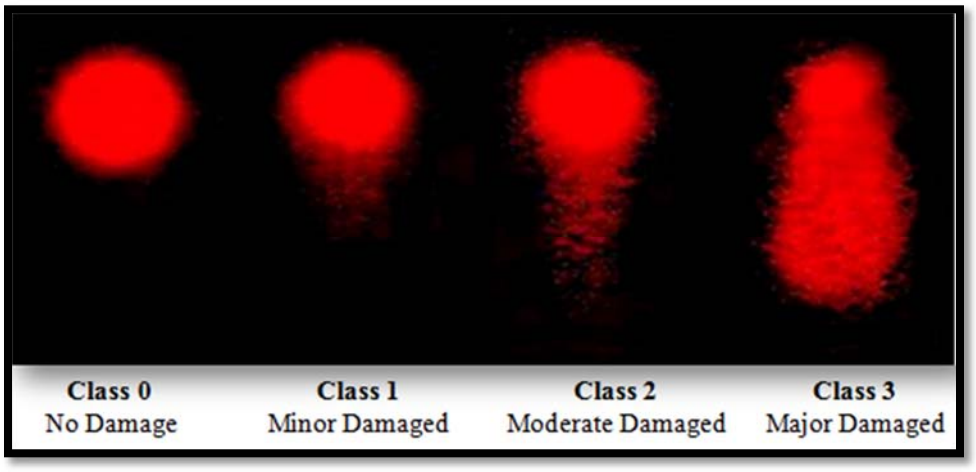

Figure 4.3 : Classification of DNA Damage

In literature DNA damage has been classified into 2 to 6 classes. In this method the total amount of DNA in the tail is estimated by eye. The benefit of using this method is that it is easy to perform and relies on human vision rather than any expensive analysis software.

The images of comets captured during the course of this research were analysed by this method and it was observed that they all mostly fall in classes 1 to 3 whereas the control falls in class 0 category. As the focus of this research was more on qualitative analysis rather than quantitative, so image analysis was done using software with focus on three indexes that are discussed below.

4.2.2. Tail Length

During the step of electrophoresis, when electric current is applied, the already damaged and unwound DNA start migrating in the direction of current towards anode. The extent of DNA to travel depends upon the level of damage occurred in the DNA. Measurement of the travelled DNA reveals the level of damage which may further be classified into low, medium, high and very high level categories. Tail length is considered to be one of the most important parameters to assess the DNA damage (Kumaravel & Jha, 2006).

Figure 4.4 depicts the relationship between DNA tail length and varying concentrations of chloroform and bromoform, with R^2 values of 0.9893 and 0.9967 respectively, indicating that the model explains the variability of the response data around its mean.

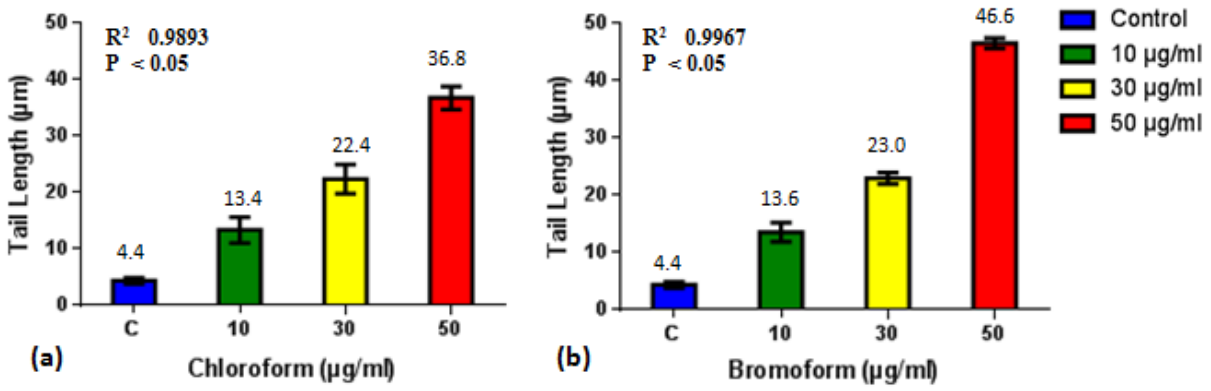

Figure 4.4 : Relationship between Tail Length and Concentration of Chloroform (a) & Bromoform (b)

The damage categorized on the basis of tail length is clearly showing an increasing trend in a dose dependant manner. The mean tail lengths values for chloroform and bromoform at the observed doses 30, 40, and 50 µg/mL were 16.2, 24.4, 36.4 µm and 13.6, 23.0, 46.6 µm, respectively. These values were quite higher than the negative control (4.4 µm).

Upon comparing the two graphs in figure 4,4 it can be observed that the damage caused by bromoform is higher than chloroform at every administered dose which indicates that it is higher in toxicity. The p value for both the compounds was < 0.05. Tail length is considered the best parameter to measure genetic damage. It can be seen in these graphs that the change is very gradual and even.

4.2.3. Tail DNA%

Another useful index of evaluation single strand DNA damage is calculating the % of DNA in tail. The graphs below in Figure 4.4 depicts the relationship between DNA tail length and varying concentrations of chloroform and bromoform with R^2 values of 0.9792 and R^2 0.9754 respectively indicating that the model explains well explains the variability of the response data around its mean.

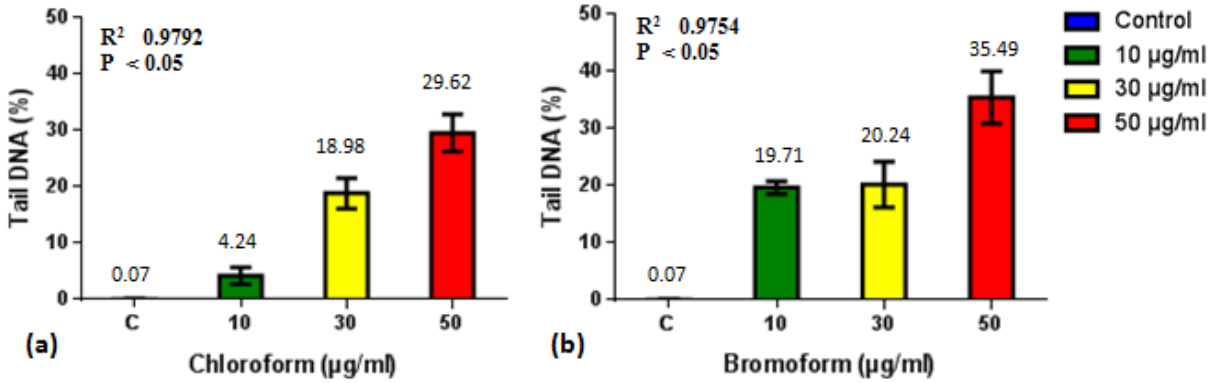

Figure 4.5 : Relationship between Tail DNA% and Concentration of Chloroform (a) & Bromoform (b)

Dose-dependent DNA damage was observed in case of %DNA in tail just like tail length. A greater DNA tail area and longer DNA tail length reflects more extensive DNA damage. The mean tail DNA values for chloroform and bromoform at the observed doses of 30, 40, and 50 µg/mL were 4.24, 18.98, 29.62% and 19.71, 20.24 and 35.49%, respectively. These values were significantly higher than the negative control (0.07%). Comparison between graphs of figure 4.5 shows that control versus 10 µg/mL dose of bromoform shows higher level of significance as compared to chloroform. The same goes for control versus 10 µg/mL and control versus 50 µg/mL as well.

4.2.4. Olive Tail Moment (OTM)

Tail moment is the product of tail length and the fraction of total DNA in the tail. It incorporates a measure of both the smallest detectable size of migrating DNA (reflected in the comet tail length) and the number of relaxed / broken pieces (represented by the intensity of DNA in the tail).

Figure 4.6 it can be clearly seen that the level of genotoxicity is increasing in a dose dependant manner, both for chloroform (R^2 0.9810) and bromoform (R^2 0.9754) in the form of single strand breaks. The OTM values for chloroform and bromoform at

doses 30, 40, and 50 µg/mL were 1.56, 5.58, 6.17 and 4.44, 6.31, 10.75 respectively, which were higher than the negative control (0.036).

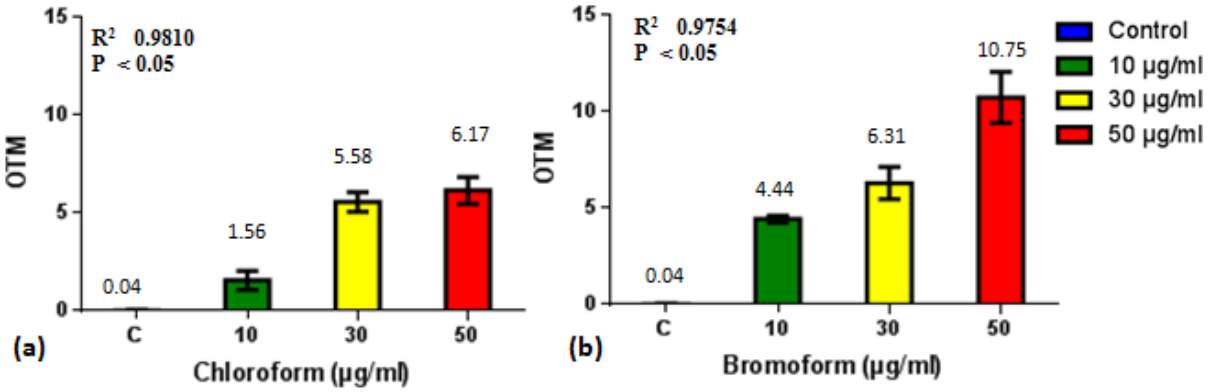

Figure 4.6 : Relationship between OTM and Concentration of Chloroform (a) & Bromoform (b)

The results of previous two parameters were further proven and strengthened by graph of olive tail movement values. The p value < 0.05 remained maintained for all parameters.

The results obtained show some similarity with the results of the research conducted by Khallef *et al.* in 2015. The high DNA damage is oxidative stress induced. The secondary genotoxicity causing potential of these agents may possibly be due to formation of highly reactive oxygen species or lipid peroxidation, or both. It is noteworthy that, for the target compounds used in this study, secondary genotoxicity is linked to high concentrations reaching the level for cytotoxicity and may possibly explain why they are only weakly positive in certain genotoxicity assays that were conducted at high concentrations (Luo *et al*., 2004).

The literature suggests that the best image analysis parameter to use is %DNA in tail. This shows a good linearity with dose of damage over a reasonable range. Tail length tends to reach a maximum at a low level of damage. According to literature tail moment

is the least informative parameter. It has no generally accepted units and has a drawback of not telling much about the appearance of the comets, whereas with % tail DNA one can immediately visualize them (Liao *et al*., 2009).

4.3. Haematological Analysis

The pharmacokinetics of these two trihalomethanes has been well studied through experimentation on laboratory rodents. The uptake and removal of volatile organic compounds from the body is not a simple phenomenon but it is controlled by a chain of active mechanisms that control the movement of compounds throughout the body and metabolize them into more water-soluble compounds. The work performed on pharmacokinetics of these volatile organic compounds (VOC) indicates that repeated and long term exposures cause bioaccumulation (Meek *et al*., 2002).

The data obtained from CBC test was statistically analysed using two- way ANOVA. The dose-dependent changes included that chloroform and bromoform have only minor effect on hematocrit (HCT) and white blood cells (WBCs) ($p>0.05$) .

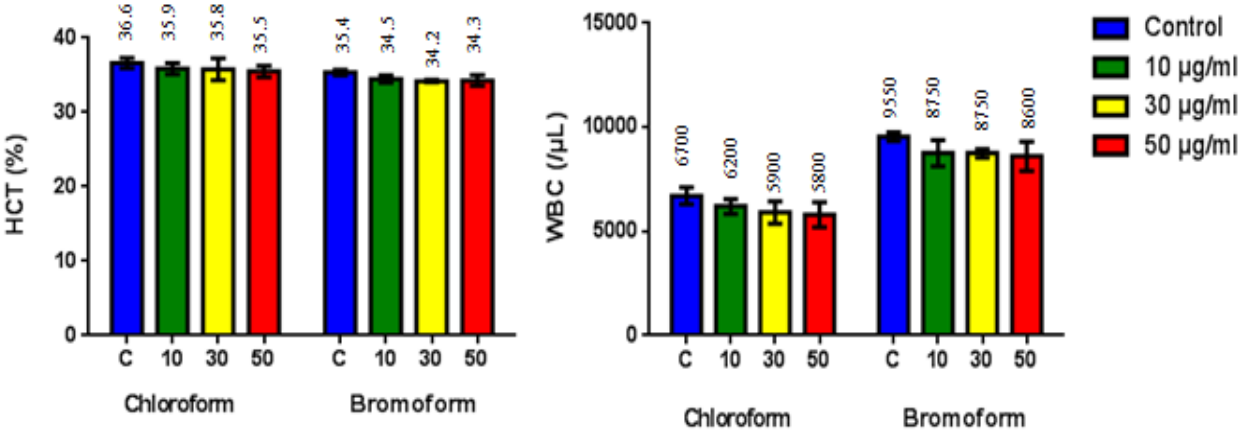

Figure 4.7 : Change in HCT. Level (a) Change in WBC level (b)

Both haemoglobin (HGB) and mean corpuscular haemoglobin concentration (MCHC) levels were significantly lowered at all administered doses of bromoform ($p<0.05$). Effect of DBPs on haemoglobin has previously been reported in literature (Sen *et al*., 2011). Bromoform is absorbed by the tissues which enhances the chemical activity and so it biotransforms into more reactive compounds. Bromoform is known to metabolize to dibromocarbonyl which is the bromine analogue of phosgene and carbon monoxide. Upon reaction with proteins phosgene is likely to cause either cell damage or cell death (USEPA, 2005).

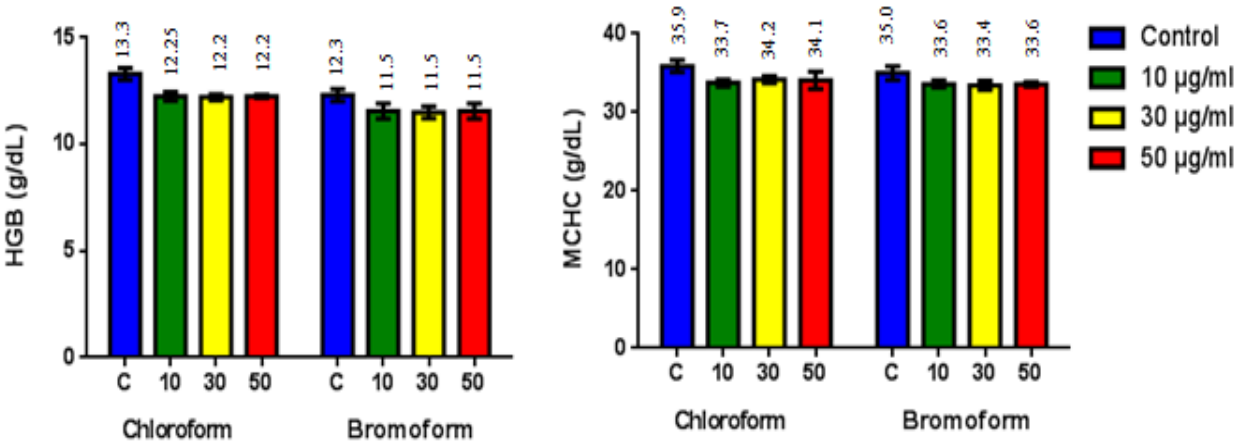

Figure 4.8 : Change in Hgb level (a) Change in MCHC level (b)

Bromoform significantly lowered platelets level at doses 30 µg/mL ($p<0.05$) and 50 µg/mL ($p<0.05$). RBC count was also lowered significantly by 30 and 50 µg/mL dose of bromoform ($p<0.05$) whereas chloroform only had a minor effect ($p>0.05$).

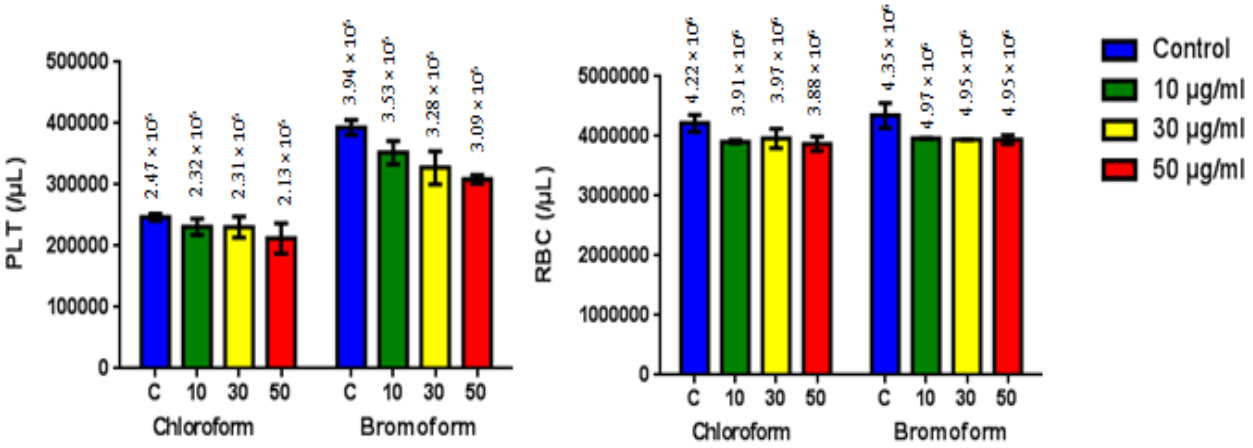

Figure 4.9 : Change in PLT level (a) Change in RBC level (b)

As mentioned above bromoform metabolizes to carbon monoxide, this carbon monoxide reacts with haemoglobin in blood stream and converts it into carboxyhaemoglobin (COHb) which unlike oxyhaemoglobin (O_2HB) prevents haemoglobin from supplying oxygen (O_2) to the body tissues. The cells die because of lack of O_2 and their number begins to decline (Andersen *et al*., 1991). This phenomenon explains the decline in RBCs.

The results showed a strong correlation between studied DBPs and their effect on haematological parameters. These results are in accordance with the literature (CEPA, 2010). Hence it was depicted that chloroform and bromoform have the potential to change haematological count. Although the haematological effects were significant but they were still well within the normal range.

The genotoxic and haematological effects of these compounds are owing to the fact that all trihalomethanes are primarily metabolized to either carbon monoxide or carbon dioxide. According to literature the toxicity of both the compounds is due to their reactive

metabolites. Metabolism of chloroform has been identified and studied in both oxidation and reduction pathways. Chloroform is metabolized to carbon dioxide that is generated via oxidative pathway. Phosgene and hydrochloric acid are produced during oxidative activation and both are very toxic and cause damage. But at these observed doses they caused no significant damage to haematological parameters (Borgert *et al*., 2015).

Chapter 5

CONCLUSIONS AND RECOMMENDATIONS

5.1. Conclusions

i. Successful cell treatment with only minor changes in spiked doses, ranging from 16.8 – 19.9% and 15.1 – 22.7% for chloroform and bromoform respectively.

ii. Comet assay results showed that DNA damages were significantly high for both compounds ($P \leq 0.05$).

iii. Mean tail lengths values for bromoform (13.6, 23.0, 46.6 μm) were higher than chloroform (13.4, 22.4, 36.8 μm) at the observed doses showing that bromoform has a higher genotoxic potential.

iv. Effect of bromoform on haematological parameters haemoglobin, red blood cells, mean corpuscular haemoglobin, platelets was statistically higher in significance ($P<0.05$) as compared to chloroform ($P>0.05$).

Pertaining to the harmful effects of these compounds, the levels of trihalomethanes must be kept to minimum in water supply. In order to do so either water must be pre-treated before chlorination or alternative methods of water disinfection must be used.

5.2. Recommendations

i. *In vivo* analysis for genotoxicity of trihalomethanes in drinking water.

ii. Sonochemical removal of trihalomethanes from aqueous solutions.

iii. Quantification of trihalomethanes in soft drinks/mineral water.

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