RISK FACTORS OF TUMORIGENESIS IN *TUIBUR* CONSUMERS

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By

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CERTIFICATE

This is to certify that the thesis entitled "*Risk factors of tumorigenesis in tuibur consumers*" submitted by ***Ms. Rebecca Lalmuanpuii,*** for the degree of ***Doctor of Philosophy*** in the Mizoram University, Aizawl, Mizoram, embodies the record of original investigations carried out by her under my supervision. She has been duly registered and the thesis presented is worthy of being considered for the award of the Ph.D. degree. This work has not been submitted for any degree in any other university.

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I, Rebecca Lalmuanpuii, hereby declare that the subject matter of this thesis is the record of work done by me, that the contents of this thesis did not form basis of the award of any previous degree to me or to do the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other University/ Institute.

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Dedication

For my family, who offer me unconditional love and support.

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GLOSSARY

Carcinogen	Any substance, radionuclide, or radiation that is an agent directly involved in causing cancer.
Carcinogenesis	Actual formation of cancer, whereby normal cells are transformed into cancel cells.
Control region (D-loop)	D-loop is also known as control region because it contains essential sequence for the mitochondrial expression.
DNA adduct	A piece of DNA covalently bonded to a (cancer-causing) chemical. This process could be the start of a cancerous cell, or carcinogenesis.
DNA Replication	The double helix is unwound and each strand acts as a template for the next strand. Bases are matched to synthesize the new partner strands. DNAreplication is the process of producing two identical replicas from one original DNA molecule.
DNA Transcription	A process that involves transcribing genetic information from DNA to RNA. The transcribed DNA message, or RNA transcript, is used to produce proteins.DNA is housed within the nucleus of our cells. It controls cellular activity by coding for the production of proteins.
Free radical	Any atom or molecule that has a single unpaired electron in an outer shell. Free radicals are highly reactive.
Gauss (G)	Unit of magnetic field strength. 1Tesla = 10^4 G.
Genotoxicity	The property of chemical agents that damages the genetic information within a cell causing mutations, which may lead to cancer.
g-value	Factor which expresses the size of magnetic moment of a magnetic moment of a paramagnetic species. Specifies the position at which the EPR spectrum of paramagnetic species occurs.
Heteroplasmy	The presence of more than one type of organellar genome (mitochondrial DNA or plastid DNA) within a cell or individual. It is an important factor in considering the severity of mitochondrial diseases.

Hyperfine splitting	Splitting in an EPR spectrum arising from the interaction of unpaired electron with the metal ion nucleus in the vicinity. Can be used to determine the unpaired electron densities on particular nuclei or to identify the ligands of a paramagnetic ion.
Lipid peroxidation	The oxidative degradation of lipids, in which free radical steal electron from the lipids in cell membrane, resulting in cell damage.
Magnetic field	Region of magnetic forces (attraction or repulsion) around a magnet.
Microsatellite instability	The condition of genetic hypermutability that results from impaired DNA mismatch repair.
Mutagen	A physical or chemical agent that changes the genetic material, usually DNA, of an organism and thus increases the frequency of mutations.
Mutation	A permanent alteration of the nucleotide sequence of the genome of an organism, virus, or extrachromosomal DNA or other genetic elements.
Polymorphism	The occurrence of structurally and functionally more than two different types of individuals, within the same organism or genetic variation or DNA sequence variation that is common in the population.
Recognition sequence	It is sometimes also referred to as recognition site, of any DNA-binding protein motif that exhibits binding specificity, refers to the DNA sequence (or subset thereof), to which the domain is specific.
Restriction enzyme	Also known as restriction endonucleaseis an enzyme that cuts DNA at or near specific recognition nucleotide sequences.
Restriction Site	Also known as restriction recognition sites, are locations on a DNA molecule containing specific (4-8 base pairs in length) sequences of nucleotides, which are recognized by restriction enzymes.
Tumorigenesis	Formation of a cancer, whereby normal cells are transformed into cancer cells.

ABBREVIATIONS

AAR	Age-Adjusted Rate
4-ABP	4-Aminobiphenyl
ACT	Aqueous cigarette tar
*Alu*I	*Arthrobacter luteus*
ANOVA	Analysis of variance
APCI	Atmospheric Pressure Chemical Ionisation
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
bp	base pair
CDRI	Central Drug Research Institute
CR	Confirmation rearrangement
dB	decibel
D-Loop	Displacement loop
DMPO	5,5-dimethyl-1-pyrroline-N-oxide
DNA	Deoxyribonucleic acid
DTPA	Diethylenetriamine pentaacetic acid
EBI	European Bioinformatics Institute
EC_{50}	Half maximal effective concentration
EDTA	Ethylenediamine tetraacetate
EI	Electron Impact
EPHX1	Epoxide hydrolase
EPR	Electron paramagnetic resonance
ESI	Electrospray Ionisation
ESR	Electron spin resonance
ETS	Environmental tobacco smoke
FAA	Flame atomic absorption
FTIR	Fourrier transform infrared spectroscopy
GABA	γ-aminobutyric acid
GC	Gas chromatography
GFAA	Graphite furnace atomic absorption
GHz	Giga hertz

GRIN	Germplasm Resources Information Network
GSH	Reduced glutathione
GSTM1	Glutathione S-transferase *mu* 1
GSTT1	Glutathione S-transferase *theta* 1
*Hae*III	*Haemophilus aegyptius*
HCN	Health Council of the Netherlands
HOMO	Highest occupied molecular orbital
HPB	4-hydroxy-1-(3-pyridyl)-1-butanone
HPLC	High performance liquid chromatography
HRMS	High resonance mass spectrometry
HVR	Hypervariable region
IARC	International Agency for Research on Cancer
ICDA	International Chromium Development Association
ICMR	Indian Council of Medical Research
ICP	Inductively coupled plasma
ICPAES	Inductively coupled plasma atomic emission spectrometry
ICPMS	Inductively coupled plasma mass spectrometry
ICPOES	Inductively coupled plasma optical emission spectrometry
IR	Infrared
ISTH	Indian Society on Tobacco and Health (Mizoram Chapter)
IUPAC	International Union of Pure and Applied Chemistry
IZiNCG	International Zinc Nutrition Consultative Group
*Kpn*I	*Klebsiella pneumoniae*
MAO	Monoamine oxidase
MEGA	Molecular Evolutionary Genetics Analysis
ML	Maximum likelihood
MnSOD/SOD2	Manganese containing superoxide dismutase
MPO	Myeloperoxide
MS	Mass spectrometry
MSCI	Mizoram State Cancer Institute
*Msp*I	*Moraxella sp*
mtDNA	Mitochondrial deoxyribonucleic acid
m/z	mass to charge ratio

nAChRs	neuronal nicotinic Acetylcholine Receptors
NCRP	National Cancer Registry Programme
nDNA	Nuclear deoxyribonucleic acid
NNAL	4-(methylnitrosamino)-4-(3-pyridyl)-butanal)
NNK	4-(methylnitrosamino)-1-(3'-pyridyl)-1-butanone)
NNN	N-nitrosonornicotine
np	nucleotide position
ns	nanosecond
ODS	Octadecyl silane
PAH	Poly-aromatic hydrocarbon
PAGE	Polyacrylamide gel electrophoresis
PAST	Paleontological Statistics
PBS	Phosphate buffer saline
PCI	Phenol chloroform isoamylalcohol
PCR	Polymerase chain reaction
PVDF	Polivinylidene fluoride
ppb	parts per billion
ppm	parts per million
ppt	parts per trillion
Q-QH2-QH	Quinone-hydroquinone-semiquinone
rCRS	revised Cambridge Reference Sequence
RFLP	Restriction fragment length polymerase
ROS	Reactive oxygen species
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SDS	Sodium dodecyl sulfate
SOD	Superoxide dismutase
SHS	Secondhand smoke
SNP	Single nucleotide polymorphism
SS	Sidestream smoke
TAE	Tris acetate EDTA
TE	Tris EDTA
tRNA	Transfer ribonucleic acid

TSNA	Tobacco specific nitrosamine
USEPA	United State Environmental Protection Agency
US ATSDR	U.S. Agency for Toxic Substances and Diseases Registry
UV	Ultraviolet
WHO	World Health Organization
WPRO	Western Pacific Regional Office

NOTATION

B_o	Magnetic field
β	Electron Bohr magneton
E	Energy
g	gyromagnetic ratio
G	Gauss
h	Planck constant
m-	*meta*-position
o-	*ortho*-position
p-	*para*-position
ν	Frequency

CHAPTER 1: Introduction

CHAPTER 1

INTRODUCTION

1.1. Background and Review of Literature

Tobacco is a value added agricultural product processed from the fresh leaves of plants of the genus *Nicotiana* of the Solanaceae (nightshade) family. The genus contains a number of species; according to the Germplasm Resources Information Network (GRIN) taxonomy for plants, there are 72 species records of *Nicotiana*. Many varieties of tobacco are grown in the world, with multifarious uses. The types of tobacco vary according to tobacco classes in countries and elements such as manipulation of nitrogen fertilization, plant density, time and height of topping, harvesting and curing are added to favorably influence the utility of cured leaves for specific products.

The name 'tobacco' is commonly used for the product manufactured from tobacco leaves which is used in cigars, cigarettes, snuff, pipe, chewing tobacco, etc. Different species of the tobacco plant, with a whole range of attributes associated with smoking (*e.g.*, fast burning, slow burning, mild, strong); have become popular in various regions of the world. The primary active ingredient of tobacco, the alkaloid nicotine, a secondary metabolite, is responsible for its narcotics and soothing qualities.

Tobacco, one of the most important cash crops of India, is native to North America. It became known to the rest of the world when European explorers in the 15^{th} and 16^{th} centuries witnessed it being used as medicine and hallucinogen by the Native Americans. The tobacco

plant was met with criticism initially; banned by Kings and Popes in Europe. Its economic effects and broad popularity forced acceptance among all cultures and became popular nonetheless. It quickly spread throughout the civilized world and became a foundation for the growth of the American economy [West and Shiffman, 2007]. Tobacco, both as the product and the plant, followed the major trade routes to major ports and markets, and then on into hinterlands. By the mid 17th century, tobacco smoking had been introduced to every major civilization and had also assimilated into the native culture, despite the attempts of many rulers then to eliminate the practice with harsh penalties of fines. The English language term smoking was coined in the 18th century; before then, the practice was called drinking smoke [Lloyd and Mitchinson, 2007].

The smoking of tobacco and various hallucinogenic drugs were used to achieve trances and to come into contact with the spiritual world. Apart from smoking, tobacco had a number of uses as medicine. As a painkiller, it was used for earache, toothache and occasionally as a poultice. In Mizoram, juices of tobacco is also used to cure pig's skin disease (similar to leucoderma) [Bhardwaj and Gakhar, 2004]. Smoking was said to be employed by the desert Indians as a cure for colds, especially if the tobacco was mixed with the leaves of a small Desert Sage, *Salvia dorrii*, or a root of Indian Balsam or Cough Root, *Leptotaenia multifida*, the addition of which was thought to be particularly good for asthma and tuberculosis [Balls, 1962].

South Asia is a major producer, consumer and net exporter of tobacco. The tobacco plant is a robust annual little branched herb with 20-30 large green leaves and long trumpet-shaped flowers which may grow over 60 cm upto 2.5 m in size depending on the variety and agroclimatic conditions. All the plant parts are sticky, covered with short viscid-glandular hairs.

Among certain number of species identified, *Nicotiana tabacum* (Figure 1.1) and *Nicotiana rustica* (Figure 1.2) are of economic significance.

General information about the tobacco plant

Kingdom: Plantae

Phylum: Magnoliophyta

Class: Magnoliopsida

Order: Solanales

Family: Solanaceae

Genus: *Nicotiana*

Species: *Nicotiana tabacum*; *Nicotiana rustica.*

Figure 1.1: *Nicotiana tabacum* (also known as Virginia tobacco).

Figure 1.2: *Nicotiana rustica* (also known as Wild tobacco).

In the late 1920s, German scientists formally identified the link between smoking and lung cancer leading the first anti-smoking campaign in modern history. The movement, however, failed to reach across enemy lines during the Second World War, and quickly became unpopular thereafter [Proctor, 2000]. In 1950, health authorities again began to suggest a relationship between smoking and cancer [Doll and Hill, 2004]. Scientific evidence mounted in the 1980s, which prompted political action against the practice. Rates of consumption from 1965 onward in the developed world have either peaked or declined [Rock *et al*., 2007]. However, they continue to climb in the developing world [WHO/WPRO, 2002].

Smoking is the most common method of consuming tobacco, and tobacco is the most common substance smoked. The agricultural product (leaves) is often mixed with other additives and then pyrolysed [Wingand, 2006]. The resulting vapors are then inhaled and the active substances are absorbed through the alveoli in the lungs [Gilman and Xun, 2004a]. The active

substances trigger chemical reactions in nerve endings which temporarily enhances the heart rate, memory, alertness [Parrot and Winder, 1989], and reaction time [Parkin *et al*., 1998]. Initially dopamine and later endorphins are released in the brain, which are often associated with pleasure [Gilman and Xun, 2004b].

As of 20th century, smoking is practiced by over 1.22 billion people. Men are more likely to practice smoking as compared to women [Guindon and Boisclair, 2003], though the gender gaps decline with younger age [WHO, 2001; Satcher, 2001]. The poor are more likely to smoke than the wealthy, and people of developing countries than those of developed countries [WHO/WPRO, 2002]. Many smokers begin the habit during adolescence or early adulthood. Usually during the early stages, smoking provides pleasurable sensations, serving as a source of positive reinforcement. After an individual has smoked for many years or when someone becomes habituated to nicotine, and then stop using it, they experience the symptoms of withdrawal, including anxiety, irritability, restlessness, shortened attention span and an intense, sometime irresistible, craving for nicotine. The avoidance of withdrawal symptoms and negative reinforcement become the key motivations to continue smoking.

It is now well known that nicotine is powerfully addictive and it is the primary reason why people continue their use of tobacco [Benowitz, 1988]. In the plant kingdom, nicotine is not restricted to tobacco but is widespread. Besides tobacco, the nightshade family also includes potatoes, tomatoes, eggplant and red peppers. It is made in roots and accumulates in the leaves of these plants. However, the concentration of nicotine in these vegetables is far lower than the level in tobacco plant [Domino *et al*., 1993]. In tobacco plant leaves, the constituent of nicotine

is approximately 0.6-3.0 % (30 mg/g) of the dry weight of the plant [Hoffmann and Hoffmann, 2012].

Nicotine exhibits powerful pharmacological effects including heart rate, heart stroke volume, and oxygen consumption by the heart muscle, as well as powerful psychodynamic effects such as euphoria, increased alertness, and a sense of relaxation. It is a potent parasympathomimetic alkaloid and a stimulant drug synthesized by tobacco plant or produced synthetically. The effects of nicotine are related to blood levels of nicotine, the kinetics of exposure and the resulting psychoactive effect. Nicotine is an alkaloid that acts as an acetylcholine analogue in humans. When a person inhales smoke from cigarette, nicotine is distilled from the tobacco and is carried along with smoke particles into the lungs, where it is absorbed rapidly into the pulmonary venous circulation. It then enters the arterial circulation and moves quickly to the brain. Nicotine diffuses readily into brain tissue, where it binds to neuronal nicotinic acetylcholine receptors (nAChRs), which are ligand-gated ion channels. When a cholinergic agonist binds to the outside of the channel, the channel opens, allowing the entry of cations, including sodium and calcium. These cations further activate voltage-dependent calcium channels, allowing further calcium entry [Laviolette and Vander, 2004; Benowitz, 1990; 2009].

Nicotine is a tertiary amine, and a bicyclic compound consisting of a pyridine (blue) and a pyrrolidine (red) ring (Figure 1.3). It is also called 3-(1-methyl-2-pyrrolidinyl) pyridine according to the International Union of Pure and Applied Chemistry (IUPAC) nomenclature. It is hygroscopic, colorless oily liquid that is readily soluble in alcohol, ether or light petroleum. Also, it is optically active as the molecule possesses an asymmetric carbon (on the red ring as indicated in Figure 1.3) and so exists in two enantiomeric compounds. The naturally occurring form of

nicotine is *levo*rotatory with a specific rotation of $[\alpha]_D$ = -166.4^o((-)-nicotine). The *dextro*rotatory form, (+)-nicotine is physiologically less active than (-)-nicotine. (-)-nicotine is more toxic than (+)-nicotine [Gause, 1941]. The salts of (+)-nicotine are usually *dextro*rotatory [Henry, 1949].

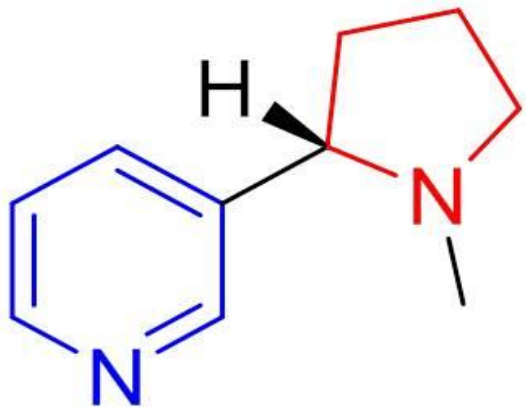


Figure 1.3: Molecular Structure of Nicotine.

Also, brain imaging studies demonstrated that nicotine acutely increases activity in the prefrontal cortex, thalamus, and visual system, consistent with the activation of corticobasal ganglia-thalamic brain circuits [Brody, 2006]. Stimulation of central nAChRs by nicotine results in the release of a variety of neurotransmitters in the brain, most importantly dopamine. Nicotine causes the release of dopamine in the mesolimbic area, the corpus striatum, and the frontal cortex. Of particular importance are the dopaminergic neurons in the ventral tegmental area of the midbrain, and the release of dopamine in the shell of the nucleus accumulates, as this pathway appears to be critical in drug-induced reward [Dani and De Biasi, 2001; Nestler, 2005]. Other neurotransmitters, including norepinephrine, acetylcholine, serotonin, γ-aminobutyric acid

(GABA), glutamate and endorphins are released as well, mediating various behaviors of nicotine.

Most of the nicotine-mediated release of neurotransmitters occurs via modulation by presynaptic nAChRs, although direct release of neurotransmitters also occurs [Wonnacott, 1997]. Dopamine release is facilitated by nicotine-mediated augmentation of glutamate release and, with long term treatment, by inhibition of GABA release [Mansvelder, 2002]. In addition to the direct and indirect stimulation of neurotransmitter release, chronic cigarette smoking (but not nicotine administration) reduces brain monoamine oxidase A and B (MAOA and MAOB) activity, which would be expected to increase monoaminergic neurotransmitter levels such as dopamine and norepinephrine in the synapses, thus augmenting the effects of nicotine and contributing to addiction [Lewis *et al*., 2007]. Inhibition of MAO facilitates acquisition of nicotine self-administration in rats; supporting the idea that MAO inhibition interacts with nicotine to reinforce tobacco dependence [Villégier *et al*., 2007].

Dopamine release signals a pleasurable experience, and is critical to the reinforcing effects of nicotine and other drugs of abuse [Nestler, 2005]. Chemically or anatomically, lesioning dopamine neuron in the brain prevents nicotine self-administration in rats. When intracranial self-stimulation is used as a model for brain reward in rats, nicotine acutely lowers the threshold for self-stimulation [Cryan *et al*., 2003]. Thus, through its effects on dopamine release, acute nicotine administration increases brain reward function. Likewise, nicotine withdrawal is associated with significant increases in intracranial self-stimulation reward threshold, consistent with deficient dopamine release and reduced reward [Epping-Jordan *et al*.,

1998]. The decrease in brain reward function experienced during nicotine withdrawal is an essential component of nicotine addiction and a key barrier to abstinence.

All over the world, a large variety of smoking and smokeless tobacco products are extensively utilized. In India, tobacco is used liberally for smoking or chewing purposes irrespective of age, sex and socio-economic status either for pleasure or for ritual purpose, or for self medication or out of habit or to satisfy addiction [Verma *et al*., 2010]. Smokeless tobacco use is very popular, among female users, in North-East India [Rani *et al*., 2003; Gupta and Ray, 2003].

In the North-Eastern corner of India, Mizoram is situated between 92°16’ to 93°26’ E longitude and 21°56’ to 24°31’ N latitude, virtually land locked bordering Myanmar in the east and Bangladesh in the west [Phukan *et al*., 2005]. It is inhabited by 1,091,014 populations (2011 census) and it covers an area of approximately 21,087 sq. km. The Mizo people have their ancestral origin in China [Thawnga, 1986]. The people of Mizoram are culturally and ethnically distinct from the other tribes and communities of India [Phukan *et al*., 2005].

Consumption of tobacco along with smoking habit is deeply embedded in the tradition and culture of the Mizos. Mizos grew tobacco to meet their requirements. Till the 1980s, there was no public awareness of the disastrous consequences of tobacco use. Both sexes, young and old indulged in it any time and place except the church and educational institutions. Tobacco was used in different form such as hand-rolled cigarette, smoking pipe-a hookah type apparatus (Figure 1.4), and a unique form of water extract of tobacco smoke, locally known as tuibur, is consumed orally.

Offering a hand-rolled cigarette, by a young girl, while being courted by a young man, was also an imperative courtesy and good gesture. Parents looking for bride also looked, among other things, if the girl was good at smoking/making tuibur to ensure sufficient supply of tuibur. A young woman's ability to smoke pipe (Figure 1.4) was a desired attribute of a good bride, mainly because the elderly people of the family who are extensively consuming the by-product (called *tuibur*) of this type of smoking. The situation was such that wherever people gathered, from celebration of marriages to mourning of the death, the room would be filled with haze of tobacco smoke. [ISTH, 2009]. This rich tradition was practiced as a great gesture of friendship.

Unlike other smokeless tobacco products, a peculiar habit of consuming tobacco (extracts of tobacco smoke) in the form of water called ''tuibur'' (tobacco smoke–infused water) has been observed in Mizoram and Districts of Manipur (known as Hidakphu in Manipur) bordering Mizoram [Sinha *et al*., 2004]. The use of tobacco in this unique form has been a cultural practice. Habitual consumption of tuibur is presumed to be one of the "safe" nicotine delivery medium. Most of the users take about 5 to 10 ml of tuibur few times a day by keeping it in the lower buccal space of mouth, for about 15 – 20 minutes, when the flavor considerably decreased, it was spat out and taken again as and when needed. Some people are addicted to the extent of consuming it constantly, and some people have the habit of swallowing tuibur occasionally [Phukan *et al*., 2005].

Figure 1.4: An elderly Mizo lady and a Mizo damsel exhibiting the traditional indigenous smoking pipe of Mizoram called tuibur.

The pipe is made of clay, and is fitted with a bamboo receptacle for water to get impregnated with the fumes of the smoke and the oil of the tobacco, then sipped from time to time through gourds or bamboo tubes and kept in the mouth of the smokers for few seconds before spitting it out. (Picture courtesy: Ralte Jimmy)

Use of smokeless tobacco (snuff, tobacco powder for oral use, gutka, etc.) or cigarette smoking is known to cause various forms of cancers as various chemical constituents of tobacco are found to be carcinogenic. A carcinogen may be defined as a chemical, physical or biological agent that causes cancer or induces the incidence of cancer. Even then, consumption of tobacco continues to be the leading global cause of preventable death. It is estimated that the use of tobacco in diverse forms led to 6 million deaths globally every year, [WHO, 2008; 2011], with a majority occurring in Asia, and this figure is expected to rise further. Within South Asia, smokeless tobacco use is very high among female users in North-East India [Rani *et al.*, 2003;

Gupta and Ray, 2003]. Although, the annual death figure due to tobacco consumption increases every year, cessation of tobacco consumption will not happen anytime in the near future. And, if this tendency continues, tobacco related mortalities will reach 8 million worldwide annually by the year 2030 [WHO, 2008; 2011].

At various concentration levels of oxygen, due to a range of heating temperatures, over 8400 chemical species have been identified as generated in processed tobacco and tobacco smoke [Rodgman and Perfetti, 2008] and many of them are presumably potential participants in the deleterious free radical generating reactions [Ghosh and Ionita, 2007]. The type and number of chemical constituents vary among a range of diverse tobacco products [Hoffmann *et al*., 2001], and the identified chemical compounds of tobacco are classified among different groups according to their health hazard index by International Agency for Research on Cancer (IARC) [Pappas *et al*., 2006; IARC, 2007; Chiba and Masironi, 1992] including nicotine based alkaloids, arising from both primary as well as secondary metabolites. Chemical compounds generated during the burning of tobacco may be capable of reacting in specific *in vivo* reactions leading to the formation of either new toxic molecules or the functional modification of some biological molecules [Calafat *et al*., 2004; Pryor *et al*., 1983a].

Among the identified chemical species, transient or stable, present in tobacco smoke, more than 80 of them are proven carcinogens [Hoffmann *et al*., 2001] as per the assessments done by the International Agency for Research on Cancer [IARC, 2007]. Each of these chemical carcinogens exhibited more than one type of the mode of activity (mechanisms) in exacerbating the risk of developing cancer [Hecht, 2002]. The majority of known carcinogens of tobacco are organic compounds, while several trace metals present in tobacco also pose health as well as

environmental hazard [Judd and Swami, 2010; Verma *et al*., 2010]. Some of these metals can act as tumor promoters in synergy with other carcinogens [Barlas *et al*., 2001]. The best way to minimize exposure to these harmful compounds is essentially discontinuation of the consumption of tobacco products. However, many users struggle to quit because of the intensity of nicotine addiction.

Cancer is one of the leading causes of death worldwide. The latest report of Indian Council of Medical Research (ICMR) showed that Aizawl district in Mizoram has the highest incidence rates of different types of cancer in the country [NCRP, 2013]. The report shows that the age adjusted rate (AAR) per 100 000 population in males ranged from 43.7 in Barshi (Expanded) to 273.4 in Aizawl District of Mizoram state followed by East Khasi Hills District (216.0) of Meghalaya. In females, it ranged from 51.6 in Ahmedabad (Rural) to 227.8 in Aizawl District of Mizoram state. Cancer is a major disease condition among this tribal populace. Mizoram records the number one position for incidence of stomach (64.2), liver (11.6) and lung (45.7) cancers among male per 10^5 population in Aizawl District of Mizoram and nasopharynx (5.2), stomach (31.2), lung (44.6) and cervix uteri (24.3) among female per 10^5 population in Aizawl District of Mizoram in India [NCRP, 2013]. In fact, the incidence is comparable to those areas in the world where these cancers were traditionally considered to be high, such as Japan, Korea, Chile and Costa Rica in stomach cancer [Mallath *et al*., 2014].

The epidemiologic investigations have identified numerous risk factors as the putative initiator(s) of stomach cancer, albeit the etiology still remains unclear. However, it has been possible to identify certain risk factors that contribute significantly to the development of cancer and death due to cancer. Most important among them are lifestyle habits, *viz*., tobacco and diet.

These two factors are especially important in context of the types of cancer prevalent in Mizoram [Mallath *et al*., 2014]. The emerging evidence strongly suggests that the pathogenesis is complex and multifactorial, ranging from environment and diet to genetic and molecular alterations; it is clear that several genetic pathways may exist in the development and progression of various cancers. [Mallath *et al*., 2014; Tipirisetti *et al*., 2014].

The state of Mizoram being a high risk/incidence region of stomach cancer in India [NCRP, 2013; Rao and Ganesh, 1998; Phukan *et al*., 2004], few epidemiological studies have been carried out earlier to find out the risk factors for stomach cancer. A matched case-control study has been carried out at the Aizawl Civil Hospital, Aizawl to investigate influence of the use of various forms of tobacco on the incidence of stomach cancer. The consumption of tuibur is prevalent among smokers as well as non-smokers and it may be one of the important reasons for the high prevalence of stomach cancer among the populace in Mizoram [Phukan *et al*., 2005] which commensurates with the study described in this dissertation. Therefore, this peculiar form of tobacco solution usage assumes importance from a public health point of view.

Putative toxic effects of tuibur were also studied using a modified version of Allium test. Microscopic features revealed the reduction of mitotic index, formation of micronuclei, lagging chromosome and c-mitosis (full form) in the root tip cells treated with a range of concentrations of tuibur. EC_{50} value (the minimum concentration of a material required to kill 50% target cells) of tuibur for root growth was also estimated to be 2.48% [Mahanta *et al*., 1998]. Moreover, a recent study showed that tuibur consumers have three times higher risk of stomach cancer than tuibur non-consumers. Persons who smoke tobacco and/or consume tuibur had increased the risk of stomach cancer if they carry GSTM1 null genotype and GSTT1 non-null genotype.

Tuibur consumers carrying GSTM1 null genotypes had 2.4 times higher risk of stomach cancer than non-consumers of tuibur having GSTM1 non-null genotype [Malakar *et al*., 2012].

Most human cells contain hundreds of mitochondria and thousands of mitochondrial DNA copies [Wallace, 1994]. The human mitochondrial DNA (mtDNA) is a 16, 569 bp circular double-stranded DNA molecule containing 37 genes, all of which are essential for normal mitochondrial function. Among these genes, Thirteen of them provide instructions for making enzymes involved in oxidative phosphorylation, 2 ribosomal RNAs (rRNAs), and 22 transfer RNAs (tRNAs) [Anderson *et al*., 1981; Ketmaier and Bernardini, 2005].

Oxidative phosphorylation is an essential metabolic process during which the abstraction of protons and reducing equivalents (electrons) from simple sugars is coupled with the reduction of molecular oxygen to water and the generation of proton gradient across the inner membrane and the matrix leading to the concomitant production of adenosine triphosphate (ATP), the cell's main energy source. About 2-3 % molecular oxygen that must be completely reduced to water during the metabolic oxidative phosphorylation process, instead undergoes a partial reduction and is converted to superoxide radical, a deleterious form of reactive oxygen species (ROS). The retarded reduction of molecular oxygen due to the leakage of electrons from the respiratory complexes I & III involved in the oxidative phosphorylation serves as the primary source of the production of superoxide species in most tissues.

Such an imbalance production of excess ROS over the existing antioxidant defense capacity of the cells, defined as oxidative stress, is implicated in a wide variety of malignant diseases. One of the primary targets of ROS is mtDNA, which encodes several enzymes and

proteins essential for the production of ATP during oxidative phosphorylation process. Thus, mtDNA is very much susceptible to inductive oxidant attack by ROS, owing to its close proximity to the oxidative phosphorylation enzyme complexes and the absence of protective histone proteins which are present in nuclear chromatins [Ritcher, 1998]. The level of oxidatively impaired nucleotide bases, such as 8-hydroxydeoxyguanosine, in mtDNA is 10-20 folds higher than in nuclear chromosomes [Wallace *et al.*, 1997]. Oxidative damage inflicted by ROS is one of the important sources of mitochondrial genomic instability resulting in mitochondrial respiratory complex dysfunction.

A mutation is defined as any permanent change of the nucleotide sequence or DNA sequence of the genome of an organism, away from normal. This implies that there is a normal allele that is prevalent in the population and that the mutation changes this to a rare and abnormal variant. Mutations result from unrepaired damage to DNA or to RNA genomes (typically caused by radiation or chemical mutagens), errors in the process of replication, or from the insertion or deletion of segments of DNA by mobile genetic elements [Bertram, 2000; Burrus and Waldor, 2004]. Mutations may or may not produce discerning changes in the observable characteristics (phenotype) of an organism. Mutations play a part in both normal and abnormal biological processes including evolution, cancer, and the development of the immune system.

Mutation can cause several different types of change in sequences. Mutations in genes may have either no effect or alter the product of a gene or alter the genetic message carried by gene or prevent the gene from functioning properly or completely. Agents which bring about a permanent alteration or damaged to the physical composition of DNA by inducing mutations in DNA are called mutagens. They could be exogenous factors or environmental factors like

sunlight, radiation, infection and smoking, etc. and also, endogenous factors like errors which occur during DNA replication leading to genetic changes and the production of certain toxic by-products of cellular metabolism, etc.

Mitochondrial DNA is prone to non-inherited (somatic) mutations. Somatic mutations occur in the DNA of certain cells during a person's lifetime and typically are not passed to future generations. Somatic mutations in mitochondrial DNA have been reported in some forms of cancer, including breast, colon, stomach, liver, and kidney tumors. Mitochondrial DNA mutations like insertions and deletions have been observed in many types of human cancer [Chatterjee *et al*., 2006]. Mitochondrial DNA (mtDNA) has been correlated with the various types of cancer due to its vulnerability towards reactive oxygen species and limited repair mechanisms compared with nuclear DNA leading to mitochondrial genomic aberration. An increase in ROS has been observed in cancer cells, which may be due to leakage of reducing equivalents from oxidative phosphorylation complexes during mitochondrial respiratory burst. These reducing equivalents combine with molecular oxygen generating transient superoxide radicals which are then converted to ROS. However, the molecular mechanisms of oxidative stress in cancer progression and metastasis processes are still not well understood.

Mitochondrion plays a vital role in energy metabolism, aging and apoptosis [Chan, 2006]. There are several biological attributes which cast mitochondria, and the mitochondrial genome, in particular, as a biological tool for early detection and monitoring of neoplasia and its potential progression. Mitochondria are also a major source for ROS generation. Besides mitochondrial DNA (mtDNA) alterations have been observed in a variety of human diseases including cancer [Govatati *et al*., 2013; Lu *et al*., 2009; Wallace, 2005]. mtDNA is more

vulnerable to oxidative damage, unlike nuclear DNA, it lacks protective histone proteins, limited DNA repair mechanisms as well as a high rate of ROS generation and hence exhibits higher mutation rate than nuclear DNA (nDNA) [Croteau and Bohr, 1997].

Mitochondrial DNA alterations have been implicated in the development and progression of cancer. Several mutations have been identified in a wide variety of human tumors, including breast, colorectal, ovarian, gastric, hepatic and esophageal cancers, as well as hematological malignancies [Gattermann, 2000; Tan *et al*., 2002]. D-loop region of the mtDNA is the most potent accumulation site for many of these mutations and numerous polymorphisms have also been reported in this region as it lacks protective histones, exhibits high oxidative stress with deficient DNA repair mechanisms [Penta *et al*., 2001]. D-loop is the only non-coding mtDNA region which possesses crucial elements for replication and transcription. Thus, the sequence alterations of this region may contribute to the altered replication or transcription properties [Liu *et al*., 2001; Penta *et al*., 2001].

The vast majority of the mitochondrial genome is under the scrutiny of selection because mutations in these areas are usually deleterious There is a region in which there are no coding sequences called non-coding region of mtDNA, also known as the control region or D-loop region [Pereira *et al*., 2004]. The displacement loop (D-loop) {nucleotide position (np) 16024-576 = 1124 base pair (bp)} of mitochondrial genome is known to accumulate mutations at a higher frequency than other regions [Michikawa *et al*., 1999]. It is a hot spot for mtDNA alterations and comprises of two hypervariable regions (HVR) which are HVR1: nucleotide position 16024–16383 and HVR2: nucleotide position 57–333. The D-loop contains crucial elements for replication and transcription of mtDNA [Clayton, 2000]. Mutation rates in HVI and

HVII are especially high on average and there is evidence that the rates vary within the region as well [Relethford, 2001]. Hence, sequence alterations in D-loop region may contribute to impaired replication and/or transcription of mitochondrial genes which may impede the overall mitochondrial function while concomitantly exacerbates cellular ROS generation. Accumulation of D-loop alterations has been implicated in several complex human diseases [Govatati *et al*., 2013; Chen *et al*., 2012; Wang *et al* 2011; Mueller *et al* 2011].

Mutations occurring in the D-loop region, therefore, might interfere with transcription of the entire mtDNA genome and might cause severe alterations in mitochondrial function. Altered mitochondrial physiology has been described in tumor cells, while large deletions of the mtDNA genome have been reported in different solid tumor types. Oxidative damage to mtDNA followed by mtDNA mutations might be one of the critical steps in carcinogenesis [Tan *et al*., 2002]. Somatic mutations of the mitochondrial DNA (mtDNA) are common in many human cancers, which maybe a reflection of altered DNA repair mechanisms and also predisposition of mtDNA to free radical damage [Hochhauser., 2000; Singh., 2006]. mtDNA mutations thus, can serve as an important biomarker for human cancers [Jakupciak, 2005; Parr *et al*., 2006]. Although, mtDNA mutation has been frequently observed in human cancer, its use as a biomarker has not been explored much.

Mutations can occur anywhere in a mitochondrial genome which makes it different from nuclear genome [Chatterjee *et al*., 2006, Kagan and Srivastava, 2005]. The wide occurrence of mtDNA mutation in cancers may have significance in cancer detection. Point mutations or deletions, resulting in mitochondrial genetic diseases other than cancer, have been identified at more than 300 positions in mtDNA. Nearly 100 other sites unrelated to known mitochondrial

diseases have been found to be mutated in tumors [Fliss *et al*., 2000]. Nuclear and mitochondrial genome instability studies have been found to be important in malignancy diagnosis. Such instability was observed in 87.5% of the breast cancers studied. [Richard *et al*., 2000].

Polymerase chain reaction (PCR) is a revolutionary method based on the ability of DNA polymerase to synthesize new strand of DNA complementary to the offered template strand. Because DNA polymerase can add a nucleotide only onto a pre-existing 3'-OH group, it needs a primer to which it can attach the first nucleotide. Heat resistant DNA polymerase isolated from *Thermus aquaticus* is one of the most popular DNA polymerase used for PCR. This requirement makes it possible to delineate a specific region of template sequence that researcher needs to amplify. At the end of the PCR reaction, the specific sequence will be in billions of copies (amplicons). The specific DNA product generated by PCR using one pair of PCR primer is called an amplicon. Primer is a short oligonucleotide complementary to target DNA and acts as the leader for DNA extension. The amount of amplified product is determined by the available substrates in the reaction, which become a limiting factor as the reaction progresses [Carr and Moore, 2012].

Restriction fragment length polymorphism (RFLP) is a molecular biology technique that exploits variations in homologous DNA sequences. It identifies difference between samples of homologous DNA molecules that exhibit differing restriction enzyme sites. In RFLP analysis, the DNA sample is nicked into pieces (digested) by restriction enzymes and the resulting restriction fragments are separated according to their lengths by gel electrophoresis.

RFLP analysis is used to identify a change in the genetic sequence that occurs at the site where restriction enzyme cuts. RFLP can be used to trace inheritance patterns, identify specific mutations and locate specific genes for genetic disorders. Moreover, RFLP analysis is an important tool in genome mapping, DNA fingerprinting, determining genetic linkage and it is also useful in the determination of risk for diseases and in the characterization of genetic diversity.

Gel electrophoresis is a method of separation and the subsequent analysis of macromolecules (DNA, RNA and proteins) and their fragments, based on their size and charge. The types of gel most typically employed are agarose and polyacrylamide gels. Each type of gel is well-suited to different types and sizes of analytes. Polyacrylamide gels are usually used for proteins, and have very high resolving power for small fragments of DNA (5-500 bp). On the other hand, agarose gels have lower resolving power for DNA but have greater range of separation, and are therefore used for DNA fragments of approximately 50-20,000 bp in size. Polyacrylamide gels are run in a vertical configuration while agarose gels are typically run horizontally in a submarine mode. They also differ in their casting methodology, as agarose sets thermally, while polyacrylamide forms in a chemical polymerization reaction.

Agarose gel electrophoresis is a simple, reliable and robust way of separating and analyzing DNA. The purpose of the gel may be to view the DNA, to quantify it or to isolate a particular band. The DNA is visualized in the gel by addition of ethidium bromide, which is a mutagen, while less-toxic proprietary dyes such as GelRed, GelGreen, and SYBR green are safe. Ethidium bromide and the other proprietary dyes bind to DNA and are consequently fluorescent, meaning that they absorb invisible UV (ultraviolet) light and transmit the energy as visible light

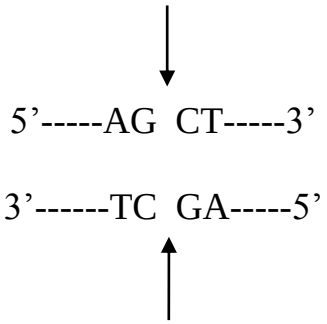
[Lewis, 2011]. Agarose gels are generated using the natural polymers extracted from the sea weeds. Agarose gels can be easily casted and handled compared to other matrices because the gel setting is a physical rather than a chemical change. Samples are also easily recovered. After the experiment is finished, the resulting gel can be stored in a plastic bag in a refrigerator.

Agarose gels do not have a uniform pore size, but are optimal for electrophoresis of proteins that are larger than 200 kDa [Smisek and Hoagland, 1989]. Agarose gel electrophoresis can also be employed for the separation of DNA fragments ranging from 50 base pairs to several megabases (millions of bases), the largest of which require specialized apparatus. The distance between DNA bands of different lengths is influenced by the percentage of agarose in the gel, with higher percentages requiring longer run times, sometimes days. Most agarose gels are made using a range of 0.7% (good separation or resolution of large 5–10 kb DNA fragments) to 2% (good resolution for small 0.2–1 kb fragments) agarose dissolved in electrophoresis buffer. Up to 3% can be used for separating very tiny fragments but a vertical polyacrylamide gel is more appropriate in this case. Low percentage gels are normally very weak and may break during the handling of gels after the experiments, as appropriate care must be exercised. High percentage gels are often brittle and do not set evenly. 1% gels are commonly employed for many applications [Lewis, 2011]. Polyacrylamide gel electrophoresis (PAGE) is used for separating proteins ranging in size from 5 to 2,000 kDa due to the uniform pore size provided by the polyacrylamide gel. Pore size is controlled by modulating the concentrations of acrylamide and bis-acrylamide powder used in preparing the gel. Care must be taken while preparing this type of gel, as acrylamide is a potent neurotoxin in its liquid and powdered forms.

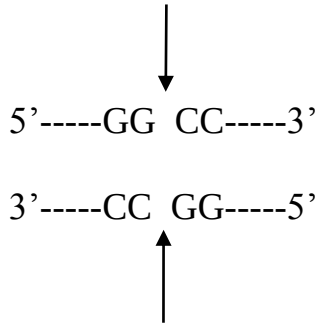
Traditional DNA sequencing techniques such as Maxam-Gilbert or Sanger methods generally employ polyacrylamide gels to separate DNA fragments differing by a single base-pair in length, so the resulting sequence could be read. Most modern DNA separation methods now use agarose gels, except for small DNA fragments. It is currently most often applied in the field of immunology and protein analysis, essentially to separate different proteins or iso-forms of the same protein into separate bands. The isolated proteins can be transferred onto a nitro-cellulose or PVDF membrane to be probed with antibodies and corresponding markers, such as in a western blot.

Restriction enzymes are enzymes that cut DNA at specific recognition nucleotide sequences [Kessler and Manta, 1990; Pingoud *et al*., 1993] depending on the particular enzyme used. Because they cut within a specific region in the molecule, they are often called restriction endonucleases. The specific site at which a restriction enzyme will cleave DNA is known as a restriction site. Enzyme recognition sites or restriction recognition sites are usually 4 to 8 bp in length which is recognized by restriction enzymes. Generally, the shorter the recognition sequence, the greater the number of fragments generated.

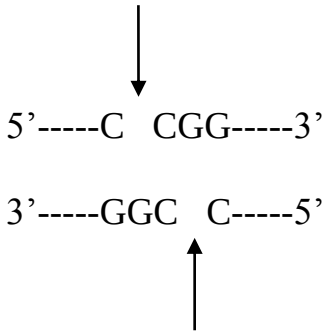
AluI (*Arthrobacter luteus*) is a restriction enzyme whose recognition site is AGCT nucleotide sequence and cleavage occurs between G and C [Nishigaki *et al*., 1985]. The enzyme cuts both strands of DNA at the same location and produces a blunt end.



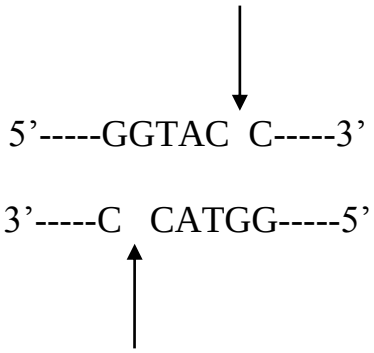
HaeIII (*Haemophilus aegyptius*) restriction enzyme recognition site is GGCC nucleotide sequence and cleavage occurs between G and C [Nishigaki *et al*., 1985]. The cut is made between the adjacent G and C across the double helix producing a blunt end.



MspI (*Moraxella sp*.) restriction enzyme recognition site is CCGG nucleotide sequence and cleavage occurs between C and C [Nishigaki *et al*., 1985]. The cut is made between the adjacent G and C across the double helix; the ends of the cut have an overhanging piece of single-stranded DNA. These are called sticky ends because they are able to form base pairs with any DNA molecule that contains the complementary sticky end. Any other source of DNA treated with the same enzyme will produce such molecules.



KpnI (*Klebsiella pneumonia*) restriction enzyme, recognition site is GGTACC nucleotide sequence [Tomassini *et al*., 1978]. The cut is made between C and C across the double helix and produces sticky ends.



Over 3000 restriction enzymes have been studied in detail, and more than 600 of these are available commercially [Roberts *et al*., 2007]. These enzymes are routinely used for DNA modification in laboratories, a vital tool in molecular cloning [Micklos *et al*., 1996; Massy and Kreuzer, 2001]. These enzymes are found in bacteria and archeae and providing defense mechanism against invading viruses [Arber and Linn, 1969; Kruger and Bickle, 1983]. Inside a prokaryote, the restriction enzymes selectively cut up foreign DNA in a process called restriction; while host DNA is protected by a modification enzyme (a methylase) that modifies

the prokaryotic DNA and blocks cleavage. Together, these two processes form the restriction modification systems [Kobayashi, 2001].

In contrast to mutation, polymorphism in biology is a discontinuous genetic variation resulting in the occurrence of two or more different phenotypes that exist in the same population of a species – in other words, the occurrence of more than one form or morph. A discontinuous genetic variation divides the individuals of a population into two or more sharply distinct forms. The term is also used somewhat differently by molecular biologists to describe certain point mutations in the genotype, such as single nucleotide polymorphism (SNP).

Polymorphism is a common feature in nature and is related to biodiversity, genetic variation and adaptation. It usually functions to retain variety of form in a population living in a varied environment. The most common example is sexual dimorphism *i.e.*, the separation of higher organisms into male and female sexes, which occurs in many organisms. Other examples are mimetic forms of butterflies and human hemoglobin and different blood types in human, the smooth graduation of height among individuals of human populations and the graduations possible between the different geographic races. If the frequency of two or more discontinuous forms within a species is too high to be elucidated by mutation, the variation – as well as the population displaying it –is said to be polymorphic.

According to the theory of evolution, polymorphism results from evolutionary processes. It is heritable and is modified by natural selection. In genetic polymorphism, the genetic make-up determines the morph. Some polymorphisms exhibit no visible manifestations and require biochemical techniques to identify the differences that occur between the chromosomes,

proteins, or DNA of different forms. A single nucleotide polymorphism, frequently called SNP (pronounced snip; plural snips), is a DNA sequence variation occurring commonly among people. Each SNP represents a difference in a single DNA building block, called a nucleotide. A single nucleotide – adenine (A), thymine (T), cytosine (C), or guanine (G) – in the genome differs between members of a biological species or paired chromosomes.

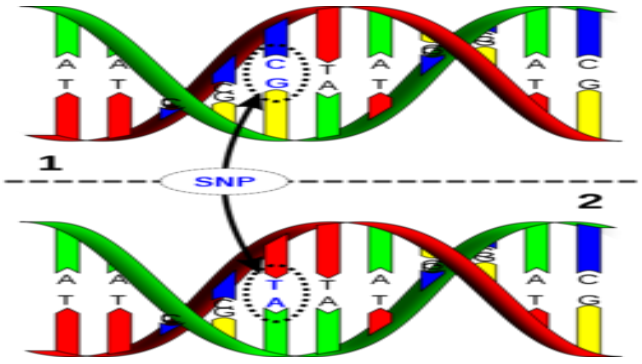


Figure 1.5: Single Nucleotide Polymorphism.

For example, two sequenced DNA fragments from different individuals, AAGCCTA to AAGCTTA, contain a difference in a single nucleotide in a certain stretch of DNA, *i.e.*, there are two alleles. Almost all common SNPs have only two alleles. The genomic distribution of SNPs is not homogenous; SNPs occur in non-coding regions more frequently than in coding regions or, in general, where natural selection is acting and fixing the allele of the SNP that constitutes the most favorable genetic adaptation [Barreiro *et al.*, 2008]. Analysis of single nucleotide polymorphisms (SNPs) has been increasingly employed in molecular biology, particularly in studying genetic determinants of complex diseases. Such studies are aided by rapid, simple, low cost and high throughput methodologies for SNP genotyping [Ye *et al.*, 2001].

Single nucleotides may be changed (substitution), removed (deletions) or added (insertion) to a polynucleotide sequence [Vignal *et al*., 2002; Yue and Moult, 2006]. Single nucleotide polymorphisms may fall within coding sequences of genes, non-coding regions of genes, or in the inter-genic regions between genes. SNPs within a coding sequence may not necessarily change the amino acid sequence of the protein that is produced, due to degeneracy of the genetic code. SNP in which both forms lead to the same polypeptide sequence is termed synonymous (sometimes called a silent mutation). However, if a different polypeptide sequence is generated, they are non-synonymous. A non-synonymous alteration may either be missense or nonsense. While a missense mutation may result in a different amino acid, a nonsense mutation may result in a premature stop codon. SNPs that are not in protein-coding regions may still have consequences for gene splicing, transcription factor binding, or the sequence of non-coding ribonucleic acid (RNA) [Komar, 2009; Cargill *et al*., 1999; Stephens *et al*., 2001].

SNPs is a normal occurence throughout a person's DNA. They occur once in every 300 nucleotides on average, which means there are roughly 10 million SNPs in the human genome. Most commonly, these variations are found in the DNA between genes. They can act as biological markers, helping scientists identify genes that are associated with a disease. SNPs occur within a gene or in a regulatory region near a gene as they may play a more direct role in the disease by affecting the gene's function.

Variations in the DNA sequences of humans can affect how humans develop diseases and respond to pathogens, chemicals, medication, vaccines, and other agents. While, most SNPs have benign effect on health or development, albeit some of these genetic differences have proven to

be very important in the study of human health. A growing body of literature evidence has suggested that SNPs may help predict an individual's response to certain drugs, susceptibility to environmental factors such as toxins, and risk of developing particular diseases. SNPs can also be used to track the inheritance of disease genes within families. Future studies will aid in the identification of SNPs associated with complex diseases such as heart disease, diabetes, and cancer.

Analysis of restriction fragments or mtDNA sequence variation can be used to construct a phylogenetic network that displays the evolutionary relationships between individual sequences. Such networks were found to be helpful in deciding between a rare polymorphism and a pathogenic mutation in patients. Phylogenetic networks are required for the purposes of both medical genetics and population genetics. A phylogenetic network is any graph/network used to visualize evolutionary relationships (either abstractly or explicitly) [Huson and Scornavacca, 2011] between nucleotide sequences, genes, chromosomes, genomes, or species [Huson *et al*., 2010]. They are employed when reticulate events such as hybridization, horizontal gene transfer, recombination, or gene duplication and loss are believed to be involved. They differ from phylogenetic trees by the explicit modeling, by means of the addition of hybrid nodes (nodes with two parents) instead of only tree nodes (nodes with only one parent) [Arenas *et al*., 2008]. Phylogenetic trees are a subset of phylogenetic networks. A phylogenetic tree is commonly defined as a leaf labeled tree that represents the evolutionary history of a set of taxa, possibly with branch lengths, either unrooted or rooted.

For the chemical constituents of tuibur, their chemical form in the aqueous solution and their mode of interaction with tissues, their potency, either individually or synergistically, in

stimulating oncogenic transformations need to be studied in detail. Furthermore, to understand their mode of interaction with tissues, their potency, either independently or synergistically, in stimulating oncogenic transformations along with the putative cellular and molecular mechanisms of induced pathophysiological processes due to the consumption of tuibur, it is pertinent to carry out the characterization of the altered end products of the cell. Hence, to examine the putative genotoxic/mutagenic effects of tuibur, in the present study, the DNA typing population analysis using a polymerase chain reaction-restriction fragments length polymorphism (PCR-RFLP) platform was performed to gain an understanding of the carcinogenic and oxidative effects of tuibur. Furthermore, the present study was undertaken to elicit the correlation between the polymorphism of D-Loop gene of mitochondrial DNA and stomach cancer risk considering various dietary habits, tobacco and alcohol habits for the first time in the high risk state of Mizoram in the North-Eastern part of India.

The composition of tobacco is multifarious as the type and number of chemical constituents varies in its different formulation [Hoffmann *et al*., 2001]. Tobacco smoke has exhibited toxic [Chiba and Masironi, 1992; Stohs *et al*., 1997], genotoxic [Husgavfel Pursiainen, 2004], mutagenic [Demarini, 2004], and carcinogenic properties [Eyre *et al*., 2004], and has been linked to adverse pregnancy outcomes [Kallen, 1999; Little *et al*., 2004]. As far back as 1930s, smoke and smokeless tobacco were identified as source of dozens of free radicals and carcinogens, including volatile organic compounds like polyphenols which are potent antioxidants [Kamisaki *et al*., 1997], *viz.*, formaldehyde, acetaldehyde, acrolein, propionaldehyde, butyraldehyde, crotonaldehyde, acetone and methyl ethyl ketone, 1,3-butadiene, isoprene, benzene, toluene, styrene, poly-aromatic hydrocarbons (PAHs), benzo-[α]-

pyrene, tobacco-specific nitrosamines (TSNAs), nitrosamino acids, hydrazine, hydrogen cyanide, acrylonitrile, phenol, o-cresol, m-cresol, p-cresol, catechol, resorcinol, hydroquinone, semi-volatile bases like pyridine, quinoline, along with inorganic compounds of cadmium, chromium, nickel, lead, cobalt, berylium, radioactive polonium-210, ammonia, nitrogen oxides, and arsenic [Baker *et al*., 2004] and also, iron, aluminium, manganese, selenium, zinc and mercury [Musharraf *et al*., 2012].

Tobacco smoke is a complex carbon-based, dynamic aerosol suspended in an equally complex organic vapor mixture. Smoke is essentially the manifestation of a combination of combustion (upto 950^{o}C), pyrolysis and distillation (at less than 600^{o}C) of the plant material [Baker, 1999]. It is a well known risk factor for several diseases, ranging from inflammatory to neoplastic diseases [Ambrose and Barua, 2004; Peto *et al*., 2000]. The deleterious effects of tobacco smoke can be caused by chemical interactions between most of the smoke compounds formed in both gas and aqueous phases, and some biological reactive molecules.

Cigarette smoke contains gas-phase smoke and particulate phase (or tar) [Pryor *et al*., 1983b]. Both these phases are very rich sources of deleterious free radicals. In addition, these two phases are highly oxidizing, as well as capable of inducing an oxidative stress in the internal organs. For example, smokers have lower concentration of vitamin C in their blood plasma and vitamin E in their lung alveoli than non-smokers. The gas phase of cigarette smoke contains more than 10^{14} low molecular weight oxygen- and carbon-centered radicals [Pryor, 1997] that are much more reactive than tar-phase radicals. These gas-phase radicals do not arise in a flame, but rather are produced in a steady state by the oxidation of NO to NO_2 [Pryor *et al.*, 1983a], which then reacts with π-electron-rich reactive species in the smoke such as isoprene [Church

and Pryor, 1985]. These species are involved in the generation of oxygen-centred radicals via the addition of nitrogen dioxide to unsaturated compounds (*i.e.,* reactive double bonds). This may lead to the generation of carbon-centred radicals, which subsequently reacts with atmospheric oxygen to form oxygen-centred radicals [Ghosh and Ionita, 2007].

Considerable effort has been expended for many years to determine the composition of particulate phase of tobacco smoke. Numerous fractionation schemes have evolved. The majority of these involve a partition of the smoke condensate into a water-soluble polar portion and a non-polar portion. The non-aqueous solution (non-polar), in general, was readily fractionated into its components. By employing modern chromatographic techniques, the water-soluble portion of the smoke was studied extensively [Schumacher *et al*., 1977].

Secondhand smoke (SHS) also known as environmental tobacco smoke (ETS) results from the mixing of two forms of smoke coming from burning tobacco; that emanates from the burning end of a cigarette, cigar, or pipe, known as side-stream smoke (SS) and which is inhaled and then exhaled by the smoker also known as mainstream smoker (MS). As side-stream and exhaled mainstream smoke enter the environment, they are mixed and diluted to form ETS. ETS is a major contributor to indoor air pollution wherever smoking occurs [Baker and Proctor, 1990; Lofroth *et al*., 1989]. Studies have shown that ETS contains a variety of genotoxic and carcinogenic compounds [Claxton *et al*., 1989; Lofroth, 1989] that are chemically similar to those in mainstream and side-stream smoke [U.S. EPA, 1993]. Epidemiological studies indicate an association between environmental tobacco smoke exposure and an increased risk for cancer [Kuller *et al*., 1986; Wells, 1988]. ETS is the only agent classified by EPA as a known human

carcinogen for which an increased cancer risk has actually been observed at typical environmental levels of exposure [U.S. EPA, 1993].

Side-stream smoke constitutes at least 85% of ETS [Baker, 1999], contributing nearly all of the vapor-phase constituents and over half of the particulate matter [U.S. EPA, 1993]. The particles in side-stream smoke are about 10 times smaller (0.01-0.1 μm) than those in mainstream smoke (0.1-1.0 μm) [Baker, 1999; Kuller *et al*., 1986], but the amount of total particulate matter in side-stream smoke is 1.3-1.9 times greater than in mainstream smoke [Guerin *et al*., 1987]. Thus, side-stream smoke particles could reach more distant alveolar spaces of the lung to a larger extent than the mainstream smoke particles [Kuller *et al*., 1986]. The amount of organic compounds in side-stream smoke is greater than in mainstream smoke [Lofroth, 1989].

Tar from side-stream smoke, like that of mainstream smoke, contains a persistent radical that can be studied directly by electron paramagnetic resonance (EPR) [Pryor *et al*., 1983a; Pryor *et al*., 1983b]. These radicals can be extracted into aqueous solutions [Stone *et al*., 1994]. These aqueous cigarette tar extracts nick plasmid DNA [Borish *et al*., 1985] and cause DNA nicking in viable mammalian cells, and this nicking follows saturation kinetics [Stone *et al*., 1994]. Studies show that the tar components in side-stream smoke produces DNA nicks in viable mammalian cells. It has also been reported that the effects of reduced glutathione (GSH), catalase, superoxide dismutase (SOD), diethylenetriamine pentaacetic acid (DTPA), and deferoxamine on the yield of DNA nicks caused by ETS tar solutions [Bermudez *et al*., 1994].

Free radicals, chemical species possessing unstable electronic configuration with one or more unpaired electrons in its highest occupied molecular orbital (HOMO) can be involved in chemical carcinogenesis. Carcinogenesis, a deleterious process in biology, is generally agreed upon to involve at least three stages: initiation, promotion, and progression. It is suggested that free radicals, sometimes, are involved in the initiation step, either in the oxidative activation of a procarcinogen (such as benzo-(α)-pyrene) to its carcinogenic form or in the binding of the carcinogenic species to DNA, or both. Yet, promotion always involves radicals, at least to certain extent, while, progression probably does not normally involve radicals [Church and Pryor, 1985].

Despite antioxidant defense mechanisms, cell damage from oxygen based free radicals known as reactive oxygen species (ROS) is ubiquitous. Mutagenesis through oxidative DNA damage is widely hypothesized to be a frequent event in the normal human cell. However, the difficulties of antioxidant intervention may be explained by the complexity of both free radical chemistry and cancer development. Thus, reducing the avoidable endogenous and exogenous causes of oxidative stress is, at present, one of the safest options [Dreher and Junod, 1996; Hecht, 2002].

Direct genotoxicity induced by cigarette smoke leads to the initiation of carcinogenesis. Furthermore, the epigenetic (non-genotoxic) effects of cigarette smoke is that it might act as modulators, thus altering normal cellular functions including cell proliferation and programmed cell death. Carcinogenetic process is usually accompanied by change in the structure and function of DNA leading to various oncogene activation and tumor suppressor gene inactivation. Genotoxic agents in cigarette smoke induce DNA damage through several mechanisms including point mutation, deletions, insertions, recombinations, rearrangements and chromosomal

aberrations [Valko *et al*., 2004]. Thus, carcinogens present in cigarette smoke act directly on DNA and may eventually lead to cancer promotion and progression, while procarcinogens require activation through oxidative enzymes to become carcinogenic [Hecht, 1998]. Most of such activation lead to the generation of electrophilic species that can attack DNA to form DNA adducts. Such adduct induction occurs at the adenine (A) or guanine (G) sites.

The molecular basis of mechanisms involved in cigarette-induced tumors was studied using aqueous cigarette tar extracts (ACT) and also by a mimic of these solutions, an aged solutions of catechol [Pryor, 1997]. Aqueous extracts of cigarette tar undergoes auto-oxidization to produce semiquinone, hydroxyl, and superoxide radicals in air-saturated buffered aqueous solutions [Zang *et al*., 1995]. Thus, ACT solutions as well as aged catechol solutions contain a quinone-hydroquinone-semiquinone system that can induce oxygen to generate superoxide radical by supplying an electron to molecular oxygen. Both the cigarette tar radical and the catechol-derived radical can penetrate viable cells, bind to DNA and cause nicks in DNA [Pryor, 1997].

Free radicals are unstable besides highly reactive species, to achieve electronic stability, they can readily react with other molecules and hence they can cause considerable damage to molecules within the cells. There are many different free radicals in tobacco smoke. The intriguing possibility is that it is the free radical itself (*i.e.*, the unpaired electron in the molecule) that is involved in the postulated biological mechanisms rather than the type of individual radical [Baker and Proctor, 1990]. Free radicals are found to be involved in both initiation and promotion of multistage carcinogenesis. These highly reactive compounds can act as initiators or promoters, cause DNA damage, and activate procarcinogens [Sun, 1990].

Cigarette smoke was known to contain gas-phase free radicals, by condensing it at very low temperature and detecting the radicals by EPR [Lyons *et al*., 1958]. The study of such deleterious radicals in tobacco smoke has since been important in biology and medicine owing to their potential implication for various disease conditions including cardio-vascular diseases, cancer, diabetes etc. It has been claimed that oxidative damage due to oxidative stress in cells might be induced by reactive free radicals [Baker, 1999; Flicker and Green, 2001; Lyons *et al*., 1958]. The damaging radical species in cigarette smoke comprised of inorganic (NO_2, $^{\bullet}OH$, $^{\bullet}OOH$) as well as organic carbon-centered ($^{\bullet}R$) or oxygen-centered ($^{\bullet}OR$, $^{\bullet}OOR$) species [Pryor, 1987; Flicker and Green, 2001]. However, it is important to note that free radicals are not only one of the groups of toxicants present in cigarette smoke. Besides, their relative toxicities are not fully understood [Baker, 1999].

It is presumed that the organic radicals formed initially in the cigarette combustion zone are carbon-centered and they react with O_2 to generate myriad oxygen-centered radical compounds. The short lifetime of most of these transient free radicals owing to the electronic configuration resulting in their high reactivity makes their detection difficult. Nevertheless, detection of oxygen-centered radicals present in smoke is a critical step towards understanding radical chemistry occurring in the system [Flicker and Green, 2001]. Many direct and indirect methods have been employed to detect, identify and quantify short-lived or stable free radicals, including those formed during the pyrolysis of tobacco.

The multitude of spectroscopic methods that are employed in the detection of these transient species include direct EPR (flow), indirect EPR of spin-trapped species, short-lived radical and stable radical coupling followed by HPLC, LC, fluorescence, GC, MS, or IR methods

[Lyons *et al*., 1958; Baum *et al*., 2003; Nishizawa *et al*., 2005; Kalai *et al*., 2002]. It is interesting to note that EPR spectroscopy provides the most useful information about the nature of free radicals and possibly the amount of radical species exist in the sample in a single experiment. This is because EPR technique is specific (it detects only unpaired electrons oblivious of the surroundings), highly sensitive (it can detect free radicals at about 10^{-9} M concentrations) and in many cases, it may provide essential structural attributes such as the nature of atoms closest to the unpaired electron bearing atom or ion (Ghosh *et al*., 2008).

The primary hazard of cigarette smoke is oxidative stress. Evidence has shown that ROS can damage DNA, initiate lipid peroxidation, and can cause inflammation. As the oxidative stress accumulates over time, it may trigger smoke-related diseases such as lung cancer, pulmonary emphysema, cardiovascular diseases, and stroke [Ou and Huang, 2006]. In 1958, Lyons and co-workers reported electron spin resonance (ESR) signals from cigarette smoke condensate, and shortly afterwards, free radicals were detected in whole cigarette smoke. Later, Pryor and co-workers further reported detection and estimation of free radicals concentrations by ESR spin-trapping method.

The radicals in gas-phase smoke are too short-lived, with an average life time of millisecond to nanoseconds, to be detectable by directly most of the molecular spectroscopic techniques, but they can be studied by spin trapping method using electron spin resonance (ESR) spectroscopy. Aqueous extract of cigarette tar (ACT) contains very low molecular weight quinone-hydroquinone-semiquinone system (Q-QH_2-$QH^{\cdot}$). These semiquinone radicals can reduce molecular oxygen to superoxide, a deleterious free radical species, eventually leading to the formation of hydrogen peroxide and hyperactive hydroxyl radicals. Unlike the tar radical

itself, the superoxide and hydroxyl radicals are highly reactive and short-lived to be observed by direct ESR measurements, but they can be detected as distinct spin adduct of organic reagents known as spin trap such as 5, 5-dimethyl-1-pyrroline-N-oxide (DMPO) [Pryor, 1997].

Electron paramagnetic resonance spectroscopy (EPR), also called electron spin resonance spectroscopy (ESR), is the best analytical technique that directly as well as unambiguously measures various free radicals in a solution. EPR spectroscopy plays an important role in understanding of organic and inorganic radicals, transition metal complexes, and some biomolecules (paramagnetic species) when placed in a strong magnetic field. EPR also provides information on a radical's geometry and the orbital of the unpaired electron. Although, it requires steady-state concentrations of free radicals in the 10^{-7} – 10^{-8} M range, EPR has been used to detect free radicals in human tissue samples obtained *in vitro*. EPR spectrometry can usually be applied for the analysis of samples *in vivo* only through the technique of spin trapping as well as oximetry. This involves the addition to samples of a diamagnetic organic nitrone compound known as spin-trap, which reacts readily with the transient free radicals to form radical based adducts known as spin-adducts, that are relatively more stable and longer-lived than the original free radical species and can therefore build up to steady-state concentrations in the detectable range.

Spin-traps have been used in experimental animals to demonstrate the production of free radicals in vivo [Buettner and Mason, 2003], but as no effective spin-traps presently exist that can be administered to humans, the technique is currently limited to samples of blood mixed with the spin-trap as soon as possible after administering them. Despite the obvious shortcomings of this approach, valuable data have been obtained, for example, relating to free radical production

during angioplasty, myocardial infarction, ischemia reperfusion etc [Holley and Cheeseman, 1993]. The advantage of ESR spectrometry over other spectroscopic techniques is that it not only provides characteristic spectroscopic features of various reactive free radical species for the detection and identification purpose, but also quantifies the amount of free radical species generated as the change in the spectral intensity as a function of time.

It is also well known that tobacco is a rich source of hazardous organic compounds as well as toxic heavy metals that get accumulated in the tobacco leaves during plant growth [Schneider and Krivan, 1993, Golia *et al*., 2007] or additives that are incorporated during the manufacturing processes turning the raw product into a value-added product for consumption. Moreover, tobacco has the ability to hyperaccumulate metals which translocates throughout aerial portions of the plant and sequesters them in its leaves on a large scale [Golia *et al*., 2007]. While some of the trace metal ions are nontoxic and play an important role in a wide spectrum of functions of life [Stihi *et al*., 2011], some of the trace metal ions are proved to be highly detrimental to humans even at very low concentration [Swami *et al*., 2009; Smith and Sneddon, 1999; Csalari & Szantai, 2002] as they can act as tumor promoters in conjunction with other carcinogens [Barlas *et al*., 2001] and needed careful consideration to ascertain their biological impact. In fact, toxic metals such as aluminium, magnesium, vanadium, chromium, manganese, iron, nickel, copper, zinc, cadmium, mercury, lead, arsenic and selenium are found in tobacco, cigarette paper, filters, and cigarette smoke [Baker *et al*., 2004; Musharraf *et al*., 2012; Chiba and Masironi, 1992]. On the other hand, consumption of tobacco causes disturbance of essential elements associated with various metabolic processes in our body which lead to serious life threatening consequences [Mudawi *et al*., 2013]. Quantity of these trace elements in tobacco also

depends upon genotype, water, type of soil, soil pH, applied fertilizers & pesticides besides the environment [Musharraf *et al*., 2012].

An Inductively Coupled Plasma (ICP) technique is a very powerful tool and an excellent analytical method for detecting and assessing trace and ultra-trace elements [Batsala *et al*., 2012; Mermet, 2005; Ramyalakshmi *et al*., 2012] which also allows monitoring multiple metals at once and offers the capability of low detection limits parts per billion (ppb) to parts per trillion (ppt). Many toxic metals are hazardous to health even at ppb concentrations. Most instruments applied for metal analysis do not possess the desired detection limits to assess elements such as lead, mercury and arsenic that are toxic even at lower concentrations. ICP spectrometers can be used for the analysis of environmental samples, contaminants in food or water, metalloproteins in biological samples, and similar studies. The technique typically analyzes materials in liquid form, in which a liquid sample is injected into argon gas plasma contained by a strong oscillating magnetic field.

Inductively coupled plasma atomic emission spectrometry (ICP-AES) or inductively coupled plasma optical emission spectrometry (ICP-OES) is a very sensitive technique for the identification and quantification of elements in a liquid sample. It is a type of emission spectroscopy that uses the inductively coupled plasma to produce excited atoms and ions as the elements in the sample become thermally excited and the electrons emit electromagnetic radiation at wavelengths characteristic of a particular element as they return to the ground state [Stefansson *et al*., 2007; Mermet, 2005]. The emitted light is then measured by optical spectrometry and the intensity of this emission is indicative of the concentration of element within the sample. Detection limits typically range from parts per million (ppm) to parts per

billion (ppb), although depending on the element and instrument, can sometimes achieve less than ppb detection [Boss and Fredeen, 1997].

In plasma mass spectroscopy (MS), the inductively coupled argon plasma (ICP) is used as an excitation source for the element of interest. However, in contrast to OES, the plasma in ICP-MS is used essentially to generate ions that are subsequently introduced into the mass analyzer. These ions are further separated and collected according to their mass to charge (m/z) ratios. The constituent elements of an unknown sample can then be identified and measured. ICP-MS offers extremely high sensitivity [Mermet, 2005] for a wide range of elements and also exhibits detection limits at the level of parts per trillion (ppt) [Batsala *et al*., 2012; Ramyalakshmi *et al*., 2012] that cannot be matched by other atomic spectroscopic methods making it far better suited for the total metal analysis in samples such as tobacco products. The ICP-MS allows the simultaneous determination of elements with atomic mass ranges 7 to 150. This encompasses F to U. Some masses are prohibited such as 40 due to the abundance of argon in the sample. Other blocked regions may include mass 80 (due to the argon dimer), and mass 56 (due to ArO), the latter of which greatly hinders Fe analysis unless the instrumentation is fitted with a reaction chamber [Batsala *et al*., 2012].

Even though, it can broadly determine the same suite of elements as other atomic spectroscopic techniques such as flame atomic absorption (FAA), graphite furnace atomic absorption (GFAA), and inductively coupled plasma – optical emission spectrometry (ICP-OES), ICP-MS has clear advantages in its multielement characteristics, speed of analysis, detection limits, and isotopic capabilities [Montaser and Golightly, 1992]. ICP-MS has the ability to scan for all the elements simultaneously which allows rapid sample processing [Ramyalakshmi *et al*.,

2012; Montaser and Golightly, 1992]. Over the past years, ICP-MS has become the technique of choice in many analytical laboratories for providing the accurate and precise measurements needed for today's demanding applications and for providing required limits of detection.

The concept of mass spectrometry is relatively simple: a compound is ionized (ionization method), the ions are separated on the basis of their mass-to-charge ratio (m/z ratio) *i.e.*, ion separation method and the number of ions representing each m/z unit are recorded as a spectrum. There are many ionization methods and many methods for separating the resulting ions [Silverstein *et al*., 2005]. It is a powerful analytical technique that is used to determine molecular weight, elemental composition and it calculates molecular formula using high resolution mass spectrometry. A mass spectrum is a plot of the ion signal as a function of the m/z ratio. The spectra are used to determine the elemental or isotopic signature of a sample, the masses of particles and of molecules, and to elucidate the chemical structures of molecules, such as peptides and other chemical compounds. The MS principle consists of ionizing chemical compounds to generate charged molecules or molecular fragments by bombarding the molecules in the gas phase with high energy electron beam and records the result as a spectrum of positive ions, which have been separated on the of their m/z ratios [Silverstein *et al*., 2005].

The high resolution mass spectrometry (HRMS) makes possible precise mass measurement to a few parts in 10^5 or better. This is sufficient to distinguish between ions having the same mass number, but different chemical composition. The precise mass of an ion is the sum of the masses of its constituent elements and the molecular formula can usually be found from the mass determination. The ability to determine the chemical composition of ion from a measurement of its mass gives mass spectrometry a new dimension [Kendrick, 1963]. The most

abundant ion formed in the mass spectrometer gives the most intense peak in the mass spectrum. This peak, the most intense peak in the spectrum is called the base peak and it is assigned a value of 100%, and the intensities of the other peaks, including the molecular ion peak, are reported as the fraction (percentage) of base peak. The most intense peak corresponds to the most stable cation as a manifestation of the fragmentation of the molecular ion as the large amount of energy it acquires after being bombarded with a high energy electron at the ionization chamber [Mistry, 2009].

In a typical mass spectrometry procedure, a sample, which may be solid, liquid, or gas, is ionized, *e.g.*, by bombarding with electrons. This may cause some of the sample molecules to break into charged fragments. These ions are then separated according to their m/z ratio, typically by accelerating them under the influence of an electric or magnetic field: ions of the same m/z ratio will undergo the same amount of deflection [Sparkman, 2000]. Ions are detected by a mechanism capable of detecting charged particles, such as an electron multiplier. Results are displayed as spectra of the relative abundance of detected ions as a function of m/z ratio. The atoms or molecules in the sample can be identified by correlating known masses of the identified masses or through a characteristic fragmentation pattern.

The time-of-flight (TOF) analyzer uses an electric field to accelerate the ions through the same potential, and then measures the time they take to reach the detector. If all the particles have the same charge, the kinetic energies will be identical, and their velocities will depend only on their masses. Lighter ions will reach the detector first and vice versa [Wollnik, 1993]. Many mass spectrometer work in either negative ion mode or positive ion mode. It is very important to know whether the observed ions are negatively charged or positively charged. This is often

important in determining neutral mass but it also indicates something about the nature of the molecules. Different types of ion source result in different arrays of fragments produced from the original molecules. An electron ionization source produces many fragments and mostly single-charged (1-) radicals (odd number of electrons), whereas an electrospray source usually produces non-radical quasimolecular ions that are frequently multiply charged. Knowledge of the origin of a sample can provide insight into the component molecules of the sample and their fragmentations.

Mass spectrometry has both qualitative and quantitative uses. These include identifying unknown compounds, determining the isotopic composition of elements in a molecule, and determining the structure of a compound by observing its fragmentation. Other uses include quantifying the amount of a compound in a sample or studying the fundamentals of gas phase ion chemistry (the chemistry of ions and neutrals in vacuum). Mass spectrometry is now a very frequent use in analytical laboratories that study physical, chemical, or biological properties of a great variety of compounds.

As an analytical technique it possesses distinct advantages such as: increase sensitivity over most other analytical techniques because the analyzer, as mass-charge filter, reduces background interference. Excellent specificity from characteristic fragmentation patterns to identify unknowns or confirm the presence of suspected compounds; information about the isotopic abundance of elements; temporally resolved chemical data. Few disadvantages of the method are that it often fails to distinguish between optical and geometrical isomers and the position of substituent in *o*-, *m*- and *p*-positions in an aromatic ring. Also, its scope is limited in identifying hydrocarbons that produce similar fragmented ions.

Fourier transform infrared spectroscopy (FTIR) is a simple and reliable technique widely used in academic research and industry. It works on the basis of vibrations of various functional groups in a molecule and provide information in the form of vibrational frequencies. It is a rapid, non-destructive, cost-effective method that can detect a wide variety of functional groups and is very sensitive to subtle change in the functional group(s) of a molecular structure. It provides useful information on the chemical composition and physical state of the whole sample [Cocchi *et al*., 2004]. Infrared radiation while passing through the sample interacts with some of the molecular constituents in the sample which either absorbs the energy or is transmitted through the molecules altogether. This is manifested as the absorption/transmitance peaks which are consistent with the frequencies of molecular vibrations of bonds between the atoms that make up the molecule. In principle, the intensity of various peaks in the spectrum is a direct indication of the amount of material present [Dowell *et al*., 2006]. IR method also has its limitation as it cannot process monoatomic gases as the atoms in the gas do not form chemical bonds with each other given that IR spectrometer measures the vibrational energies of the bond lengths which will not show up on the final spectra.

Infrared spectroscopy has been applied in the analysis of mainstream cigarette smoke, which comprises smoke emitted from the filter end of the cigarette [Vilcins and Lephardt, 1976; Williams, 1980; Shi *et al*., 2003]. Maddox and Cueto [Maddox and Mamantov, 1977; Cueto *et al*., 1989; Cueto, 1990] demonstrated analysis of gas phase components of mainstream smoke by FTIR spectroscopy. Cole and Martin measured the emission of several gas phase components of sidestream cigarette smoke [Cole and Martin, 1996] , *i.e.*, smoke arising from the hot-cold end of the cigarette, by FTIR techniques with univariate calibrations. FTIR analysis demonstrated that

cigarette smoke that is known to contain thousands of compounds and the amounts of vapor phase components in the sidestream smoke are emitted at levels two to ten times the mainstream amounts [Dube and Green, 1982; Baker, 1999].

CHAPTER 2:

Materials and Methods

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MATERIALS AND METHODS

As very limited literature is available on the health related effects of the prolonged consumption of tuibur, it is the most opportune time to carry out a detailed characterization of tuibur, identify the chemical constituents present in it and their potentially adverse effects on human health. Here, we provide the details of spectroscopic studies applied in the characterization of tuibur as it represents the first step towards a much needed systematic investigation into the carcinogenic properties of tuibur solution.

2.1. Tuibur manufacturing protocol

Unlike other smokeless tobacco products, tuibur, the unique tobacco smoke-infused aqueous solution is made locally in and around many towns of Mizoram, including Aizawl city, on a cottage industry scale (Figure 2.1). Tuibur is produced by passing smoke, generated from smoldering tobacco leaves, leaf veins, leaf petioles and leaf stalks, through water treated with tobacco ash till the preparation turns cognac in color. This solution has a smell similar to a pungent saturated ammoniacal nicotine smell. Indigenous vessels and devices (Figure 2.2 – 2.4) are used for the production of tuibur on a cottage industry basis [Sinha *et al*., 2004]. Manufacturing process begins with treating the water being used for the preparation of tuibur, with spent tobacco stalk (ashes) which converts water into alkaline solution (pH ~ 8 - 11) that could extract much higher concentration of nicotine (Figure 2.3) as neutral species from the gas phase tobacco smoke [Hurt and Robertson, 1998]. Once tuibur production is completed, in the steel container, there are two layers. The upper (aqueous) layer which is larger in quantity is

called tuibur separated from the denser layer which is, very small in quantity, also immiscible with tuibur layer (organic layer). Thus, habitual consumption of tuibur is considered as one of the 'safe' solvated nicotine delivery medium.

At one instant, approximately 8 kg tobacco stalk were normally smouldered, usually over a period of 12 hr to completely burn the whole 8 kg heap of tobacco stalk (Figure 2.2). The alkaline filtrate was kept in the lower container (Figure 2.3) and the tobacco smoke gets its polar constituents dissolved in the aqueous filtrate which further augments the concentration of the filtrate into a more concentrated tobacco smoke solution. One complete cycle of about 12 hr period using a 20 l feedstock normally generates the so called 'lowest' quality of tuibur (most diluted tuibur). Usually, three different concentrations of tuibur were prepared. The concentrated tuibur were manufactured by doubling-up the quantity of tobacco for augmenting the lower quality tuibur and hence demands adequately longer duration for the augmentation process.

Once the production cycle is complete, the tobacco ashes were collected and kept in another container with perforation at the bottom. The perforated bottom was covered with a thick jute bag in order to prevent the particulate matter of ash escaping into the aqueous solution during the filtration process (Figure 2.3). The prepared tuibur was taken out of the bottom container subsequently filtered through tobacco stalk ash and the final filtrate was collected for human consumption, which is called tuibur. The quality, concentration as well as 'taste' of tuibur depend very much on the quality and quantity of tobacco besides the uninterrupted production cycle. The quality is graded according to the amount of tobacco used and the duration of smoke infusion through water, *i.e.*, higher the smoke content, better the quality [Sinha *et al*., 2004].

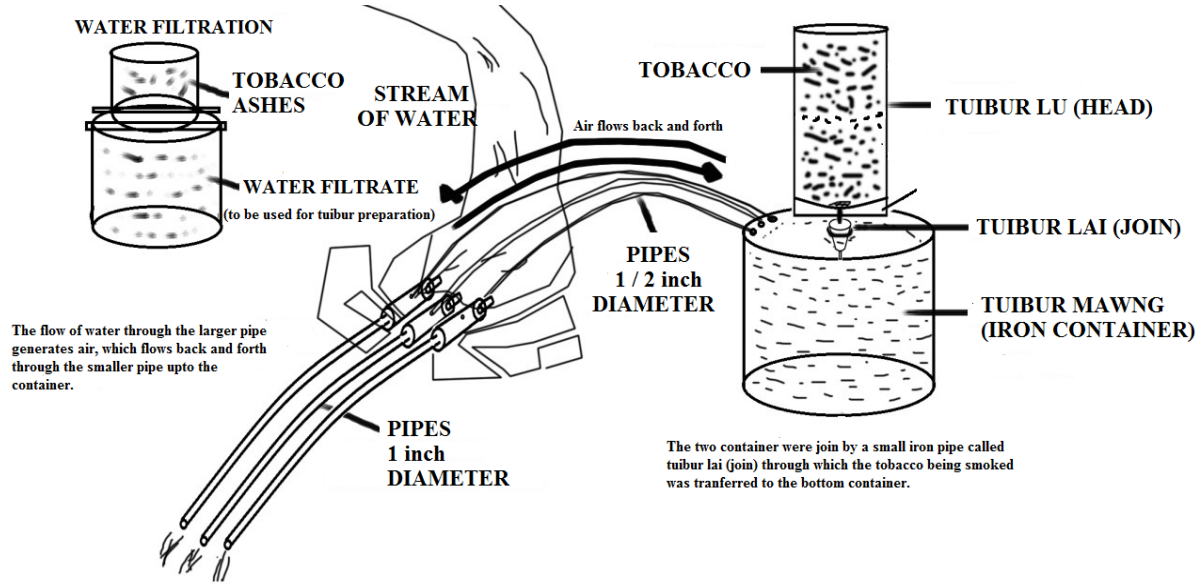


Figure 2.1: Preparation of Tuibur.

Briefly, water from nearby stream or rivulet may be diverted through large pipes to water checkpoint (Figure 2.1). It must be ensured that water checkpoint or waterhole is located at the edge of the waterfall or rapid-stream so that we will have intense running/flowing water in the pipe leading to a good circulation of air (aspiration) through the containers holding the tobacco stalk, petiole and vein.

The iron container (Tuibur Mawng – Figure 2.1) would be filled with water. Tuibur Lai (Figure 2.1) will be vertically inserted on top (within the hole made in the middle of the top). One end of the connector (Tuibur Lai) will be dipped into alkaline water/diluted Tuibur in the container, while the other end of Tuibur Lai would be inserted in such a manner that Tuibur Lu will be kept on top of it that would enable Tuibur Mawng connected to Tuibur Lu through Tuibur Lai (Figure 2.1).

(A) (B)

Figure 2.2: Fresh heap of tobacco stalk (A); Spent tobacco stalk (ash and 'charcoal') (B).

(A) (B)

Figure 2.3: Filtration of water through the spent tobacco stalk for generating feedstock (A); Aqueous filtrate (feedstock) (B).

(A) (B)

Figure 2.4: Generation of tobacco smoke for the manufacture of tuibur (A); Release of spent tobacco smoke into the rapids (B).

Once all three components are connected together, the water rapidly flowing through the plastic pipes will generate air circulation (back and forth similar to aspiration) through the small iron pipes up to the container (Tuibur mawng - Figure 2.1). At the top of iron vessel (tuibur lu), a heap of tobacco stalk will be heated up using a smoldering fire with the help of the air flowing (back and forth) through iron pipes into the container and the resulting tobacco smoke will be sucked through tuibur lai and concomitantly passed over the water (bubbled) stored in Tuibur Mawng and finally tobacco smoke from tuibur mawng is released towards the large pipe in the stream through the small pipe (Figure 2.4). At one instant, 5-8 kg of tobacco stalk mixture is normally used, which will be put inside tuibur lu. Once the tobacco started smoldering, it usually takes ~ 12 hr as air circulation takes that long to completely burn the whole batch of tobacco stock, due to the indigenously conceived rudimentary aspiration process. The smoke while

ventilated through the tuibur mawng, helped in transforming the water stored in the container (tuibur mawng) from a mere H_2O/diluted Tuibur to Tobacco smoke saturated solution (Tuibur) which can be consumed.

Mixtures of varieties of tobacco are being used for manufacturing tuibur. The locally grown Mizo tobacco plant (*Nicotiana tabacum*) harvested by the farmers were treaded with barefoot and sun dried. As the tobacco leaves turns reddish brown, the stalk, the petiole and the central vein were separated from the lamina. Lamina is being used for making local cigarette (Meizial); the stalk, the petiole and the vein were used for preparation of tuibur. But the locally grown Mizo tobacco plant is not considered a good choice for manufacturing tuibur (due to flavour) and hence it is rarely used.

The mixture of tobacco stalk, petiole and vein, packed in jute bags, is generally imported from Myanmar. The tobacco processing process is completely different in rural Myanmar. Normally, around the month of September, the leaves were plucked, leaving the main stalk of the tobacco plant. A pitch of about 3 - 4 feet deep was dug in the soil where tobacco leaves were piled on top of each other. Before spreading tobacco leaves into the pit, brine solution or slaked lime solution was sprinkled all over the pitch, and again over the leaf pile to prevent fungal growth. The size and depth of the pitch depends on the quantity of the tobacco. As the tobacco leaves were carefully piled up on top of each other, the pitch was covered with soil to protect the leaves from sunlight and to facilitate an anaerobic fermentation of the leaves. After about a month or two, the leaves were taken out of the pitch. This way, the leaves completely changed colour from green to the preferred golden brown colour and becomes flexible in such a way that it does not break easily. Then the lamina of foliage, separated from the veins was sold for the

manufacture of commercial cigarette in Myanmar and China. The main stalk of the tobacco plant were also cut down and dried under the sun; then the stalk, the petiole and the veins, henceforth collectively called as tobacco stalk, were fed into a machine that cut the stalk into uniform size, subsequently packed and exported to Mizoram mainly for tuibur production.

The tobacco varieties mainly grown in Myanmar are bamasayu (*Nicotiana rustica*) and Virginia (*Nicotiana tabacum*). The former is harsh and more expensive while the latter is light and less expensive, hence the stalk of the two varieties are usually mixed together in a ratio 1:3 (bamasayu:Virginia) for the purpose of tuibur preparation. The tobacco stalk imported from Myanmar perceptibly makes the best quality of tuibur. Tobacco coming from Shillong, Meghalaya was the main stalk and the leaf. The quality of tuibur using this tobacco is better than the locally grown Mizo tobacco but inferior than those imported from Myanmar. The cost is also much cheaper compared to the Myanmar tobacco. When the supply of Myanmar tobacco is scarce during rainy season, only then these tobacco is being used, even then, it was usually mixed with other variety of tobacco.

2.2. Collection of tuibur samples

Commercially available tuibur sample was collected for this study. 14 different samples of tuibur were collected for spectroscopic characterization from different regions of Mizoram as given in table 2.1. The pH measurements were made for all tuibur samples using a pH meter (Eutek model 510) prior to analysis.

Table 2.1: Place of sample collection

S.No	Place of Sample collection
1	Champhai
2	Khawzawl
3	Lunglei Site1*
4	Saiha
5	Lunglei Site2*
6	Tawipui Site1A**
7	Tawipui Site1B**
8	Aizawl Site1A**
9	Aizawl Site1B**
10	Aizawl Site2A***
11	Aizawl Site2B***
12	Aizawl Site2C***
13	Kolasib Site1A**
14	Kolasib Site1B**

* *Site1 and site2 are of same district but different location.*

** *Site1A and Site1B are of same location but different concentration (from Tawipui, Aizawl district and Kolasib district).*

*** *Site2A, Site2B and Site2C are of same location but different concentration.*

2.3. Analysis of free radicals by EPR

Electron paramagnetic resonance spectroscopy (EPR), also called electron spin resonance (ESR), is a technique used to study chemical species with unpaired electrons. EPR spectroscopy plays an important role in the understanding of organic and inorganic radicals, transition metal complexes, and some biomolecules. With 14 different tuibur samples, an EPR spectrometry analysis was performed to investigate chemical species with unpaired electrons.

Unlike most spectroscopic techniques, EPR spectrometers measure the absorption of electromagnetic radiation as the function of magnetic field. A phase-sensitive detector is used in the EPR spectrometer which converts the normal absorption signal to its first derivative in order to enhance the sensitivity of the spectrometer. Then the absorption signal is presented as its first derivative in the spectrum, thus, the magnetic field is on the x-axis of EPR spectrum: $d\chi''/dB$, the derivative of the imaginary part of the molecular magnetic susceptibility with respect to the external static magnetic field in arbitrary units is on the y-axis. In the EPR spectrum, where the spectrum passes through zero corresponds to the absorption maximum of absorption spectrum as this facilitates the determination of the center of the signal. On the x-axis, the magnetic field is represented in the units of gauss (G) most of the time instead of tesla (T) as one tesla is equal to 10000 gauss.

Room temperature solution Electron Paramagnetic Resonance Spectral measurements at Central Instrumentation Centre, IIT-Guwahati were carried out using ESR spectrometer, JEOL, Model – JES-FA200. Room temperature solution EPR spectra were also recorded on a Bruker-ER073 spectrometer (situated at the University of Hyderabad) equipped with an EMX microX

source (Microwave Bridge) for the measurement in the X-band region using Xenon1.1b60 software provided by the manufacturer.

2.4. Trace metal analysis of Tuibur samples

ICP-MS spectrometer in which a high-temperature ICP (Inductively Coupled Plasma) source is coupled with mass spectrometer. The sample is typically introduced into the ICP plasma as an aerosol, either by aspirating a liquid or dissolved solid sample through a nebulizer or using a laser to directly convert solid samples into an aerosol (laser ablation). Once the aerosol sample is introduced into the ICP torch, it is completely de-solvated and the elements in the aerosol are initially converted into gaseous atoms, subsequently ionized towards the end of the plasma. Once the constituent elements in the sample are converted into ions, these ions are further separated and eventually detected by the mass spectrometer.

All samples were filtered through 0.22 mm mixed cellulose ester membrane (Millipore, Ireland) and diluted with de-ionised water to a proper extent (below 1 mgl^{-1}) into 15 ml – polypropylene tubes (VIOLAMO, Japan) prior to the elemental analysis using the ICP-MS spectrometer. The samples were then analyzed by a fully quantitative analytical method in a standard mode. Analysis of selected trace metals concentration: *viz*., chromium, manganese, nickel, copper, zinc, arsenic, cadmium and lead were conducted using an ICP-MS (Thermoelemental, UK; Model X-series).

Similarly analysis of selected trace metals with high concentration such as magnesium (Mg), calcium (Ca), iron (Fe), were conducted using ICP-AES (Perkin Elmer, USA; Model Optima-8300).

2.4.1 Statistical analysis

Statistical analysis of ICP-MS data was performed using SPSS statistical Package V. 18.0 (IBM; Armonk, New York, USA) for the correlations between the concentration of various metal ions and the amount of tobacco (kg/l) used for the manufacture of tuibur.

2.5. Mass Spectrometry

Mass spectrometry (MS) analyses were performed on an Agilent 6520 QTOF-MS/MS system coupled with an Agilent 1200 HPLC (Agilent technologies, USA). The Agilent 1200 HPLC system consisted of a quaternary pump (G1311A), online vacuum degasser (G1322A), auto sampler (G1329A), and diode-array detector (G1315D). HPLC was connected to ESI source of mass spectrometry through a guard column. The mobile phase, which consisted of a 0.1% formic acid aqueous solution (A) and methanol (B), was delivered at a flow rate of 0.2 ml/min under an isocratic program: 40% (A) and 60% (B) for 2 min. The sample injection volume was 1 µl. Agilent 6520 accurate mass QTOF-MS/MS system consisted of an ESI source operating in both positive and negative ion mode. In the ESI source, nitrogen was used as drying and collision gas. The heated capillary temperature was set to 310 °C and nebulizer pressure to 35 psi. The drying gas flow rate was 10 lit/min. VCap, fragmentor, skimmer and octapole RF peak voltages were set to 3500 V, 150 V, 65 V and 750 V, respectively in the ion source parameters. Detection was carried out within a mass range of m/z 10-1500 and resolving power above 10000 (FWHM). The chromatographic and mass spectrometric analyses, including the prediction of chemical formula and exact mass calculation were performed by using Mass Hunter Software version B.04.00 build 4.0.479.0 (Agilent Technology).

Preliminary work was done to isolate and study the 'bio-active' xenobiotics of tuibur using High Performance (Pressure) Liquid Chromatograph – Mass Spectrometry [HPLC-MS] methods using a Waters LC-MS instrument, where MS is a low-resolution instrument.

Centrifugation of tuibur is done to remove all suspended particles and other particulate matters. All the solvents are purchased as HPLC grade (Merck) and used as such. The separation of xenobiotic compounds was also performed using Waters LC with MS as a detector system. The column was zorbax C-18. For MS study, MM-APCI ionization method is utilized. For gradient elution, the following compositions of solvents were used (A& B): A - 0.1% HCOOH in Water: B - Methanol at a flow rate of 1.2 ml/min. The injection volume for all samples was 5 µl.

Elution time (min.):	**0 – 3.5**	**3.5 – 5.0**	**5.0 - 5.5**	**5.5 – 7.0**
A B:	**10 - 95**	**95**	**95 - 10**	**10**

For HPLC method, a single 7 minute chromatographic run was performed and analyzed by MS. The chromatograms showed that the compounds are isolated in accordance with the polarity of the solvents used. The APCI permits the observation of $[M+H]^+$ molecules, and their fragment ions. In the positive ion mode, tuibur produced mostly protonated cation molecules.

2.5.1. High Resolution Mass Spectrometry

The separation of xenobiotic compounds present in tuibur was also performed on an Agilent6520 LC with MS/MS (Q-TOF) as a detector system. The column employed for the separation was ODS C-18. For MS study, ESI ionization method is utilized.

For HPLC method, a single 9 minute chromatographic run was performed and analyzed by HRMS (CDRI, Lucknow). The chromatograms showed that the compounds are isolated in accordance with the polarity of the solvents used. The ESI permits the observation of $[M+H]^+$ molecules, and their fragment ions. In the positive ion mode, tuibur produced a protonated cation molecule.

2.6. FTIR (Fourier Transform Infrared Spectroscopy)

Fourier Transform Infrared Spectroscopy (FTIR) analyses were performed on Thermo Avatar 370 FTIR spectrometer. A small quantity of the sample is added to KBr in the ratio 1:100 approximately. The matrix was ground for 3-4 minutes using mortar and pestle. The fine powder was transferred into 13 mm diameter die and made into a pellet using a hydraulic press by applying a pressure of 7 ton/sq. mm. The resulting thin and semi-transparent pellet was subjected to FTIR analysis using an universal pellet holder. Infrared spectral data were collected over a range of 4000-400 cm^{-1} with an interferogram of 32 scans. It may be also possible to characterize the immiscible as well as denser distillate (immiscible layer) and its effect on human health as it is occasionally used as insecticide for household gardens.

2.7. Genotoxic effects of tuibur – mtDNA Study

In addition, it was aimed to evaluate the mutagenic and carcinogenic potency of tuibur by measuring the type, the frequency and the extent of polymorphism(s) in mtDNA from a pool of donor samples, within the Mizo tribal population from the state of Mizoram, classified according to their life style habits, *viz.*, consumption of tuibur, smoking status, diet, etc. Using the information from the single nucleotide polymorphisms (SNPs) and restriction fragment patterns, it could be possible to predict the genetic predispositions leading to the development of cancer, for early diagnosis and optimal medical treatment. Thus, it is of significant interest to investigate the toxicity of *tuibur* and other tobacco product on genomic level. The main purpose of the current study was to understand the genetic etiology of the tribal population and evaluate the mutagnetic effects of tuibur and other tobacco products in inducing mitochondrial gene (D-Loop) polymorphism among the Mizo population using DNA typing study.

The genetic material, DNA molecule is made up of a sequence of four smaller molecules called nucleotides. The four nucleotides are purines, adenine (A), and guanine (G), besides pyrimidines, cytosine (C), as well as thymine (T). The sequence of these nucleotides is extremely important, as it determines the characteristic attributes of an individual. Differences in individuals result from small variations in the DNA, called mutations, in the sequence of DNA. A whole range of mutations are possible in DNA. Insertions are regions of DNA where nucleotides have been added to a DNA sequence. Deletions are regions where nucleotides have been removed. Point mutations, known as polymorphisms, may also occur in DNA. This is simply the replacement of a single nucleotide by a different one.

2.7.1. Collection of biological samples

For the present study, buccal swab samples were collected from 40 tuibur consumers and 40 unrelated healthy (without records of cancer or other disease) non-tuibur consumer serving as a control group. As for the stomach cancer group, buccal swab samples with the matching blood samples were collected from 40 cancer patients. All stomach cancer patient blood samples were confirmed histopathologically in the Mizoram State Cancer Institute (Zemabawk, Aizawl, Mizoram). The age distribution of the three groups (tuibur consumer, control, and cancer patients) is from 19 to 95, 19 to 74, and 40 to 78 yrs, respectively. All volunteers were fully informed about the study and participated with their full consent. Detailed information on demographic factors, dietary habits, previous disease history, physical activity, tobacco habit, alcohol consumption, weight, and family history were collected from the study subjects using a standardized structured questionnaire. The undertaken protocol were reviewed and approved by the Institutional Review Board of all institutes involved in the study. All samples were immediately stored under appropriate condition until further use.

In order to identify and evaluate the genetic abnormalities, the collection of good quality DNA is necessary. The commonly used method is to obtain genomic DNA from nuclear genes of peripheral red blood cells from blood. Other alternative sources of DNA isolation include buccal cell, hair with follicle, urine, etc., which are easier than the blood collection. Buccal swabs also offer a simple method of obtaining cells that are yielding sufficient quantity of relatively pure DNA for polymerase chain reactions in which only ng quantities of DNA are needed. Although most DNA is packed tightly within chromosomes inside the nucleus, mitochondria also contain a small amount of their own DNA. In humans, mitochondrial DNA spans about 16,500 DNA

building blocks (base pairs), representing a small fraction of the total DNA in cells. The integrity of DNA may be assessed by resolving DNA extracts on a 0.8% agarose gel by electrophoresis, followed by visualization with ethidium bromide staining. Each DNA sample can be graded according to the electrophoretic migration of DNA in comparison to a known molecular weight marker ladder.

2.7.2. DNA extraction from the buccal swab

Extraction of DNA was done from these samples by following the modified protocol of Ghatak *et al*., 2013. The buccal swab samples were suspended in 500 µl of extraction buffer [(50 mM tris-HCl (pH 8.0), 50 mM ethylenediaminetetraacetate (EDTA), 10% sodium dodecyl sulfate (SDS) and 0.5 M of NaCl], followed by 10-15 µl of 10 ng/ml Proteinase K (Hi-media) were added. The samples were incubated 1-3 hr at 56ºC; until the tissue is totally dissolved. After incubation, the sample tubes were vortex for 30 sec. The DNA was then extracted from each sample with an equal volume (500 µl) of phenol: chloroform: isoamylalcohol (25:24:1) and mixed gently by inverting the tubes for few minutes. The samples were centrifuged (Eppendorf 5415R) for 10 min at 10000 rpm (4ºC) and right after centrifugation, the upper aqueous layer was transferred to a fresh sterilized microcentrifuge tube. 500 µl of chilled isopropanol (Merck) and 1/10 sample volume of 3 M sodium acetate were added to the transferred sample and it was kept at –20 ºC for 1 hr for precipitation. After one hr, the sample was centrifuged (Eppendorf 5415R) at 10000 rpm (4 ºC) for 10 min. The supernatant was decanted and 250 µl of 70% ethanol (Merck) was added to dissolve the pellet, then the mixture was tapped for thorough mixing. The mixture was again centrifuged at 14000 rpm for 10 min and the supernatant was decanted gently. The pellet was air dried under laminar air flow and the dried pellet was re-

suspended in 30 µl Nuclease free water or 1X TE buffer (10 mM Tris-base + 1 mM EDTA, pH 7.6) and stored in –20 ºC for long preservation.

2.7.3. DNA extraction from the blood sample

The lymphocytes from whole blood were separated using a hypotonic lysis buffer [ammonium bicarbonate and ammonium chloride (Hi-media)] to remove RBC with minimal lysis effect on lymphocytes. Three volumes of RBC lysis buffer was added to the blood sample and mixed well using rocker or vortex and inverting thoroughly for 5 min and centrifuged (Eppendorf 5415R) at 4000 rpm for 10 min. The supernatant was discarded and the pellet was kept. This step was repeated 2 to 3 times until a clear supernatant and a clean white pellet was obtained. After the final wash, the supernatant was discarded completely and the pellet was re-suspended in 500 µl Phosphate buffer saline (PBS) and the mixture was centrifuged at 4000 rpm for 10 min for thorough mixing. Then 500 µl extraction buffer (50 mM Tris-HCl, 50 mM EDTA, 10 % SDS, pH 7.5) and 10-15 µl of Proteinase K (10 mg/ml stock) (Hi-media) was added. The sample was vortexed to dissolve the pellet completely and it was incubated for 2 hours/overnight at 56 °C in water bath (Jeio-Tech, CW-30G) for better lysis. After incubation, the sample tubes were vortexed for 30 sec. An equal volume (500 µl) of Phenol:Chloroform: Isoamylalcohol (25:24:1) was subsequently added to the tube and it was mixed well by inverting for 1 min. The tube was centrifuged at 10000 rpm (at 4 ºC) for 10 min, right after centrifugation; the aqueous upper layer was transferred to a fresh tube. This step was repeated 2 to 3 times for consistent result. The supernatant was then transferred to a fresh tube and 10 µl of 10mg/ml RNase A (Fisher Scientific, Fermentas, Germany) was added.

The sample was incubated at 37ºC for 30 min prior to the addition of an equal volume of Chloroform: Iso-amyl alcohol (24:1) and mixed by inverting the tube for 1 min and centrifuging at 10000 rpm (at 4ºC) for 10 min. The supernatant was transferred to a fresh tube and twice the volume of absolute alcohol (Merck) was added and inverted gently a few times and kept it at −20 ºC, followed by centrifugation at 10000 rpm at (4°C) for 20 min. After decanting the supernatant, 250 µl of 70% ethanol was added and the pellet was gently tapped, followed by centrifugation at 10000 rpm for 10 min and gently decanting the supernatant. The pellet was air dried in a laminar air flow and the dried pellet was re-suspended in 50µl of Nuclease free water or 1X TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.6) buffer and stored in −20ºC or −80ºC for long preservation.

2.7.4. PCR amplification of the mtDNA D-Loop region

The polymerase chain reaction (PCR) method is applied to amplify a single or a few copies of a piece of DNA (target) across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. The amplified product is known as an amplicon. The amplification product can be detected using gel electrophoresis where the visualization of a band containing DNA fragments of a particular size indicates the presence of the target sequence in the original starter DNA sample. Similarly, absence of a band may indicate that the target sequence was not present in the original starter DNA sample. PCR permits early diagnosis of malignant diseases such as leukemia, lymphoma and carcinoma. PCR assays can be performed directly on genomic DNA samples to detect translocation-specific malignant cells at a sensitivity that is at least 10,000 fold higher than that of other methods.

DNA extracts was amplified for the mtDNA D-loop region by using primers HMt-F (5′-CACCATTAGCACCCAAAGCT-3′) and HMt-R (5′-CTGTTAAAAGTGCATACCGCCA-3′),

as described by Salas *et al*. 2001 for the HVI region. PCR (vapo.protect; Eppendorf) was carried out in 25 µl total reaction volumes, each containing 100 ng template DNA, 0.2 pM of each primer, 2.5 µl 10× PCR buffer (final 1× PCR buffer), 1.5 mM $MgCl_2$, 200 mM dNTPs, and 1 unit Taq DNA polymerase. The reaction mixture was heated to 94°C for 5 min, followed by 40 cycles, each consisting of 1 min denaturation at 94°C, 1 min annealing at 63°C, 90 sec extension at 72°C, and a final 10 min extension at 72°C. The PCR amplification products (10 µl) were subjected to electrophoresis (Bio-Rad) on 1.2% agarose gel in 1× Tris-acetate-EDTA buffer at 80 V for 30 min and stained with ethidium bromide (Himedia), and images were obtained in gel documentation (G-Box; Syngene, Cambridge, UK) systems.

2.7.5. Restriction digestion of the mtDNA D-Loop region PCR product

Restriction enzymes also known as restriction endonucleases cut DNA molecules at specific locations. PCR products may be digested with a restriction enzyme. Each enzyme recognizes a unique sequence of nucleotides in the DNA strand and cuts the double-stranded or single-stranded DNA at specific sequences in the genome. The restriction enzyme *HaeIII* recognizes the sequence GCGC and it cleaves the bond between middle cytosine and guanine. *AluI* is another restriction enzyme and its recognition site is AGCT, nucleotide sequence cleavage occur between G and C. *MspI* recognition site is CCGG, nucleotide sequence cleavage occur between C and C. *KpnI* recognition site is GGTACC, nucleotide sequence cleavage occur between C and C. These restriction enzymes are found in many different strains of bacteria where their biological role is to participate in cell defense. These enzymes “restrict” foreign (e.g. viral) DNA that enters the cell, by destroying it. The host cell has a restriction-modification

system that methylates its own DNA at sites specific for its respective restriction enzymes, thereby protecting it from cleavage.

In order to study fragment length differences among individuals due to mutations, RFLP techniques have been employed to determine distinctive differences in gene sequences and restriction cleavage patterns in certain areas of the genome. Mutations within the DNA, known as polymorphisms, result in strands of different lengths restriction enzyme digestion and subsequently electrophoresis is applied to separate the strands according to the variation in their length. RFLP is used as part of DNA fingerprinting, to detect genetic diseases and to determine genetic relationships between species.

Restriction fragment-length polymorphism (RFLP) of the mtDNA D-loop region was performed to check the polymorphism pattern. PCR products were digested with *AluI*, *HaeIII*, *MspI* and *KpnI* (Fermentas, Thermo Scientific) in a total volume of 10 µl (2 µl PCR products, 1 µl enzyme buffers, 1U enzymes, and 6.8 µl distilled water) and placed in the incubator at 37°C for 4 hr. The restriction products were analyzed by electrophoresis (Bio-Rad) on a 10% polyacrylamide gel, and the molecular weight of restricted fragments was analyzed by gel documentation systems (G-Box; Syngene) after ethidium bromide (Himedia) staining.

2.7.6. Sequence analysis

PCR products (selected) were sequenced from the opposite direction to ensure reading accuracy. The sequences are submitted in the EBI repository database (Table 1). Sequences and chromatograms obtained were examined using chromas software version 2.13 and aligned by BLAST (http:// www.ncbi.nlm.nih.gov/blast). Sequences were aligned using ClustalW and

mutations were noted by using MEGA software ver 5.0. All sequences were compared with the latest version of Revised Cambridge Reference Sequence (rCRS) of the human mitochondrial DNA (NC_012920) and subsequently analyzed for the variation in sequences using Mito Tool Programming. The results of the DNA sequence analysis were compared with the published Cambridge Sequence using Mutation Surveyor version 1.4 DNA mutation analysis software (Softgenetics, State College, PA). Sequence differences between tuibur consumer, stomach cancer and healthy blood samples were recorded as mtDNA polymorphisms. Each polymorphism was then verified against the Mitomap database (http://www.mitomap.org/) and further classified as novel or reported, depending on whether or not it is recorded in the database. Those not recorded in the database were categorized as novel mutations, and those that appeared in the database were reported as polymorphism or common mutations.

2.7.7. Phylogenetic relationships

Phylogenetic analyses based on the RFLP product of stomach cancer, control, tuibur consumer were performed using NETWORK 4.6.1.2. and DARwin5 (version 5.0.158). Phylogenetic analyses were also performed on the resulting stomach cancer, control and tuibur consumer sequences using MEGA 5.05. All mtDNA sequences were aligned by ClustalW program according to the rCRS. MEGA 5.05 program was used to calculate the distance matrix, followed by the creation of a Maximum Likelihood (ML) phylogenetic tree using Hasegawa–Kishino–Yano (HKY) [Tamura *et al*., 2011].

2.7.8. D-Loop secondary structure determination

Secondary structures of mitochondrial control region were inferred from D-loop sequences [Zuker, 2003]. The application predicts the structure based on free energy parameters,

the enthalpies were measured at 37^{o}C. However, they are assumed to be constant within the range of temperatures that might occur in vivo or in the laboratory. This enables the server to extrapolate free energies to other temperatures and to fold at these temperatures. D-Loop secondary structure determination was performed by online software MFOLD (RNA Secondary Structure).

2.7.9. Statistical Analysis

One-way ANOVA was performed using SPSS statistical package (V 16.0) for testing the statistical significance to check whether demographic, food habit, tobacco habit and haplotyping are significantly related with the number of D-loop mutation by a binary data. The P value >0.05 indicates a significant relationship as determined by the one-way ANOVA test within patient samples by PAST package [Hammer *et al*., 2001].

CHAPTER 3:

Identification and characterization of chemical constituents in tuibur.

CHAPTER 3

IDENTIFICATION AND CHARACTERIZATION OF CHEMICAL CONSTITUENTS IN TUIBUR

3.1. pH of Tuibur solution

The pH values were measured for tuibur samples collected at different sites, using a pH meter prior to further analysis. The pH values revealed that tuibur solutions of varying concentrations were alkaline in nature and they are in the range of 10.00 – 10.70 (Table 3.1).

Table 3.1: pH of tuibur solution collected from different region of Mizoram.

S.No	Place of Sample collection	pH
1	Champhai	10.7
2	Khawzawl	10.13
3	Lunglei Site1*	10.3
4	Saiha	10.12
5	Lunglei Site2*	10.59
6	Tawipui Site1A**	10.3
7	Tawipui Site1B**	10.2
8	Aizawl Site1A**	10.5
9	Aizawl Site1B**	10.6
10	Aizawl Site2A***	10.5
11	Aizawl Site2B***	10.4
12	Aizawl Site2C***	10.5
13	Kolasib Site1A**	10.07
14	Kolasib Site1B**	10.1

* *Site1 and site2 are of same district but different location.*

** *Site1A and Site1B are of same location but different concentration.*

*** *Site2A, Site2B and Site2C are of same location but different concentration.*

3.2. Analysis of free radicals by EPR

Figure 3.1, 3.2 and 3.3 depict various EPR spectral data arising from the putative paramagnetic species present in the tar components of tuibur (water insoluble organic compounds of tobacco smoke) that was extracted in acetonitrile solution (Figure 3.1) and extracted in chloroform solution (Figure 3.2 and 3.3), Figure 3.4 also showed the EPR spectral features arising from the alkaline feedstock solution (aqueous filtrate from which tuibur is being made). While, at room temperature, both aged as well as fresh tuibur samples have not exhibited any EPR characteristic spectral features. The g-value of the putative paramagnetic species present in the tar (water immiscible dense layer) that was extracted into acetonitrile solution (Figure 3.1) can be calculated using the following equation, where h is Planck's constant (6.626 x 10^{-34} J) and β=electron Bohr magneton (9.274x10^{-24} JT^{-1}):

$g_{exp} = h/\beta\ [\ \nu\ (GHz)/B_o\ (Gauss)]$

$= h/\beta$ [9.44073 GHz / 3369.6 Gauss]

= 2.00179~2.0018

The EPR feature exhibited by the acetonitrile soluble fraction of tar component of tuibur solution is relatively a single narrow line with no indication of hyperfine splitting features and it is centred at 3369.6 Gauss and it has a line width of 80 Gauss.

In general, organic radical species tends to exhibit narrow line width compared with EPR spectral features arising from transition metal ion/coordination complex species. In addition, the g-value exhibited by the paramagnetic species (*vide supra*) has indicated that it is probably arising from a stable organic radical species.

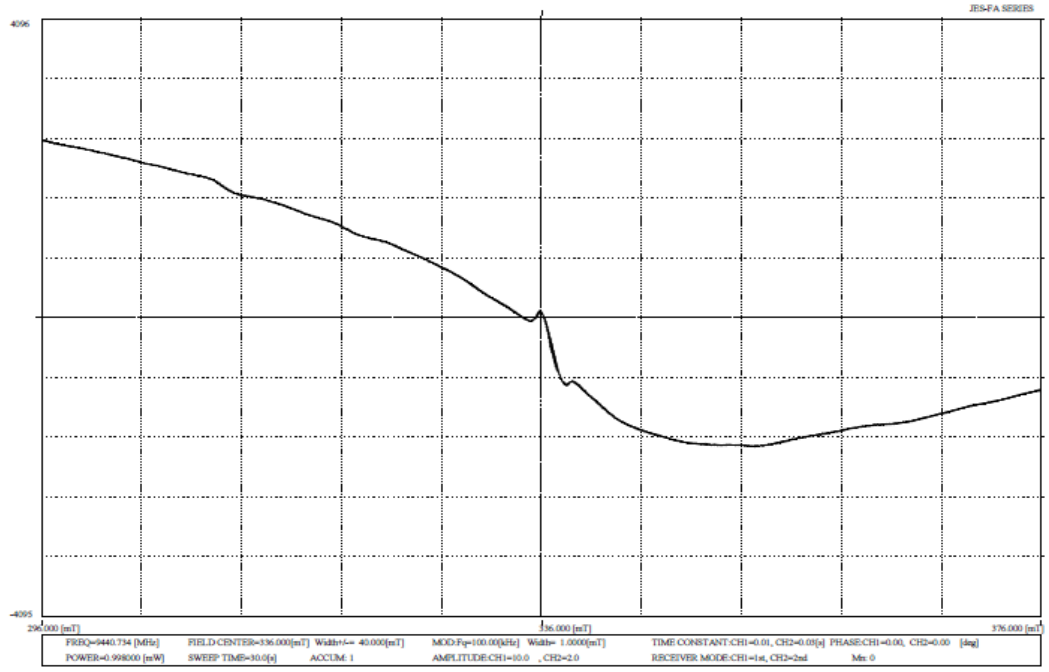


Figure 3.1: EPR spectra of tar (extracted with acetonitrile) present in tuibur.

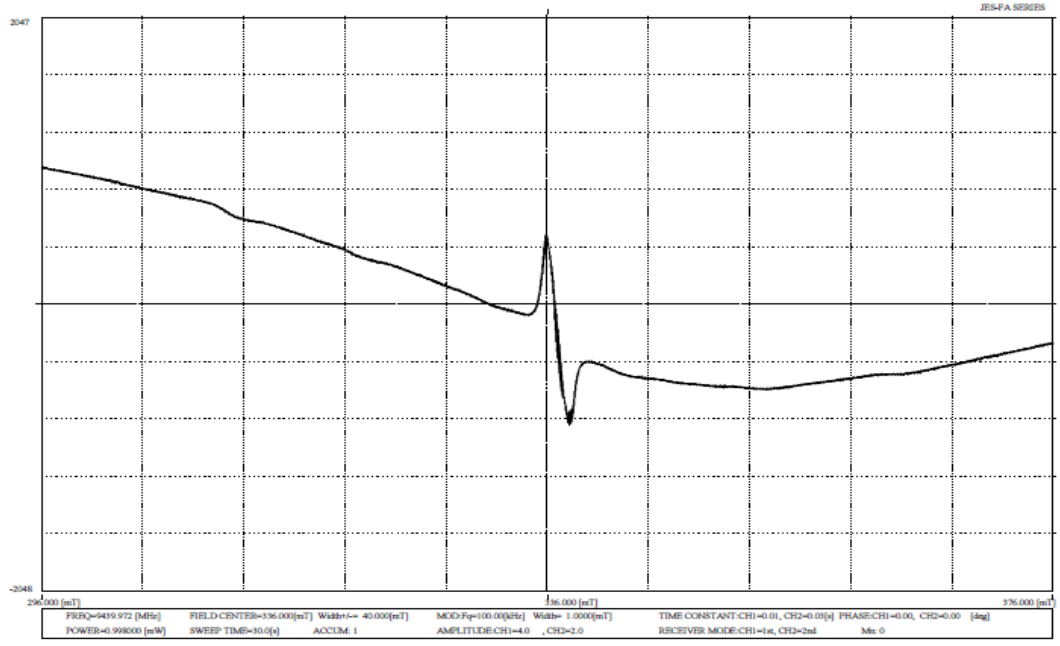


Figure 3.2: EPR spectra of tar (extracted with chloroform) present in tuibur.

Due to the higher modulation amplitude applied to observe the EPR active species in the acetonitrile solution, the EPR spectral feature is devoid of any weak hyperfine coupling splitting. A characteristic attribute of an organic radical is the observation of hyperfine couplings. Hence, at this moment, it is possible to identify the nature of this paramagnetic species except that possibly it may be an organic radical species. The g-value of the putative paramagnetic species present in the tar that was extracted into chloroform solution (Figure 3.2) can be calculated using the following equation:

$g_{exp} = h/\beta$ [9.43997 GHz / 3369.6 Gauss]

= 2.00163

The EPR feature exhibited by the chloroform soluble fraction of tar component of tuibur solution is also relatively a single narrow line with no indication of hyperfine splitting features and it is centred at 3369.6 Gauss and it has a line width of 80 Gauss.

The EPR spectral feature exhibited by the chloroform soluble fraction of the tar component of tuibur resembles very similar to the spectral feature shown in Figure 3.1, albeit the spectral intensity much higher than the acetonitrile soluble fraction and hence with better accuracy for the determination of the g-value (the centre magnetic field). This observation indicates that the putative organic radical present in the tar portion of tuibur (immiscible layer) is presumably an aromatic radical species that would commensurate with the stability of the observed organic free radical species as the polarity of acetonitrile is less than the polarity of chloroform and the solubility of the aromatic radical species is probably less in acetonitrile in comparison to the solubility of this species in choloroform.

The g-value of the putative paramagnetic species present in the tar that was extracted into chloroform solution (Figure 3.3) can be calculated using the following equation:

$g_{exp} = h/\beta$ [9.674978 GHz / 3454 Gauss] = 2.00132

The experimentally observed g-value indicates the presence of an organic free radical species and the g-value may be indicative of the admixture of excited state spin levels with the ground state levels. Moreover, the identity of the free radicals could not be ascertained due to the poor signal-to-noise ratio of the spectrum, which prevented the observation of the hyperfine lines. As a result, identity of the paramagnetic metal ion may not be possible, at present. When C_{60} and Al-doped C_{60} are deposited as thin films, an EPR signal at g = 2.001 is observed. A g-value of 2.00133 is characteristic of a weakly-bound carbon based radical besides the observed g-value is too low for an oxygen-centered radical. The data strongly suggest that the radical species are associated with C_{60}–oxygen adduct with a cage-centered radical in which the electron is delocalized over the molecular architechture [Głowacki *et al*., 2011].

The g-value of the broad EPR spectrum (Figure 3.4) of alkaline feedstock solution (tobacco ashes-treated water) used for preparation of tuibur can be obtained as follows:

$g_{exp} = h/\beta$ [9.674978 GHz / 3200 Gauss] = 2.16018 ≅ 2.1602

The observed g value indicates that the spectrum (Figure 3.4) may be due to the presence of a paramagnetic first row transition metal ion with more than half-filled 3d orbitals besides spectral broadening renders no hyperfine splitting, this may be arising from a relatively concentrated solution where the magnetic dipolar interaction between adjacent spin centres would broaden the hyperfine lines of the metal ion.

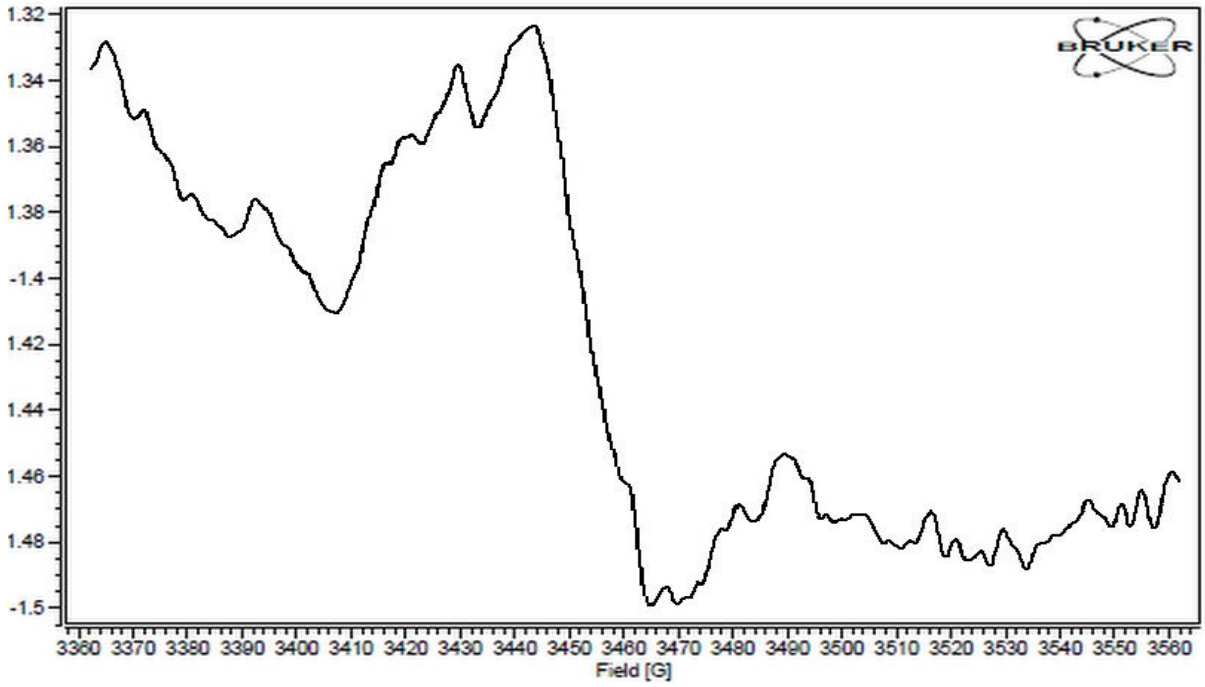


Figure 3.3: EPR spectra of tar component in tuibur.

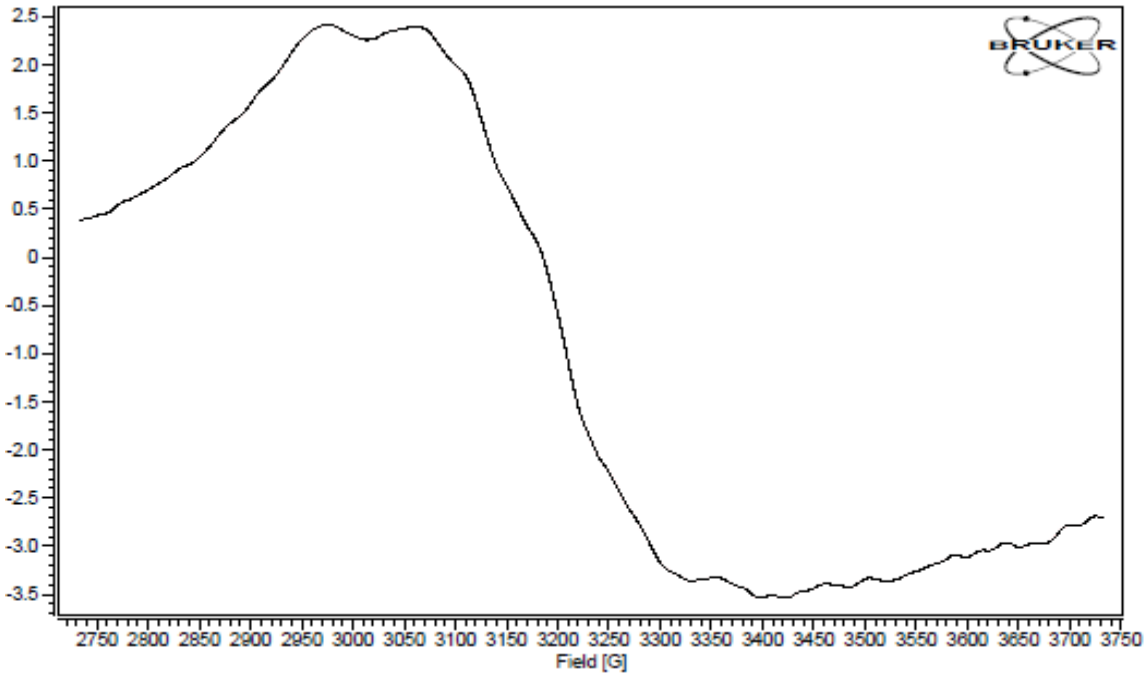


Figure 3.4: EPR spectra of alkaline feedstock solution (tobacco ashes-treated water) used for preparation of tuibur.

Furthermore, the observed EPR spectrum was measured at Instruments Facility Centre at School of Chemistry, University of Hyderabad, with higher modulation amplitude (10 Gauss) coupled with lower microwave power (30 dB) that renders an EPR spectrum with a poor signal-to-noise ratio along with a lower spectral resolution.

3.3. Trace metal analysis of Tuibur

Tuibur manufactured at different locations in Mizoram, India, were collected and various heavy metal concentrations were quantitatively determined. The concentrations of various trace metal for 14 tuibur samples are presented in Table 3.3 and 3.5 with the amount of tobacco (kg) used per litre, for the manufacture of tuibur.

Many of these metals play an essential role in human physiology. For example, the enzyme that synthesizes DNA and RNA contain zinc ions, and calcium is an important constituent of bones and teeth. Depending upon the dietary and lifestyle habits, it is possible to have either deficient levels of these metals, or to have optimal or damaging or lethal levels. However, nonessential elements such as chromium, lead, arsenic and cadmium have no beneficial role in the human body, and the daily intake of these metals is leading to bioaccumulation of these elemental species often with toxic or lethal effects.

The concentration levels of various heavy metal species in tobacco products may also vary widely and the variations may also depend on country specific and product specific [Verma *et al*., 2010] as the agroclimatic factors such as production practices and soil characteristics besides environmental conditions such as humidity, temperature, sunlight, rainfall etc., reportedly influence the accumulation of metals, in the leaf [IARC, 2004].

3.3.1. ICP-AES Analysis

With the help of ICP-AES, the concentrations of trace metals *viz.*, Ca, Fe and Mg in different tuibur samples were analyzed. The concentrations of these three metal ions exist in lower concentrations in comparison with the reference material *i.e.,* US EPA, 2012, as shown in table 3.2. Table 3.3 shows that Mg was the major metal species among all the analyzed elements and it was found in the range of 69 – 307 µg/ml, while Ca was found in the range of 9.03 – 14 µg/ml as the second most abundant element present in all the samples. As Fe was expected to be among the highest in concentration, it was found to be mostly below the anticipated concentrations, in the range of 0 – 0.5 µg/ml, even though tuibur is manufactured using steel containers.

Table 3.2: Permissible level of essential elemental concentrations (US EPA, 2012).

Elements	Permissible Level (µg/ml)
Fe	0.3
Ca	None
Mg	None

Two alkaline earth metals, calcium and magnesium belongs to group 2A in the periodic table, because of their similarity in the electronic configuration (ns^2), they closely resemble in their physical and chemical properties. They are two of the major essential elements present in the human body. Magnesium ions serve as a cofactor of many enzymes involved in numerous intracellular processes. It is responsible for more than 300 essential metabolic reactions and most importantly it forms a complex with ATP to facilitate an enzyme assisted hydrolysis of the

phosphate bond for the energy production. In addition, it is also involved in the synthesis of essential molecules along with structural roles, ion transport across cell membranes, cell signaling, besides cell migration [Rude and Shils, 2006]. Moreover, it is also essential for the transmission of impulses along nerve fibers. Calcium ions are important for the structural integrity of bones along with teeth as well as in blood clotting; besides they are required for normal growth and development. Furthermore, it is required to trigger the contraction of muscles along with the regular functioning of the heart and other metabolic processes [Lee, 2006].

They work in tandem to facilitate the heart muscle contractile process, in rhythm. Smoking causes mineral disturbances which may lead to severe and life-threatening metabolic abnormalities such as coronary heart disease, liver disease, lung infection, kidney failure, and disorders of endocrine system [John, 2007]. Smoking causes magnesium deficiency with suppressed appetite (decreased supply) in addition to the attenuated absorption imposed by the disturbance in digestive system functions [Winiarczyk *et al*., 2008]. Furthermore, magnesium deficiency results in calcium/magnesium imbalance which in turn causes tachyarrhythmia, stroke, besides it also exacerbates blood cholesterol and high blood pressure levels. Also, it is important to note that disturbances calcium metabolism have been implicated in most of the major chronic diseases, including osteoporosis, kidney disease, obesity, heart disease, and hypertension [Tordoff, 2001]. One of the important attributes of the pathological effects of magnesium depletion is the modulation of potassium fluxes and its involvement in the metabolism of calcium [Al-Ghamdi *et al*., 1994].

Biologically, iron is ubiquitously the most important transition element in all living organisms. It is an essential elemental species required nutritionally in small amounts as human

body has many metabolic enzymes and electron transfer proteins with iron at the active sites (Fe-S clusters and heme proteins) in addition to oxygen carrier proteins, *viz.*, as hemoglobin, the oxygen carrier in the blood of mammals along with myoglobin for oxygen storage, for iron scavenging and storage (ferritin and transferrin) [Lee, 2006]. It is involved in several important life processes and it is an essential component of hundreds of proteins and enzymes [Wood and Ronnenberg, 2006; Lee, 2006]. Functions of iron include the involvement in the energy metabolism, gene regulation, cell growth and differentiation, oxygen binding and transport, muscle oxygen use and transport [Provan, 1999; Beard, 2001]. However, it is toxic in large quantities; it is also known to catalyze highly reactive hydroxyl radical formation from superoxide ion and hydrogen peroxide by the two-step Fenton reaction, when present in free form as Fe^{2+} species [Halliwell and Gutterridge, 2006]. As a result, inhaled iron species contributes to the free radical-induced lung injury [Iwuoha *et al*., 2013]. Smoking causes inadequate oxygenation of hemoglobin in blood circulation leading to tissue hypoxia [El-Zayadi, 2006]. Chronic oxidative stress may modulate iron uptake and storage leading to a self sustained and ever-increasing spiral of cytotoxic and mutagenic events [Emrit *et al*., 2001].

Metal precipitation is primarily dependent upon two factors, *i.e.*, the concentration of metals and the pH of the solution. As the pH of the solution increases, the level of precipitation of these two alkaline earth metals also increases. Calcium in the form of calcium carbonate ($CaCO_3$) started to precipitate around pH 7.5. Magnesium in the form of magnesium hydroxide ($Mg(OH)_2$) started to precipitates around pH 9. It has recently been suggested that (Xinchao *et al*., 2005) hydroxides of ferrous ions precipitate at pH > 8.5 or iron can be effectively removed by adjusting the pH between 9 and 11.

Table 3.3: ICP- AES analysis of essential elemental concentrations (µg/ml) of tuibur.

Place of Sample collection	**Amount of Tobacco** (kg/l)	**Ca** (µg/ml)	**Fe** (µg/ml)	**Mg** (µg/ml)
Champhai	0.3125	13.1	0	307
Khawzawl	0.4	10.9	0.5	113
Lunglei Site1	0.375	9.03	0	69
Saiha	0.5	10.4	0	223
Lunglei Site2	0.375	10.3	0	103
Tawipui Site1A	0.375	12.4	0	127
Tawipui Site1B	0.4	10.8	0	138
Aizawl Site1A	0.8	11.6	0	195
Aizawl Site1B	0.4	13.1	0	172
Aizawl Site2A	0.357	11.5	0	206
Aizawl Site2B	0.4166	11.9	0	208
Aizawl Site2C	0.625	14	0	187
Kolasib Site1A	0.357	14	0	195
Kolasib Site1B	0.4166	13.7	0.14	180

3.3.2. ICP-MS Analysis

With the help of ICP-MS, the concentration of trace metals *viz*., Cr, Mn, Ni, Cu, Zn, As, Cd and Pb in different tuibur samples were analyzed. The concentration and the maximum permissible contamination level of these trace metals were compares with the reference material *i.e.,* US EPA, 2012, as shown in table 3.4. Table 3.5 shows that these eight elements were found mostly in the range of 1.8 –297.8 ng/ml.

Table 3.4: Permissible level of elemental concentrations

(US EPA, 2012).

Elements	Permissible Level (ng/ml)
Cr	100
Mn	50
Ni	100
Cu	1000
Zn	5000
AS	10
Cd	5
Pb	15

There was no significant difference in Cd contents of different samples; it was in the range of 14.0 – 30.0 ng/ml. The highest abundant metal species was Zinc and it was found in the range of 11.9 – 297.8 ng/ml and Pb was detected in the range of 8.6 – 228.3 ng/ml as the second most abundant element in tuibur samples. Ni, As and Cu were also found in the range of 2.8 – 84.2 ng/ml, 1.8 – 80.3 ng/ml and 2.5 – 77.5 ng/ml, respectively. Cr and Mn were found in the range of 11.0 – 91.3 ng/ml, and 5.6 – 36.6 ng/ml, respectively.

Table 3.5: ICP- MS analysis of elemental composition (ng/ml) in different tuibur samples.

Place of Sample Collection	**Amount of Tobacco** (Kg/L)	**Cr** (ng/ml)	**Mn** (ng/ml)	**Ni** (ng/ml)	**Cu** (ng/ml)	**Zn** (ng/ml)	**As** (ng/ml)	**Cd** (ng/ml)	**Pb** (ng/ml)
Champhai	0.3125	33.4	12.7	27.2	37.5	297.8	30.2	16	228.3
Khawzawl	0.4	67.3	36.6	84.2	19.5	183.6	23.5	22	144.2
Lunglei Site1	0.375	11	5.6	2.8	2.5	11.9	1.8	17	18.4
Saiha	0.5	13.3	10.8	25.6	15.6	208.3	53.8	17	18.6
Lunglei Site2	0.375	30.6	20.3	41.4	7.6	97.7	42.6	14	77.4
Tawipui Site1A	0.375	21.5	16.8	24.1	77.5	257	49	17	82.5
Tawipui Site1B	0.4	29.4	18	38.1	51.9	179.3	80.3	20	36
Aizawl Site1A	0.8	72.4	14.1	41	56.8	138.3	35.9	16	29.1
Aizawl Site1B	0.4	70.5	15.7	36.9	23.5	185.8	46.2	21	25.4
Aizawl Site2A	0.357	61.5	17.5	16.9	6.7	159.3	27.3	19	21.2
Aizawl Site2B	0.4166	52	15.5	17.4	6.9	197.4	30.7	21	40.2
Aizawl Site2C	0.625	91.3	19.5	21.7	147.9	231.7	78.9	27	24.4
Kolasib Site1A	0.357	15.2	9.5	7.8	30.4	65.4	38.7	28	9.1
Kolasib Site1B	0.4166	27.4	11.5	10.5	22.4	63.6	62.4	30	8.6

Variation in heavy metal content in tuibur suggests diverse potential origins for the heavy metal species of tobacco stalk including industrial pollution in growing region (Magway Division of Myanmar) and possibly rampant application of fertilizers [Pappas *et al*., 2006]. Though, it is expected that most of the toxic metal ions would be precipitated out due to the alkaline pH of tuibur solution, yet trace amount of heavy metals still retained in tuibur. It should be noted that as the pH level increases, the heavy metal concentrations tends to decrease. ICP-MS analysis of tuibur indicated a wide distribution of concentrations of metals in tuibur depending on the amount of tobacco consumed during the manufacture of tuibur.

Heavy metals, when present in significant concentrations, may elicit detrimental health effects. Cadmium, one of the heavy metals for instance, extremely toxic, [WHO, 1977, 1989] is being considered as one of the adverse health effects of smoking. Tobacco plants have an ability to absorb cadmium from soil and accumulate it in unusually high concentrations. Thus smoking tobacco is one of the important source of exposure [WHO, 2010], and a large portion of cadmium contained in tobacco passes into smoke thus posing a risk to passive smokers as well [Chiba and Masironi, 1992]. If cadmium is ingested, it is mainly stored in the bone, liver and kidneys [Kjellström, 1979; Nigaru, 1981]. In these organs, cadmium is bound to a small transport protein (chaperones) called metallothionein [Sanchez *et al*., 1995] rendering malfunction of the kidneys, while Cd replaces Zn in some enzymes, thus preventing them from functioning [Lee, 2006]. Furthermore, kidney being the main critical target for cadmium toxicity, its accumulation may lead to renal tubular dysfunction, the formation of kidney stones as well as disturbances in calcium metabolism [WHO, 2010]. Softening of the bones and induction of osteoporosis may occur in those exposed through living or working in cadmium-contaminated areas [WHO, 2010]. The most notorious toxicological property of cadmium is its exceptionally long half-life in the

human body [Tribowo *et al*., 2014]. It has been further suggested that cadmium may also accentuate ROS production [Manca *et al*., 1994; Bagchi *et al*., 1997; Liu and Jan, 2000] which in turn causes elevation of lipid peroxidation, ultimately results in DNA damage [Liu *et al*., 2009].

There is sufficient evidence that long-term occupational exposure to cadmium contributes to the development of various types of cancer. Therefore, the international agency for research on cancer (IARC) has classified cadmium as Group 1 carcinogens to humans [IARC, 2004]. Table 3.5 depicts the concentration of cadmium contents of different tuibur samples, while it is important to note that as per the specifications of US EPA, the maximum permissible contaminant level for cadmium in drinking water is 5 ng/ml. The form of cadmium encountered depends on the solution and soil chemistry. The most common forms of cadmium include Cd^{2+}, cadmium-cyanide complexes, or $Cd(OH)_2$ solid sludge [Smith *et al*., 1995]. Hydroxide ($Cd(OH)_2$) and carbonate ($CdCO_3$) solids dominate at high pH whereas Cd^{2+} and aqueous sulphate species are the dominant forms of cadmium at lower pH (< 8). Under reducing conditions when sulphur is present, the stable solid CdS(s) is formed. Cadmium will also precipitate in the presence of phosphate, arsenate, chromate and other anions, albeit the solubility will vary with pH and other chemical factors. Precipitation of cadmium as hydroxide occurs at pH ranges from 8 (solubility: 1 ppm) to 11 (solubility: 0.05 ppm), alternatively it can be said that at pH = 9 will be cadmium successfully removed.

Zinc, the highest abundant metal species in tuibur, was found in the range of 11.9 – 297.8 ng/ml. Table 3.5 showed the concentration of zinc contents of different tuibur samples. It is one of the most mobile heavy metals in surface waters and groundwater because it is present as

soluble compounds at neutral and acidic pH values. It is amphoteric in nature at neutral pH thus it is unable to undergo reduction or oxidation [IZiNCG, 2004; Sensi *et al*., 2009; King, 2011].

Unlike other metals, zinc is virtually non-toxic owing to a tight cellular homeostatic control of entry into, distribution in and excretion from cells; physico-chemical properties of zinc permit stable association with macromolecules through coordination flexibility. It always prefers tetrahedral complex formation and readily complexes with amino acids, peptides, proteins and nucleotides. Zinc, being a soft acid, also has an affinity for thiols, ligands with electron-rich nitrogen donors and hydroxyl groups. Thus, zinc participates extensively in the metabolism of protein, nucleic acid, carbohydrate and lipid [Nair and Choudhury, 2013].

Zinc serves as an essential catalytic and structural cofactor for many enzymes as well as other proteins, while cell differentiation, proliferation and gene expression are also regulated by zinc [Eide, 2011]. In addition, zinc facilitates the folding of proteins into three-dimensional configurations, for their biological activity. This involves chelation of zinc with the thiol sulphur of cysteine and the imino nitrogen of histidine to form a zinc "finger" in zinc finger proteins. Zinc performs its regulatory functions through zinc finger motif which is a protein and is known to bind DNA, RNA or protein [Nair and Choudhury, 2013]. It has been known for many years that zinc deficiency causes increased oxidative stress and, consequently, increased oxidative damage to DNA, proteins, and lipids [Eide, 2011].

Zinc eventually becomes toxic due to the excessive intake that is likely to be involved in the induction of pathological conditions that have been associated with oxidative stress [Ajab *et al*., 2008]. However, there is evidence that under special circumstances, like pregnancy, mononuclear cells and may be other cell types have a reduced Zn content [Chiba and Masironi,

1992]. Cadmium inhaled from smoking attenuates zinc levels, which induces the expression of metallothioneins, a transport protein rich with cysteine residues which binds zinc, thus reducing its availability for intestinal absorption and placental transfer [Bernhard *et al*., 2005; Kuhnert *et al*., 1988]. This type of peculiar cadmium-zinc interaction may occur in pregnant smoking women at the maternal-placental-fetal unit level, thus resulting in a less favourable zinc status in the neonate [Kuhnert *et al*., 1988]. Reduced zinc levels have been demonstrated to constitute a pro-carcinogenic factor [Prasad and Kucuk, 2002] as well as affecting immune system function [Rink and Kirchner, 2000].

At higher pH values, zinc can form carbonate and hydroxide complexes which control zinc solubility. Zinc readily precipitates under reducing conditions as well as in highly polluted systems when it is present at very high concentrations, and may co-precipitate with hydrous oxides of iron or manganese while, sorption of zinc increases as pH increases and salinity decreases whereas precipitation of zinc often started to occur at a pH of 7 or above [Smith *et al*., 1995].

Lead, the second most abundant element in tuibur samples, was detected in the range of 8.6 – 228.3 ng/ml. The international agency for research on cancer (IARC) has classified lead as Group 2A carcinogen to humans [IARC, 2004] which indicates there is sufficient evidence in animals to prove that lead is carcinogenic, albeit there is limited evidence in humans. Table 3.5 showed the concentration levels of lead contents in different tuibur samples, while the maximum permissible contaminant level for lead is only 15 ng/ml according to the US EPA. It is also important to note that for tuibur samples collected at Champhai and Khawzawl, the observed contaminant level of lead is exceptionally high.

Similarly like cadmium, lead is presumably highly toxic metal [WHO, 1977, 1989], even in smaller concentrations and they do not have biological function in humans and plants, yet it has been found to be an accumulative metabolic poison (bioaccumulation) with adverse physiological and neurological effects [Petrucelli *et al*., 2007; Andrade *et al*., 2009; Regassa, 2007]. They are also capable of causing serious effects on the brain, kidneys, nervous system and red blood cells [Pourkhabbaz and Pourkhabbaz, 2011; Kazi *et al*., 2009; WHO, 1977 and 1989]. The mechanism of lead-induced oxidative stress involves an oxidative imbalance imposed by ROS in tissues and cellular components causing damage to membranes, DNA and proteins [Patra *et al*., 2011].

Furthermore, lead plays a significant role in tobacco toxicity; it contributes to lung cancer through the release of radiation from Pb-210, with possible minor contributions to the development of some other types of cancers. It plays a direct role in cardiovascular problem, possibly increasing lead deposition during bone formation associated with smoking cigarettes. Lead toxicity has also been reported to cause anemia, headache, irritability, and renal damage [Goyer, 1993]. It is more hazardous for the younger ones, as its chronic exposure plays role in the lowering of intelligence quotient (IQ) levels and it has been also implicated in the impaired foetal growth and brain development among infants [Goyer, 1993; Neuspiel *et al*., 1994]. Consumption of tuibur, women can more easily avoid during nursing, may be leading to newborn as well as infants exposed to lead toxicity risks from breast milk ingestion.

The primary industrial sources of lead contamination include ore processing and smelting, secondary metals production/recovery, lead battery manufacturing, pigment and chemical manufacturing, and lead-contaminated e-wastes. Widespread contamination due to the

former use of lead (oxygenate) in gasoline is also of grave concern. Lead released to groundwater, surface water and land is usually in the form of elemental lead, lead oxides and hydroxides, and lead-metal oxyanion complexes [Smith *et al*., 1995].

Lead occurs most commonly with an oxidation state of 0 or 2^+ with the ionic species Pb^{2+} is the more common and reactive form of lead [Smith *et al*., 1995]. Most lead that is released to the environment is retained in the soil [Evans, 1989]. Precipitation of lead as hydroxide (lime) occurs at pH 11.5 with the effluent concentration ranging from 0.02 to 0.2 ppm. Precipitation of lead as sulfide occurs between pH 7.5 to 8.5. Precipitation of lead as carbonate occurs between pH 7.5 and 8.5 with the effluent concentration which is comparable to that obtained through hydroxide precipitation at high pH. Lead can be removed efficiently if the pH is raised significantly above the acid breakpoint *i.e*., a pH of 9 will facilitate the successful removal of lead.

Nickel was found in the range of 2.8 – 84.2 ng/ml in tuibur samples. Table 3.5 showed the concentration of nickel contents of different tuibur samples and according to the US EPA, the maximum permissible contaminant level for nickel is 100 ng/ml. Tobacco plants have the ability to absorb nickel from the soil and sequester it in the leaves [Bache *et al*., 1985]. Nickel has been found to be responsible for quite a number of ailments, a number of different forms of cancer, especially of the respiratory tract including dermal, lung, and nasal sinus cancers [Bernhard *et al*., 2005; Awofolu *et al*., 2005]. The main mechanism responsible for this activity is that nickel is mutagenic [Werfel *et al*., 1998] and has been reported to induce sister chromatid exchanges [Bernhard *et al*., 2005].

Nickel is usually present in high concentrations in the liquid wastes which are released directly into the environment without any pre-treatment [Olayinka *et al*., 2009]. It is one of the stable and persistent environmental contaminants since it cannot be biologically or chemically degraded or destroyed unlike many other organic toxic pollutants. Therefore, the metal has become a serious worldwide environmental problem, albeit nickel is a trace element required for living organisms, it is toxic when ingested in large amounts. Nickel can form a toxic carbonyl compound and because of the high carbon monoxide level in tobacco smoke, nickel carbonyl thus produced is considered to be a potential carcinogen [Sunderman, 1961; Chiba and Masironi, 1992]. Epidemiological and experimental studies of nickel related cancer have implicated that nickel compounds are also well recognized as carcinogens [Shakya, 2007]. Moreover, the international agency for research on cancer (IARC) has recently defined nickel as a Group I carcinogenic to human [IARC, 2004].

Furthermore, nickel causes increased level of endogenous cellular hydrogen peroxide and its associated short lived and hyper-reactive oxygen species [Lynn *et al*., 1997; Das *et al*., 2001]. Nuclear protein damage caused by nickel reduces the enzyme activity needed for DNA replication, transcription, recombination and repair [Das *et al*., 2008]. In general, precipitation of nickel as hydroxide ($Ni(OH)_2$) takes place at pH ranging from 10 to 11 (solubility: 0.12 ppm).

Arsenic was also found in the range of 1.8 – 80.3 ng/ml. Table 3.5 represents the concentration of arsenic levels of different tuibur samples; according to US EPA, the maximum permissible contaminant level for arsenic is 10 ng/ml. The international agency for research on cancer (IARC) has classified arsenic as Group 1 carcinogens to humans [IARC, 2004]. Arsenic always has been one of the most concerns for both the environmental and human health because

it is very toxic even at trace levels as its toxicity causes skin cancer, mouth ulceration, low hemoglobin content, leukemia, acute renal failure and neuronal damage [Byrd *et al*., 1996]. Epidemiological evidence has implicated that smoking and occupational exposure to arsenic act synergistically to increase the incidence of lung cancer in smelter workers [Xu *et al*., 1989; LaPaglia *et al*., 1996; Chen *et al*., 2004].

Arsenic is said to exert its toxicity through oxidative stress by generating reactive oxygen species [Sharma *et al*., 2006]. It has been further demonstrated that reactive oxygen species are directly involved in oxidative damage to lipids, proteins and DNA in cells exposed to arsenic, which can ultimately lead to cell death [Boulikas, 1991]. The attack of mitochondrial enzymes by arsenic compounds which results in impaired tissue respiration can be related to arsenic cellular toxicity. Similarly, its reaction with thiol groups (-SH) especially the enzymes or cofactors which possess two thiols (example, dihydrolipoic acid), resulting in the alteration of various enzymes including those related to tissue respiration is yet another mechanism of its toxicity [Obinaju, 2009].

Arsenic exhibits fairly complex chemistry as it exists in inorganic and organic forms, while it can be present in several oxidation states (3^-, 0, 3^+, 5^+) [Hughes, 2002; Smith *et al*., 1995]. Toxicologists are primarily concerned with arsenic in the trivalent (arsenite) and pentavalent (arsenate) oxidation state with respect to the environmental exposure [Hughes, 2002]. Both oxidation states are considered toxic, however, arsenite is the more toxic form and arsenate is the most common form. The solubility of inorganic arsenic compounds vary depending on the compound and the pH of the water [US ATSDR, 2005; US EPA, 2000]. Inorganic arsenic has been shown to readily migrate through soil to groundwater [US ATSDR,

2005; WHO, 1981], and arsenite, being charged, does not migrate as readily [US ATSDR, 2005]. Inorganic arsenic has been shown to persist in soil over 45 years [Wolz *et al*., 2003]. Alkaline pH *i.e*., pH above 7 can remove to a significant extent, but not all of arsenic contents.

Copper was found in the range of 2.5 – 77.5 ng/ml. Table 3.5 shows the concentration of copper levels of different tuibur samples; according to the US EPA, the maximum permissible contaminant level for copper is 1000 ng/ml. Copper is essential to the life process and adult human contains about 100 mg. About 4-5 mg of copper is required daily in the diet, and the deficiency of copper results in the inability to absorb iron and the subsequent storage in the liver. Like other trace elements, such as zinc, copper also possess an underlying role for the function of different enzymes and other cellular proteins with copper ion is bound to proteins in the body either as metalloproteins in electron transport or as enzymes.

However, too much intracellular accumulation may results in toxicity. Copper becomes toxic due to excessive intracellular accumulation and it plays a role in initiating the generation of reactive oxygen species and apoptotic processes [Santon *et al*., 2004]. Copper poisoning in humans, particularly by chewing the tobacco formulation called gutka is a major source of fibrosis in mouth cavities [Trivedy *et al*., 2001]. The cupric ion (Cu^{2+}) is the most toxic species of copper [LaGrega *et al*., 1994]. Solution and soil chemistry strongly influence the speciation of copper in ground-water systems. The lowest possible dissolved concentration of Cu is approximately 0.001 mg/l which occurs at pH value of 8.1 [Ayres *et al*., 1994]. Precipitation of copper often started to occur at a pH of 7, yet it may not remove all of copper contents.

Chromium was found in the range of 11.0–91.3 ng/ml, Table 3.5 presents the concentration levels of chromium in different tuibur samples. According to US EPA, the maximum permissible contaminant level for chromium is 100 ng/ml. Chromium in its trivalent form as Cr^{3+} is an essential elemental species [Mertz and Reginsky, 1987] as it mimics insulin like effects in modulating carbohydrate levels of the body. In recent years, contamination by another form of chromium, *i.e*., hexavalent chromium (Cr^{6+}) has become a major concern as Cr^{6+} species is highly toxic with significant carcinogenic effects, it is recognized by International Agency for Research on Cancer (IARC) as a group I carcinogen [Bernhard *et al*., 2005], and its elevated level in human may cause death [Zayed and Terry, 2003]. Ingestion of chromium (VI) compounds exacerbates oxidative stress as the generation of mostly hydroxyl radicals ($OH^{\bullet}$) induce DNA-damage (single strand breaks) [Liu *et al*., 1999] and have potential cell altering effects [Petrilli and De Flora., 1982]. Other, non cancerous adverse effects of chromium on the respiratory tract include ulceration, chronic rhinitis and pharyngitis, impaired lung function and emphysema [ICDA, 1997]. Precipitation of chromium often occurs at a pH of 7 or a pH of 9 will successfully remove chromium.

Manganese was found in the range of 5.6–36.6 ng/ml. Table 3.5 depicts the concentration levels of manganese of different tuibur samples. Although manganese is required nutritionally at lower concentrations as manganese containing superoxide reductase, MnSOD or SOD2 is an essential antioxidant enzyme in the mitochondrial matrix, while it is also a potent neurotoxin at high concentrations. High concentrations of manganese cause psychiatric syndromes like hallucinations, emotional liability, and other Manganese Madness [Barceloux, 1999]. According to US EPA, the maximum permissible contaminant level for manganese is 50 ng/ml. Precipitation of manganese depends on the oxidation number of cation, but usually takes place at

pH from 9.0 to 9.5. Sometimes the pH 10.5 is necessary for complete removal of manganese [Sheremata and Kuyucak, 1996].

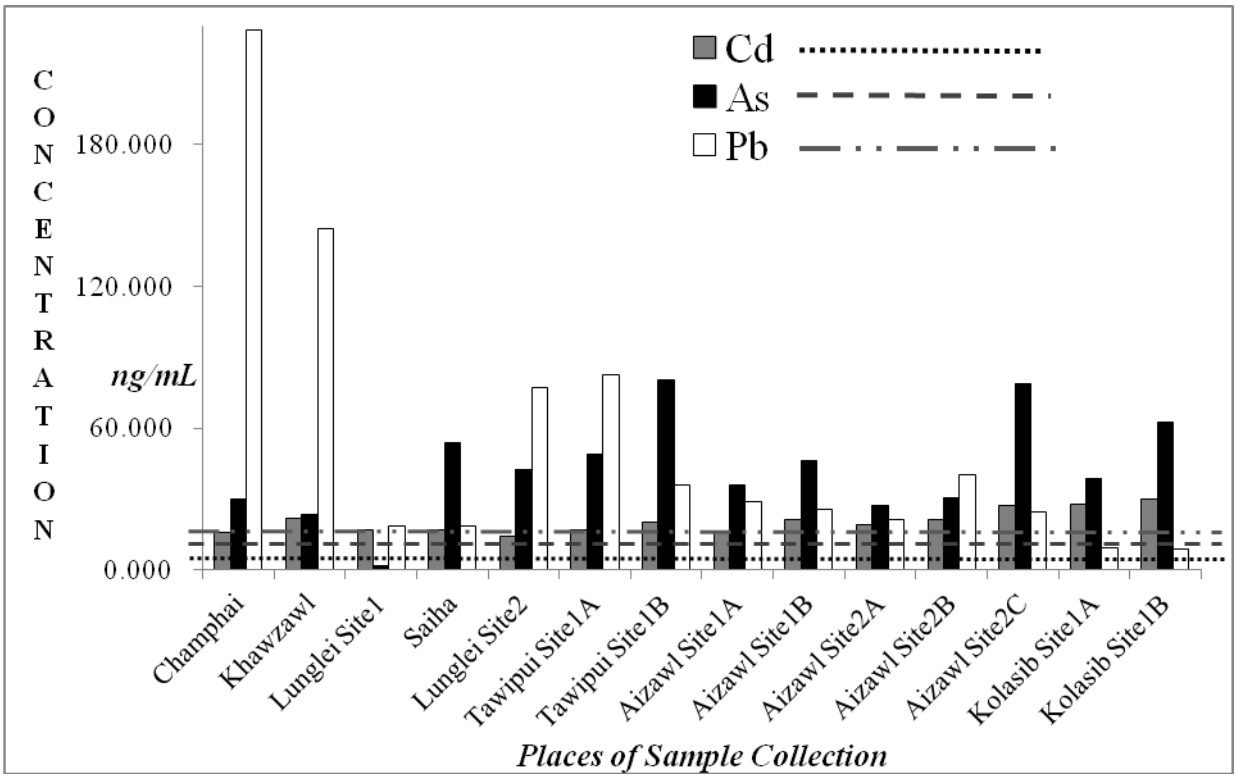


Figure 3.5: Distribution of metallic elements (As, Cd and Pb) in tuibur solution collected at multiple sites.

The concentration of Cr, Mn, Ni, Cu, and Zn were below the USEPA permissible limits (Table 3.4) [USEPA, 2012]. However, higher concentration of As, Cd and Pb, above the USEPA permissible levels (Table 3.4) [USEPA, 2012], was detected in tuibur solutions analyzed (Figure 3.5). Thus, our findings reflecting the distribution of various heavy metal species in tuibur samples suggest that tuibur consumption may be one of the major sources of abnormal levels of heavy metal uptake by the population in Mizoram and Manipur in India.

3.3.3. Statistical Analysis

The correlation coefficients were applied to measure the significance level of relationship among the variables, *i.e.*, the concentration of metallic species in terms of tobacco (kg) used per litre, for the manufacture of tuibur. Table 3.6 shows that the coefficient values for Cr (0.52), Cu (0.50), As (0.26), Ni (0.14), were positively correlated, while the concentration of Zn (0.03), Mn (0.006), and Cd (0.003) were insignificantly correlated to the amount of tobacco used and it is negatively correlated for Pb (–0.32)

Table 3.6: Correlation matrix for the concentration of heavy metal species in terms of tobacco (kg) used per litre, for the manufacture of tuibur.

	Amount of Tobacco (Kg/L)	***Cr*** (ng/ml)	***Mn*** (ng/ml)	***Ni*** (ng/ml)	***Cu*** (ng/ml)	***Zn*** (ng/ml)	***As*** (ng/ml)	***Cd*** (ng/ml)	***Pb*** (ng/ml)
Amount of tobacco (kg/l)	1								
Cr (ng/ml)	0.519	1							
Mn (ng/ml)	0.006	0.535	1						
Ni (ng/ml)	0.139	0.421	0.875	1					
Cu (ng/ml)	0.499	0.437	0.131	0.002	1				
Zn (ng/ml)	0.036	0.332	0.329	0.308	0.442	1			
As (ng/ml)	0.261	0.145	0.075	0.006	0.623	0.297	1		
Cd (ng/ml)	0.003	0.164	0.012	-0.228	0.272	-0.235	0.392	1	
Pb (ng/ml)	-0.323	0.016	0.399	0.473	-0.010	0.563	-0.254	-0.368	1

From the above data (Table 3.6), it can be seen that the variables having positive value indicates positive correlation and those negatives value also indicates negative correlation. Since the significance correlation values are less than 70 per cent (70%), the significance value of

relationship are not strong. As such it can be concluded that there is a positive correlation among the variables like, amount of tobacco with Cr, Mn, Ni, Cu, Zn, As and Cd that means if one variable increase the others also increase, albeit the rate of increase will not be the same since the correlation does not measure the rate of changes. It can also be seen that Pb has negative correlation with the amount of tobacco, Cu, As, and Cd. Cd also has negative correlation with Ni and Zn. It may likely to be possible that those with negative value like Pb, the rate of increase or decrease does not affect other variable like amount of tobacco, Cu, As and Cd. Likewise, the rate of increase or decrease for Cd does not affect other variables like Ni and Zn.

The present study has few limitations as we have only estimated the potential heavy metal content of the generated tobacco smoke as well as feedstock using tuibur solution, primarily to provide a sense of scale, while we did not measure concentrations of metal ions in tobacco stalk or the tobacco ash directly. Furthermore, the consumer expects a 'smooth taste' with 'mellowed texture' for tuibur, tuibur is mostly prepared from the tobacco stalk (stem, petiole and vein) rather than the tobacco leaves that are utilized for the manufacture of many smoke and smokeless products of tobacco (in Myanmar and China). This results in the difficulty of tracking the precise 'origin' of tobacco stalk as it is obtained, mostly, from *the Pokokku region of Magway Division of Myanmar*. Furthermore, the study identified only trace amount of heavy metal ions dissolved in alkaline tuibur solution implying that people, both in Myanmar and China, are consuming various tobacco products with significantly higher levels of heavy metal content. Secondly, the extent of distribution of heavy metals being sequestered in various parts of plant is unknown as that would shed light on the concentration of metal species in tobacco smoke, which is currently under investigation along with the determination of nicotine content in tuibur.

It is interesting to note that the analysis of tobacco ash showed that 65-75% of the mass of the total metal content of tobacco (leaves) was retained in cigarette ash [Ajab *et al*., 2014]. Ashes are, presumably, also the potential source of contaminants requiring their 'safe' disposal in the environment with appropriate precautions. Thus, tobacco smoke and ash could be significant contributor to the metal load in the soil, air, and water systems in addition to the adverse human health effects via direct tobacco consumption [Verma *et al*., 2010]. The stream water being utilised for the preparation of tuibur (water being used for the preparation of feedstock) is yet to be explored thoroughly as it may be a minor contributing factor to the metal content of the tuibur solution. These variables still need to be evaluated in order to ascertain the source of toxic metal species present in tuibur.

3.4. MS analysis

Mass spectrometry, due to its versatility, is ideally suited for both the qualitative and quantitative applications - identifying unknown compounds using its precise molecular weight, evaluating the isotopic composition of elements in a molecule, and determining the potential molecular structure of a compound by observing its fragmentation.

While, in a preliminary investigation, LC-MS (low resolution) with APCI ionization method (low energy ionization leading to the formation of charged molecular ions) for the alkaline tuibur solution has showed that the prominent base ion (the most intense peak) as well as molecular ion peak belongs to Nicotine with an (m/z) = 163.2, while other peaks which are too clustered and molecular mass of those cluster molecular species or potentially fragmented daughter ions could not be ascertained (data is not shown).

3.4.1. High Resolution Mass Spectrometry (HRMS)

The commercially available alkaline tuibur solution was then subjected to LC-HRMS study with ESI method as the ionization method. In the "hard" ionization such as EI (electron impact) method, where high-energy electron (70 eV) is used for the ionisation of molecules in the analyte leading to more fragmentation of molecular ions while the molecular weight of molecular ions are hardly observed. While, ESI and APCI methods are considered as relatively "soft" methods, since the fragmentation is observed to a much lesser extent and significant proportions of molecular ions are generally observed. Moreover, in ESI method, most of the time, $[M-H]^+$ species, but not always, are observed. In the present study, similar to APCI method, by applying ESI mode, the most prominent base ion peak (the most intense peak) as well as the molecular ion peak at 163.12 corresponds to nicotine was observed (Figure 3.7). Nicotine is the main constituent of all types of tobacco products and tobacco smoke. It is addictive, thus it activates the brain's reward system, eliciting cravings for continued tobacco consumption [Xue *et al*., 2014] besides it is also capable of activating various signaling pathways related to tumour initiation and promotion [Xue *et al*., 2014; Warren and Singh, 2013].

In addition, for tuibur sample, 23 different compounds have also been identified, as illustrated in Figure 3.6 along with their structure and m/z values. Tobacco-specific nitrosamines, NNN (N-nitrosonornicotine), NNK (4-(methylnitrosamino)-1-(3'-pyridyl)-1-butanone) and NNAL (4-(methylnitrosamino)-4-(3-pyridyl)-butanal) have been detected in the tobaaco smoke using GC-MS methods [Hecht, 1999]. In tuibur sample also, using HRMS method, molecular ions corresponding to NNN (m/z = 178), NNK (m/z = 207) and NNAL (m/z = 210) have been detected (Figure 3.8 and 3.9). In addition to these alkaloids and alkaloids derived combustion products, there are few other ion species are present whose identification is currently underway.

The HRMS spectral data of tuibur sample exhibited a peak with m/z = 159 (Figure 3.10) indicates the presence of β-nicotyrine (Figure 3.11). It is an alkaloid metabolite of nicotine as well as a major product of thermal decomposition of nicotine [Swain *et al*., 1949; Clayton *et al*., 2010]. It is speculated that β-nicotyrine is generated during the curing process as well as the burning of tobacco. Although β-nicotyrine is very much present in the processed tobacco and tobacco smoke, yet the physiological implications, if any remain unknown. In animal tissues, β-nicotyrine is reported to possess some weak nicotine-like pharmacological activities [Clark *et al*., 1965]. No work has been done probing the physiological effects of β-nicotyrine in humans.

In addition, the observation of a molecular ion peak with an m/z = 94 value indicates the presence of phenol (Figure 3.10), while m/z = 122 corresponds to nicotinamide (Figure 3.12) in tuibur sample. A molecular ion peak with an m/z = 149 detected in the HRMS indicates the presence of nornicotine $[M+H]^+$ (See Figure 3.13). It is chemically similar to nicotine, but does not contain a methyl group. Nornicotine is a secondary tobacco alkaloid produced by the N-demethylation of nicotine (Figure 3.14). Identification of nornicotine is significant as the aerial nitrosylation of nornicotine during the combustion of tobacco leading to the generation of group I carcinogens, NNN, NNK and/or NNAL [Hecht, 1998]. Tobacco-specific nitrosamines are not usually present in freshly harvested green tobacco. They are formed during tobacco curing, processing, and storage by nitrosation of tobacco alkaloids [Hecht *et al*., 1979].

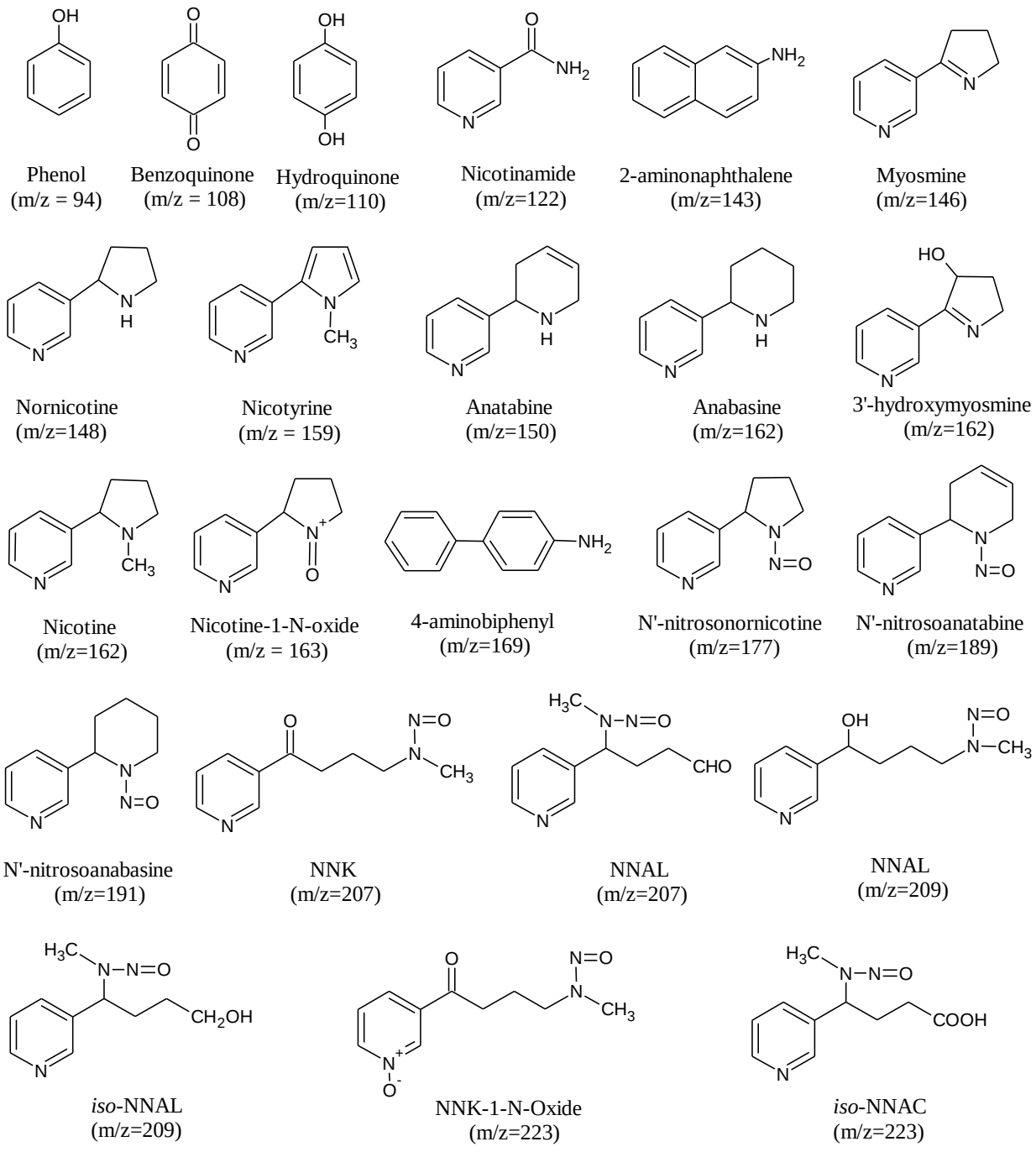


Figure 3.6: Alkaloids and alkaloids derived products present in tuibur.

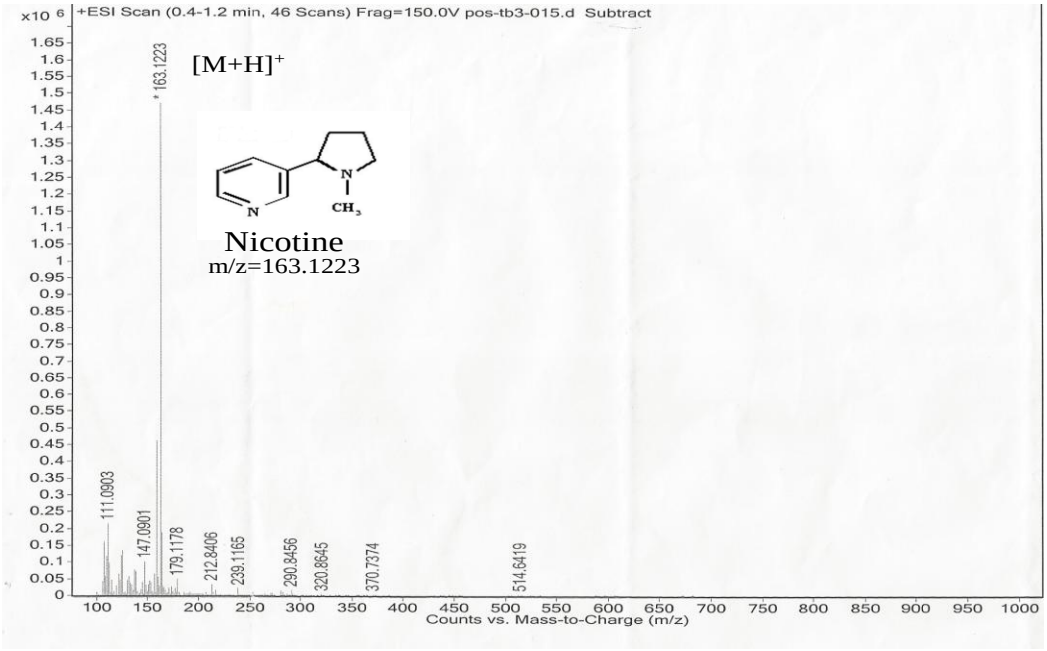


Figure 3.7: Molecular ion peak belongs to nicotine (m/z = 163.1223).

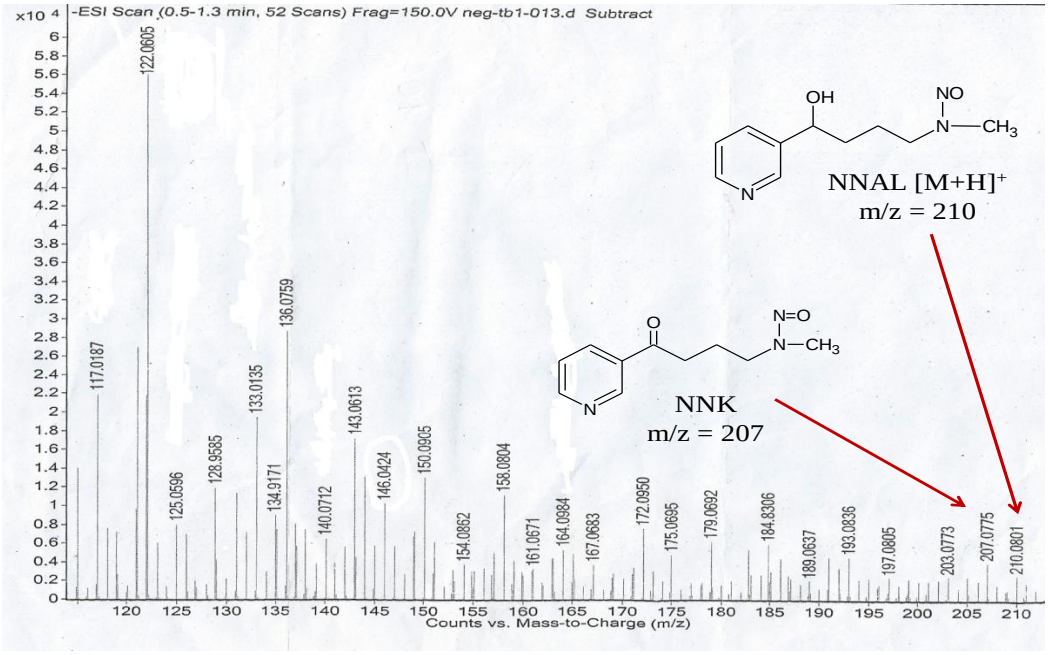


Figure 3.8: NNK (m/z = 207) and NNAL (m/z = 210).

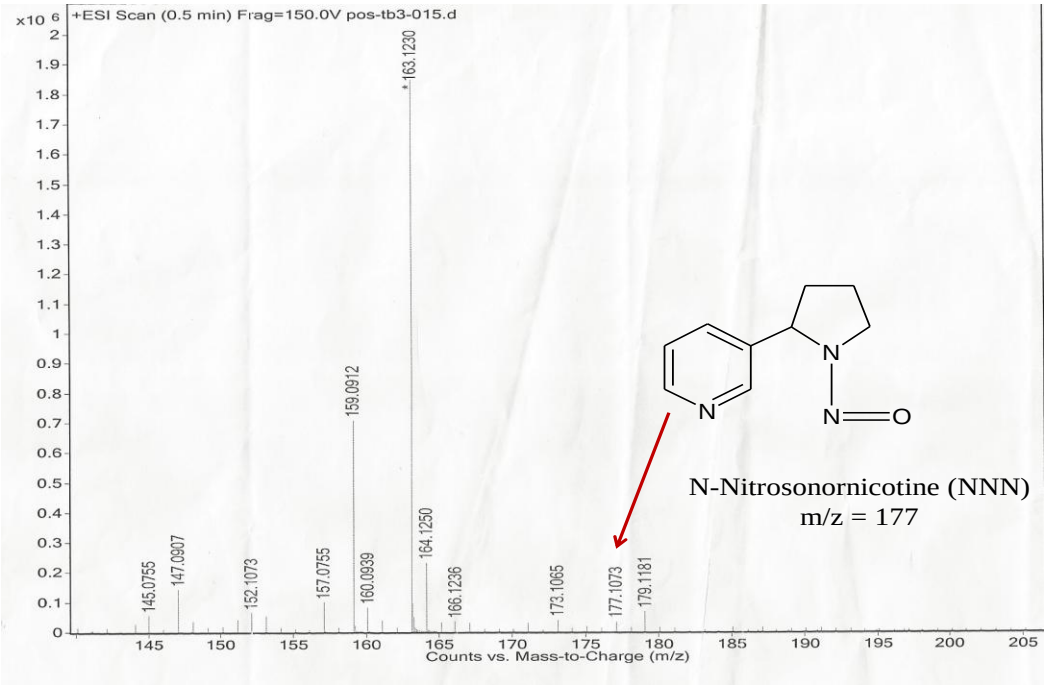


Figure 3.9: N-nitrosonornicotine (NNN) with m/z = 177.

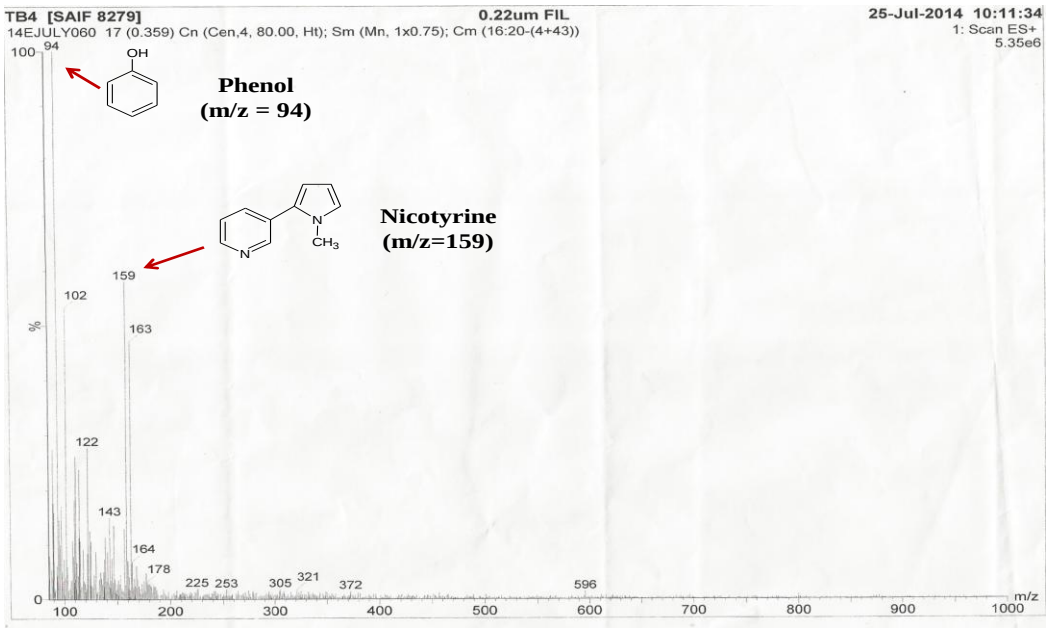


Figure 3.10: Molecular ion peak belongs to phenol (m/z = 94) and nicotyrine at m/z = 159.

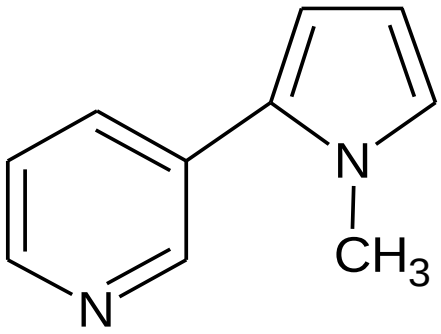


Figure 3.11: Structureof β-nicotyrine.

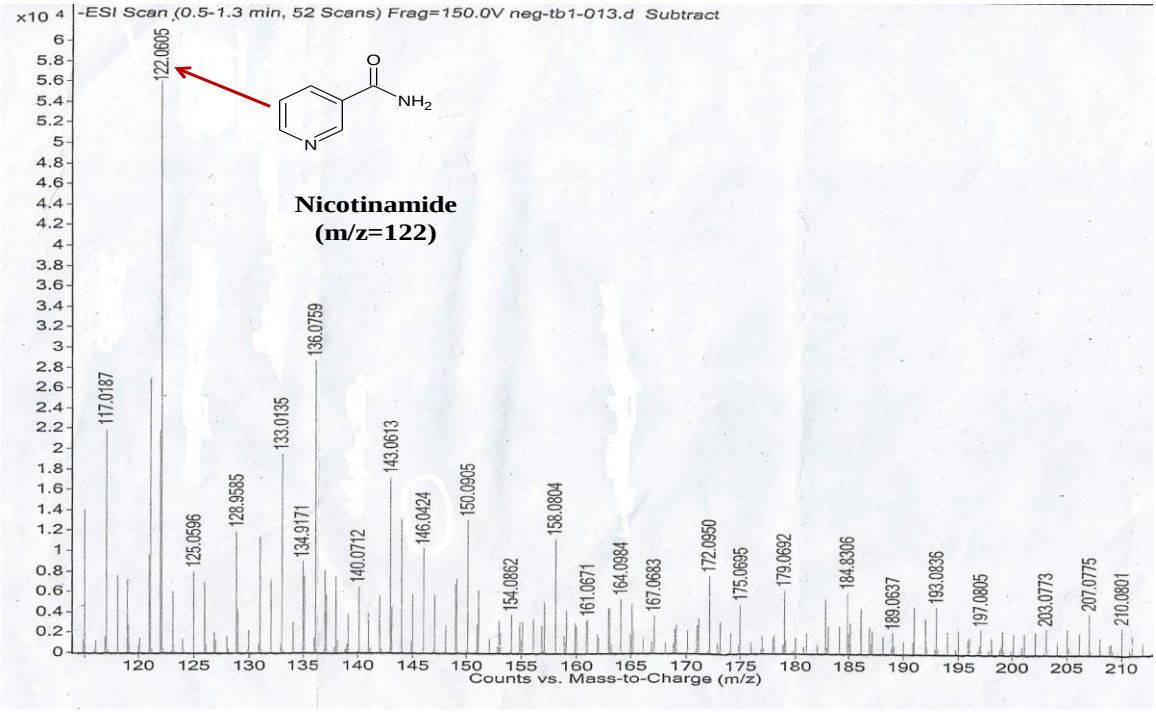


Figure 3.12: Molecular ion peak belongs to nicotinamide (m/z = 122).

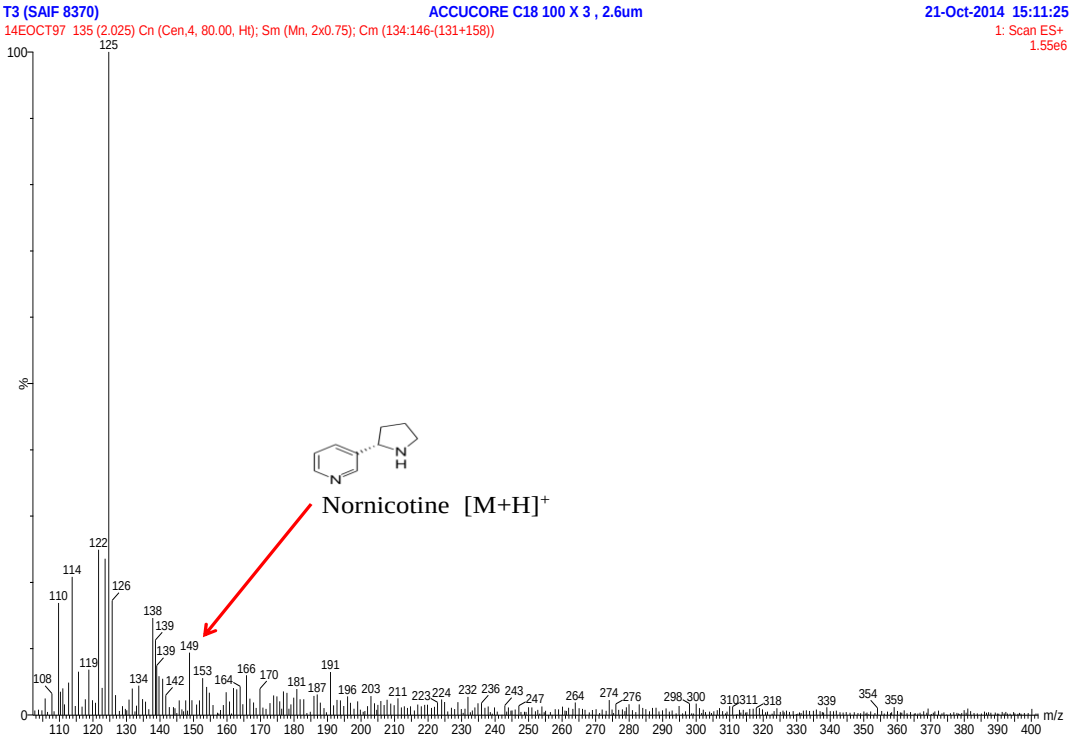


Figure 3.13: Nornicotine (m/z = 149).

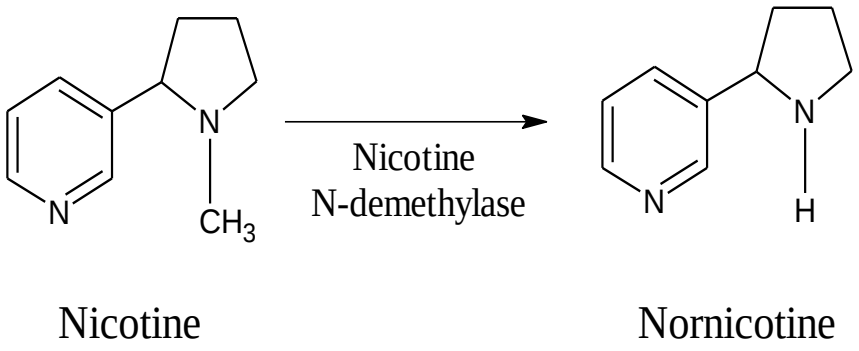


Figure 3.14: Demethylation of Nicotine to Nornicotine .

The possible pathway of metabolism of nicotine in the formation of TSNA is shown in Figure 3.15 and 3.16. Tobacco-specific N-nitrosamines occur in all commercially and non-commercially prepared tobacco products including smoke and smokeless products. N-nitrosamines occur in a wide variety of both food and non-food products, but the amount of TSNAs in all tobacco products exceed the levels of other N-nitrosamines in other commercial products by several orders of magnitude. In fact, the highest levels of TSNAs are measured in smokeless tobacco products. These compounds are also present in side-stream smoke also known as secondhand tobacco smoke. The degree of exposure to TSNAs depends not only on the levels of these compounds in tobacco products or smoke, but also on the manner in which the products are used [IARC, 2007].

Moreover, nornicotine production and accumulation in tobacco are undesirable, research reports also documents that nicotine and nornicotine serves as the precursor in the synthesis of the well characterized carcinogen N-nitrosonornicotine (NNN) and other tobacco-specific nitrosamines (TSNA). The nitrosating agent is nitrite, derived from tobacco nitrates by bacteria and enzymes during curing. TSNA levels in tobacco vary widely and are significantly correlated with the amount of nitrate present in tobacco [Fischer *et al*., 1989].When the tobacco is ignited, some of the nitrosamines transfers to smoke, some decomposes and some TSNA are also formed pyrosynthetically in the burning zone [Hoffmann *et al*., 1984].

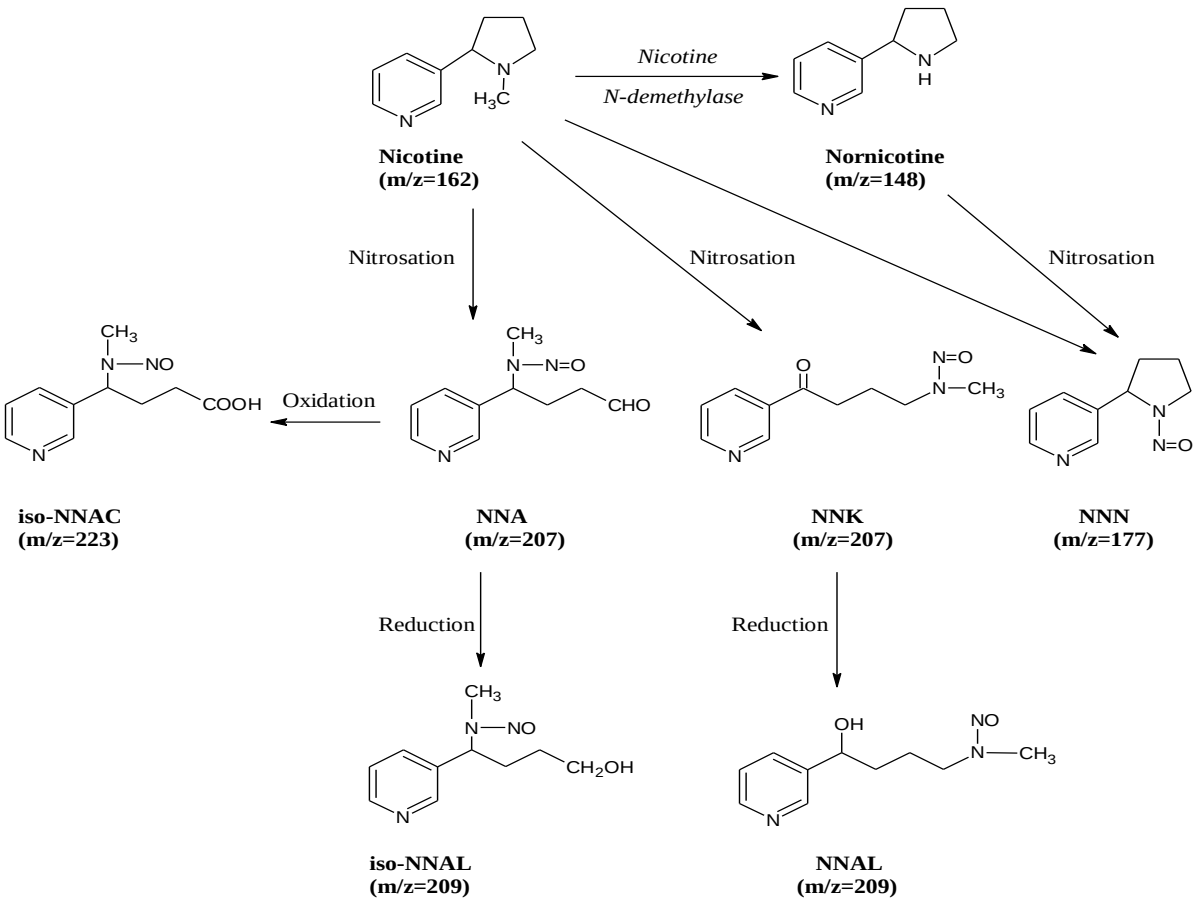


Figure 3.15: Pathway of nicotine metabolism.

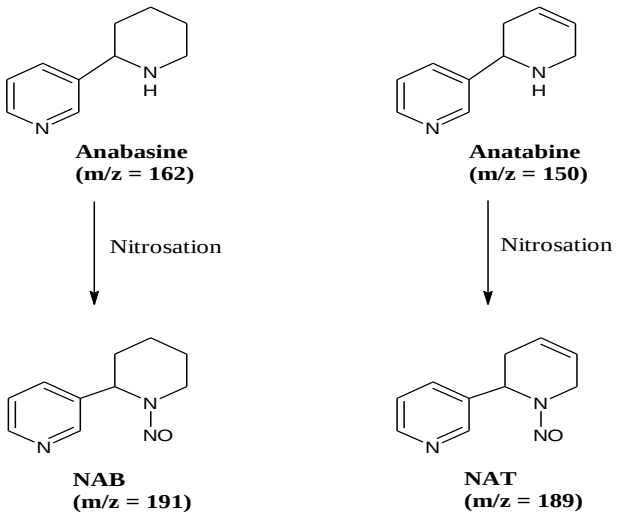


Figure 3.16: Formation of tobacco-specific nitrosamines NAB and NAT.

The primary biochemical mechanism of NNN formation is the N-nitrosation of nornicotine, an alkaloid produced through the N-demethylation of nicotine (see Figure 3.14) by the enzyme nicotine N-demethylase [Bush *et al*., 2001]. N-nitrosonornicotine (NNN) is one of the tobacco-specific nitrosamines (TSNAs), (Figure 3.15) which is mainly formed from nornicotine produced during the curing and processing of tobacco [Brunnenmenn and Hoffmann, 1991; Hecht *et al*., 1981; Wiernik *et al*., 1995]. NNN is classified as a group I carcinogen by the International Agency for Research on Cancer [IARC, 2007]. Based on its occurrence in tobacco products and its carcinogenic activity in laboratory animals, NNN is believed to play an important role in causing oesophageal cancer in smokers [Hecht and Hoffmann, 1989; Hecht, 2003] and oral cancer in smokeless tobacco consumers [IARC, 2007]. Besides, NNN induces primarily papilloma and carcinoma of the nasal cavity [Hoffmann and Hecht, 1985].

NNK is another key ingredient in various tobaccos and it plays an important role in carcinogenesis. NNK is a known mutagen as it causes a lot of polymorphisms in the human genome. Studies have indicated that NNK induced gene polymorphisms in cells that involve in cell growth, proliferation and differentiation. NNK also plays a very important role in gene silencing, modification and functional disruption which cause the early development of carcinogenesis [Akopyan and Bonavida, 2006]. Virtually all commercial tobacco products contain NNN and NNK, and they are always occurring together. But there is a great variation in levels of these compounds in smoke and smokeless tobacco products which is mainly due to differences in tobacco types used for various tobacco products, in agicultural practices, curing and processing methods, and in manufacturing processes [IARC, 2007].

As mentioned earlier, NNN and NNK are strong carcinogens, thus they provide a link between nicotine, the habituating factor in tobacco, and tobacco-related cancers [Hoffmann and Hecht, 1985]. NNN and NNK induce benign and malignant tumours in mice, rats and hamsters. NNK is the most potent carcinogen among the tobacco-specific nitrosamines. Studies showed that it induces lung tumours in mice, while causing nasal cavity, tracheal, and lung tumours in hamsters besides nasal cavity, lung, and liver tumours in rats. They induce carcinogenesis by causing DNA adducts and mutations as well as promoting tumour growth through receptor-mediated effects [Hecht, 2003; Takahashi *et al*., 2010].

Based on currently available data from studies of metabolism as well as structure-mutagenicity and structure-carcinogenicity correlation studies, α-carbon hydroxylation appears to be the major pathway of metabolic activation of NNK and NNN [Castonguay *et al*., 1983; Hecht *et al*., 1983a; Hecht *et al*., 1983b; Hecht and Young, 1982; Hecht *et al*., 1980]. Initially NNK in tobacco smoke is a procarcinogen, an inert form that requires metabolic activation to exert its carcinogenic effects [Sturla *et al*., 2005; Kiyohara *et al*., 2005; Kiyohara *et al*., 2006; Hecht *et al*., 2004]. The activation of NNK is done by enzymes of the cytochrome pigment (CYP) multigene family. These enzymes catalyze hydroxylation reactions (cytochrome P_{450}s). Beside the CYP family, NNK can also be activated by metabolic genes, like myeloperoxidase (MPO) and epoxide hydrolase (EPHX1). NNK can be activated by two different routes, the oxidative path and the reductive path. In the oxidative metabolism, NNK undergoes α-hydroxylation catalyzed by cytochrome P_{450}. This reaction can be carried out by following two pathways namely by α-methylhydroxylation or by α-methylenehydroxylation. Both pathways generate the carcinogenic, metabolized isoform of NNK, known as NNAL (Figure 3.15). In the

reductive metabolism NNK undergoes either a carbonyl reduction or a pyridine N-oxidation, both producing NNAL [Wiener *et al*., 2004].

From a lifetime study in rats, it was found that NNAL was equally as effective as NNK in inducing lung tumours [Rivenson *et al*., 1988]. In male rats, a study showed that NNAL, which was administered in the drinking-water, induced adenomas, adenocarcinomas and adenosquamous carcinomas of the lung and benign and malignant pancreatic tumours. Another studies in female mice, intraperitonial injection of NNAL induced lung adenomas, adenocarcinomas were also observed [IARC, 2007].

A further study in female mice showed that intraperitoneal injection of NAB induced lung adenomas. Another study in rats also showed that NAB, which was administered in the drinking-water, induced oesophageal carcinomas and/or papillomas in males and females whereas NAT is inactive when tested in rats [IARC, 2007]. There is limited evidence in experimental animals for the carcinogenicity of NAB and inadequate evidence in experimental animals for the carcinogenicity of NAT. NAB and NAT (Figure 3.16) are not classifiable as to its carcinogenicity to humans [IARC, 2007].

A molecular ion peak with an m/z = 147 value indicates the presence of myosmine $[M+H]^+$ (Figure 3.17), a pyrolytic product of nicotine from tobacco. For a long time, myosmine was considered as one of the minor tobacco-specific alkaloids present in the tobacco mainstream smoke. The mutagenic potential of myosmine was confirmed by the detection of DNA damage in human lymphocytes and nasal mucosa cells by the Comet assay [Kleinsasser *et al*., 2003]. Myosmine is easily nitrosylated, yielding N'-nitrosonornicotine (NNN) (m/z = 178) (Figure 3.18) and to lesser extent, 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB) (m/z = 166) (Figure 3.13).

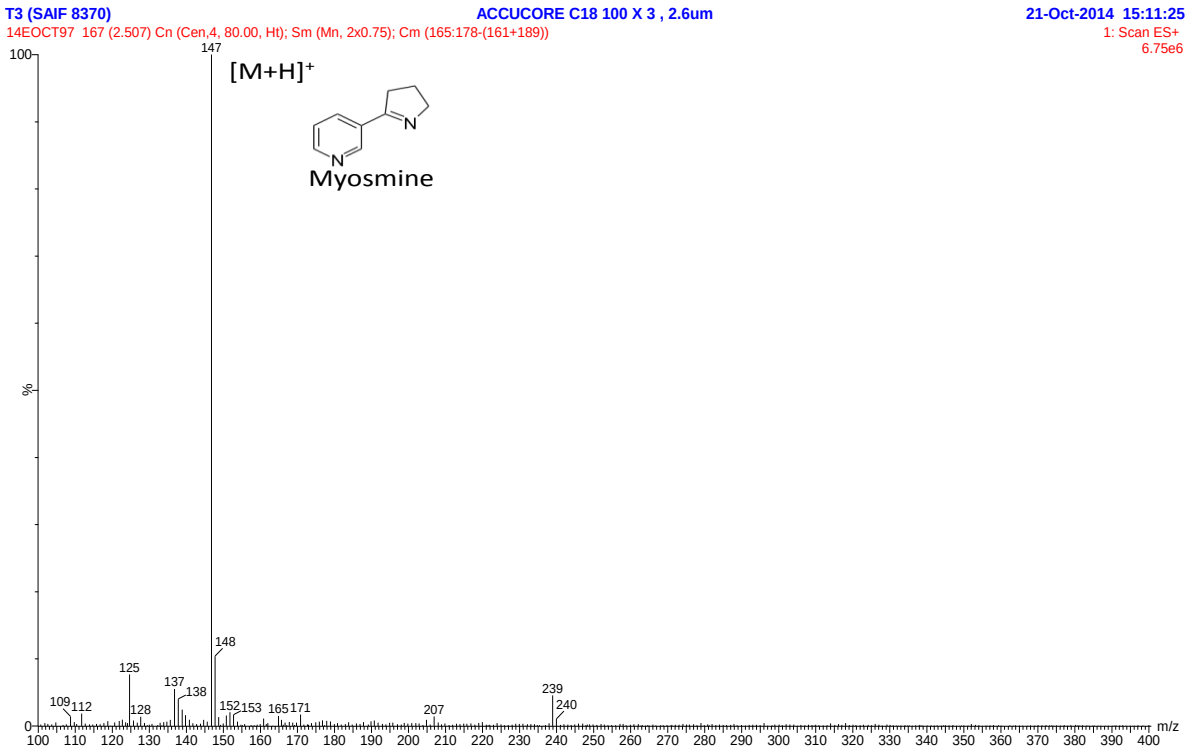


Figure 3.17: Myosmine (m/z = 147).

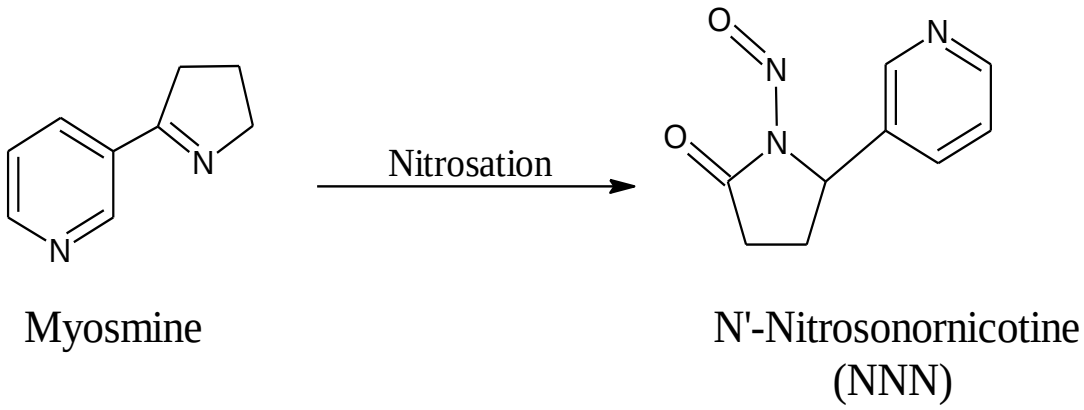


Figure 3.18: Nitrosation of myosmine to NNN.

In the ESI-HRMS analysis of tuibur sample, presence of aromatic amine such as 2-aminonaphthalene (also known as 2-naphthylamine) and 4-aminobiphenyl were observed. These carcinogenic species have been identified in the GC-MS analysis of main-stream tobacco smoke as well as side-stream tobacco smoke [Smith *et al*., 2003]. The MS spectral data with m/z = 143.0613 corresponds to 2-naphthylamine, it is an aromatic amine (Figure 3.19); it is found in tobacco products and is known carcinogen specifically for bladder cancer [IARC, 1974; 1987].

The MS spectral data with m/z=169.1303 (Figure 3.20) is identified as 4-Aminobiphenyl (4-ABP), it is an amine derivative of biphenyl and it is a known human carcinogen. It is a major etiological agent of human bladder cancer, and its metabolites are able to form DNA adducts that may induce mutation and initiate bladder carcinogenesis [Feng *et al*., 2002]. 2-Naphthylamine and 4-aminobiphenyl are known to have inflicted genetic damage in various test systems, the observed genetic damage included DNA strand breaks, chromosomal aberrations, micro-nucleus formation, aneuploidy, sister chromatid exchange, and cell transformation [IARC 1987, Gene-Tox, 1998].

2-naphthylamine and 4-aminobiphenyl are metabolized to their activated form either *via* N-hydroxylation (by cytochrome P_{450} liver enzymes) or detoxified *via* pathways such as N-acetylation. The N-hydroxylamine metabolites concomitantly can form adducts with blood-serum proteins (such as hemoglobin), which circulate freely, or they can undergo subsequent metabolism (conjugation) to form reactive compounds that can be transported to the bladder and can bind to DNA [Yu *et al*., 2002]. 2-naphthylamine-DNA adducts have been detected in bladder and liver cells from exposed dogs [IARC 1987].

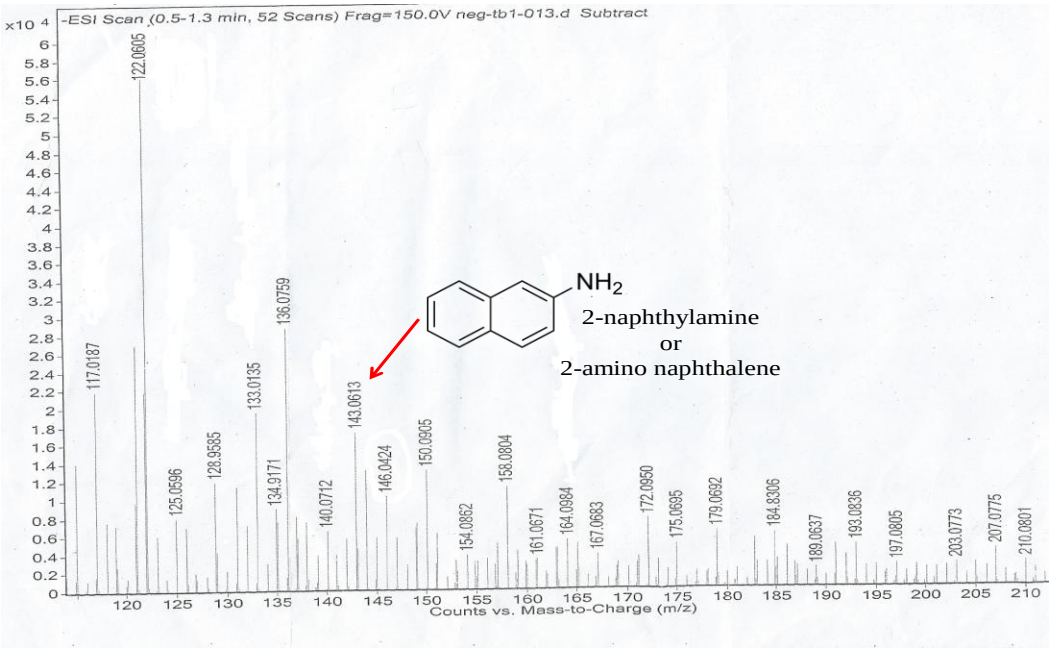


Figure 3.19: 2-amino naphthalene (m/z = 143).

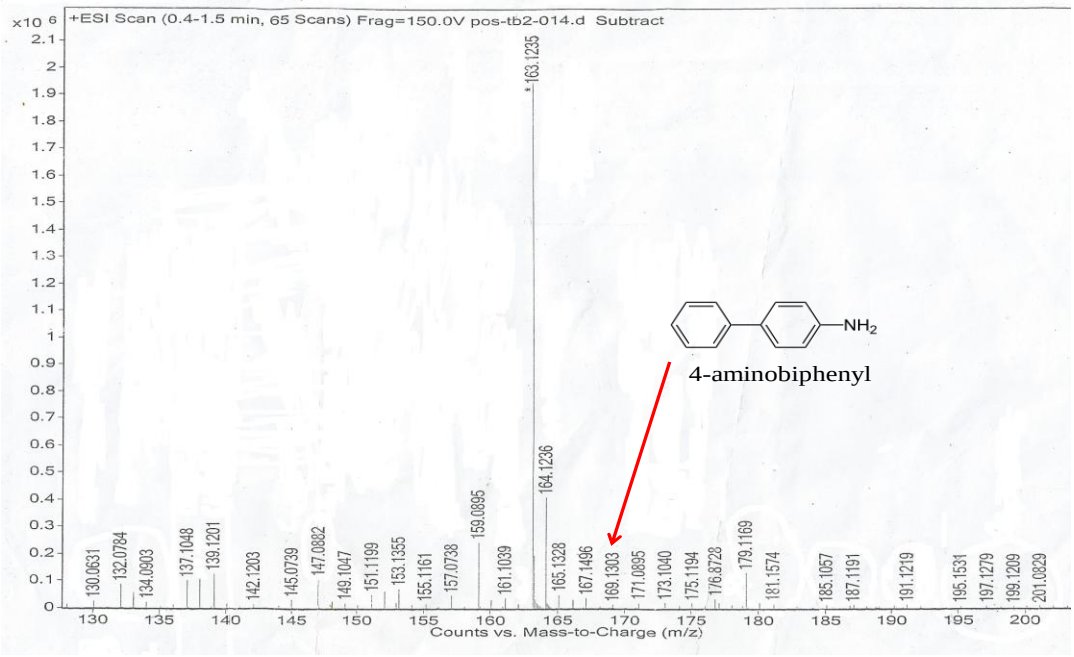
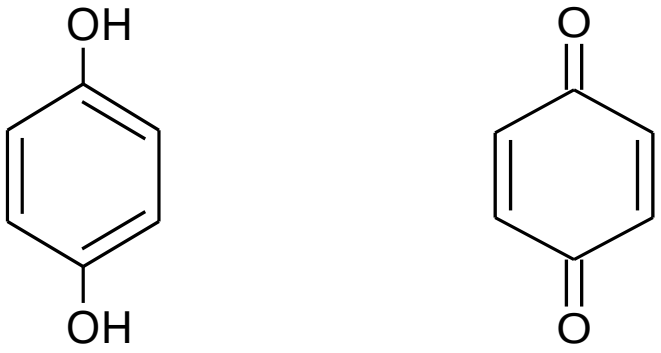


Figure 3.20: 4-aminobiphenyl (m/z = 169).

4-Aminobiphenyl–DNA adducts have been found in urinary-bladder epithelial cells from exposed dogs and humans, and 4-aminobiphenyl–protein adducts have been found in serum albumin from exposed rats and in hemoglobin from humans exposed via cigarette smoking [IARC 1987; Feng *et al*., 2002].

The MS spectral data with m/z =110 (Figure 3.13) is identified as hydroquinone (Figure 3.21(A)). When present in aqueous solution, hydroquinone is susceptible to both redox cycling and acid-base transformations, leading to the formation of benzoquinone and semiquinone, and also various reactive oxygen species (ROS) [HCN, 2012].



(A) (B)

Figure 3.21: Structure of Hydroquinone (m/z = 110) (A) and Benzoquinone (m/z = 108)(B).

The MS spectral data with m/z = 108 (Figure 3.13; also as a $[M-H]^+$ = 109 species, see Figure 3.17) is identified as benzoquinone (Figure 3.21 (B)). Since benzoquinone and hydroquinone are metabolites of each other (interconverted at reaction rates dependent on prevailing local conditions), the toxicity observed with one of the two is also relevant for the other, although there may be a variation in potency. As far as available animal data permit, toxicity profiles of hydroquinone and benzoquinone have indeed a lot in common: both

substances are irritating to eyes and skin, act as skin sensitizers, and have a comparable genotoxicity profile.

The conversions between benzoquinone, hydroquinone and the semiquinone (SQ) can occur both spontaneously and enzymatically. Redox cycling between hydroquinone and benzoquinone as well as of their gluthathione conjugates leads to generation of ROS, including superoxide anion ($O_2^{-\bullet}$) and H_2O_2. ROS potentially caused lipid peroxidation and membrane damage, cytotoxicity, DNA damage, mutagenicity, and carcinogenicity [Jeong *et al*., 1999]. Benzoquinone and hydroquinone have been demonstrated to induce double strand breaks when incubated with naked DNA in a cell free system.

Topoisomerase II is essential for the maintenance of proper chromosome structure and segregation. Several *in vitro* studies have shown that hydroquinone and benzoquinone inhibit the funtionality of topoisomerase II and enhance DNA cleavage [Lindsey *et al*., 2005; Smith, 2010]. *In vitro*, the presence of glutathione protected topoisomerase II from inhibition [Chen and Eastmond 1995]. Bioactivation of hydroquinone by peroxidase to benzoquinone enhanced topoisomerase II inhibion [Eastmond *et al*., 2005]. In a cell-free system, benzoquinone is a more potent inhibitor of topoisomerase II than hydroquinone [Hutt and Kalf 1996; Baker *et al*., 2001].

Unlike hydroquinone, the reactive benzoquinone along with the unstable and redox active semiquinone are capable of direct covalent binding to macromolecules to form DNA- and protein adducts *in vitro* [Gut *et al*., 1996; Jeong *et al*., 1999]. Potentially, macromolecular binding may also cause membrane damage, lipid peroxidation, cytotoxicity, DNA damage, mutagenicity, and carcinogenicity [Jeong *et al*., 1999; Funari *et al*., 2011]. Benzoquinone can bind to critical thiol groups of tubulin resulting in inhibition of microtubule formation. As a

consequence, the formation of a functional spindle apparatus in the mitotic cell may be disturbed, leading to abnormal chromosome segregation and aneuploidy [Pfeiffer and Metzler, 1996].

3.5. Fourier Transform Infrared Spectroscopy (FTIR)

The present study investigates the application of high resolution FTIR spectroscopy for identification of the functional group(s) that may be present in the unknown components of tuibur sample. For this purpose, commercially available tuibur sample was collected and the vibrational spectroscopic study using FT-IR was carried out. The vibrational frequencies shift to lower energies as the mass of atoms in a molecule increases. In general, but not always, metal-ligand stretching vibrations in transition metal complexes usually occur below 450 cm^{-1}, while metal-ligand vibrational frequencies, *i.e.*, ν(M-C), occur over the region 400 – 600 cm^{-1} [Nakamoto, 1997]. The vibrational motions that involve bending, wagging or deformation of metal-ligand bond angles shift the experimentally observed vibrational frequencies at even lower frequencies.

The region below 1000 cm^{-1} (the far-infrared region) is normally considered to be the region, where the presence of stretching and bending vibrations of bonds between metal atoms and both inorganic and organic ligands is observed. From the ICP anlysis, in the present study (*vide supra*), it has been observed that various metal ions including transition metal ions such as Zn, Cu, Ni are present in tuibur samples. Halide bonds such as aliphatic alky chloride and iodide were detected in their stretching mode below 830 cm^{-1} - often in the range of 400 – 600 cm^{-1} and 215 – 650 cm^{-1}, respectively. Likewise, aliphatic-Br was found in the range of < 700 cm^{-1}. While, many transition metal ions and nitrogen containing organic compounds that may act

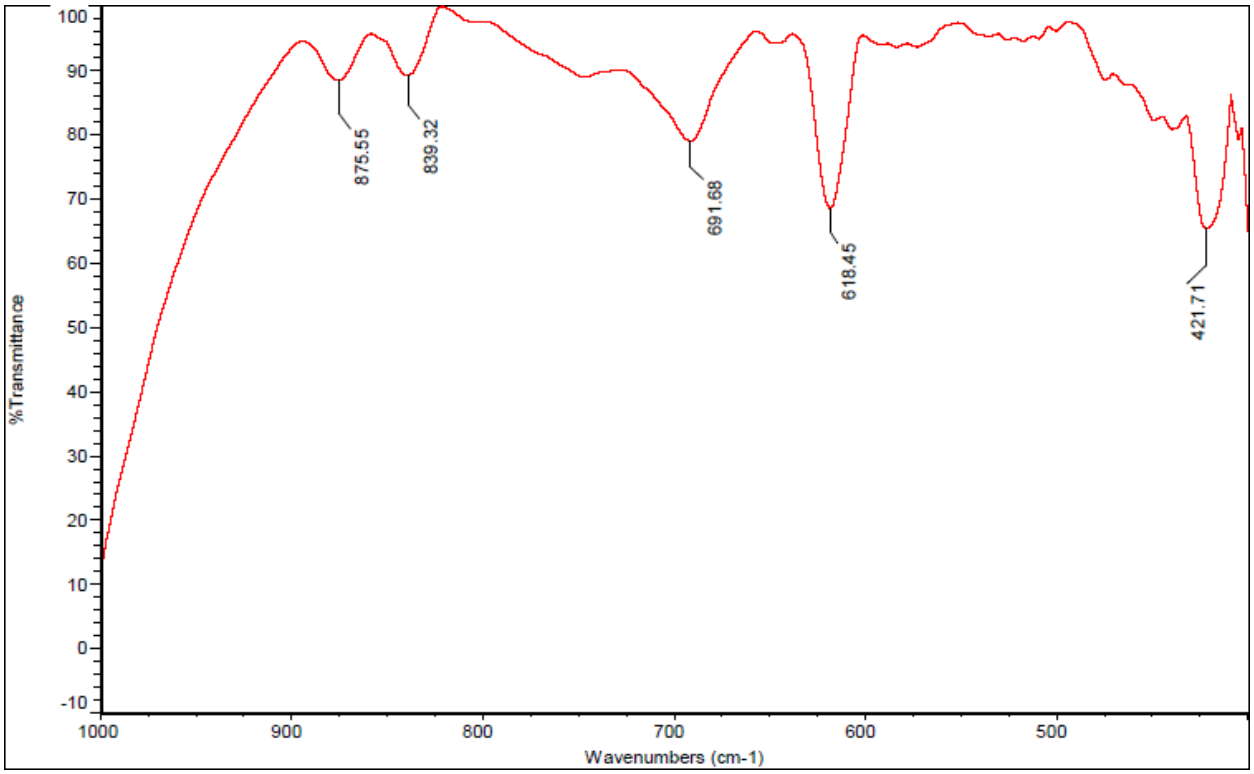


Figure 3.22: Wavelength range 1000 – 400 cm^{-1}.

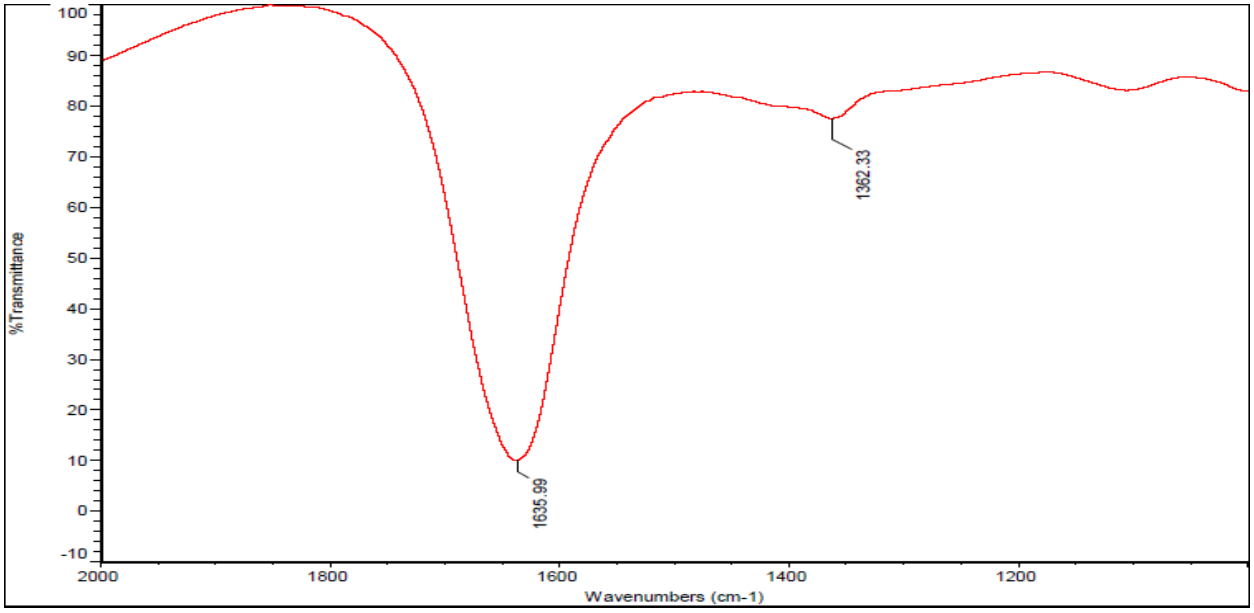


Figure 3.23: Wavelength range 2000 - 1000 cm^{-1}.

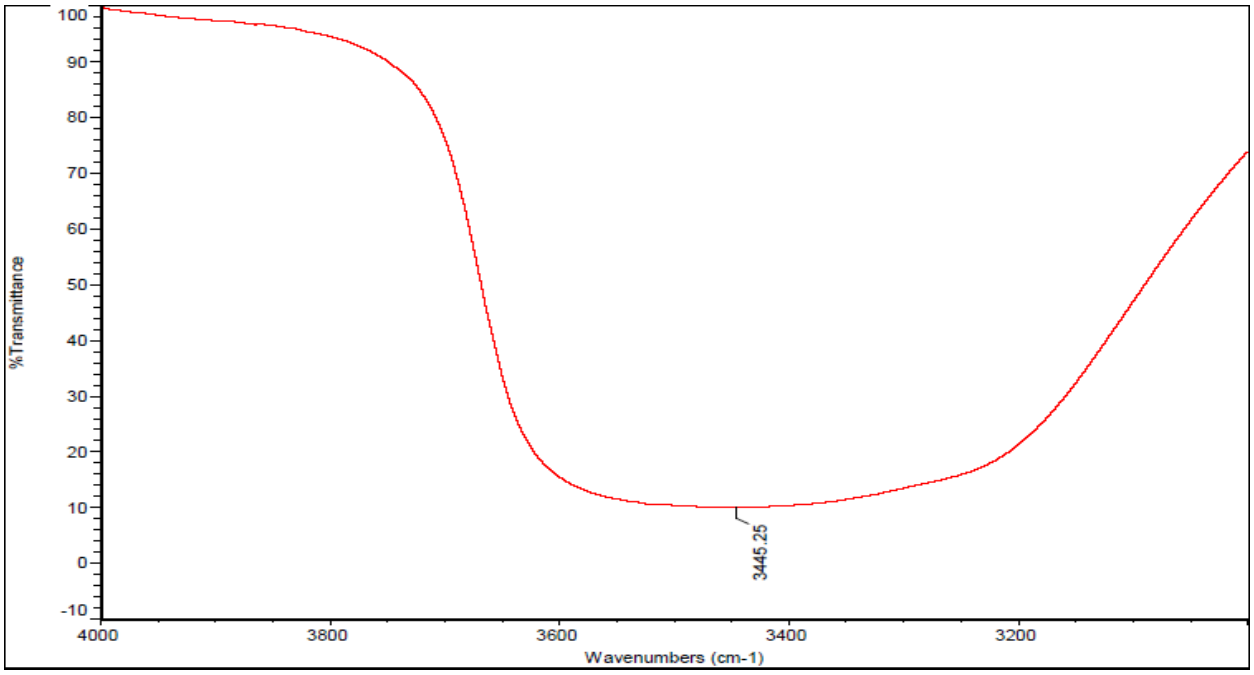


Figure 3.24: Wavelength range 4000 - 3000 cm^{-1}.

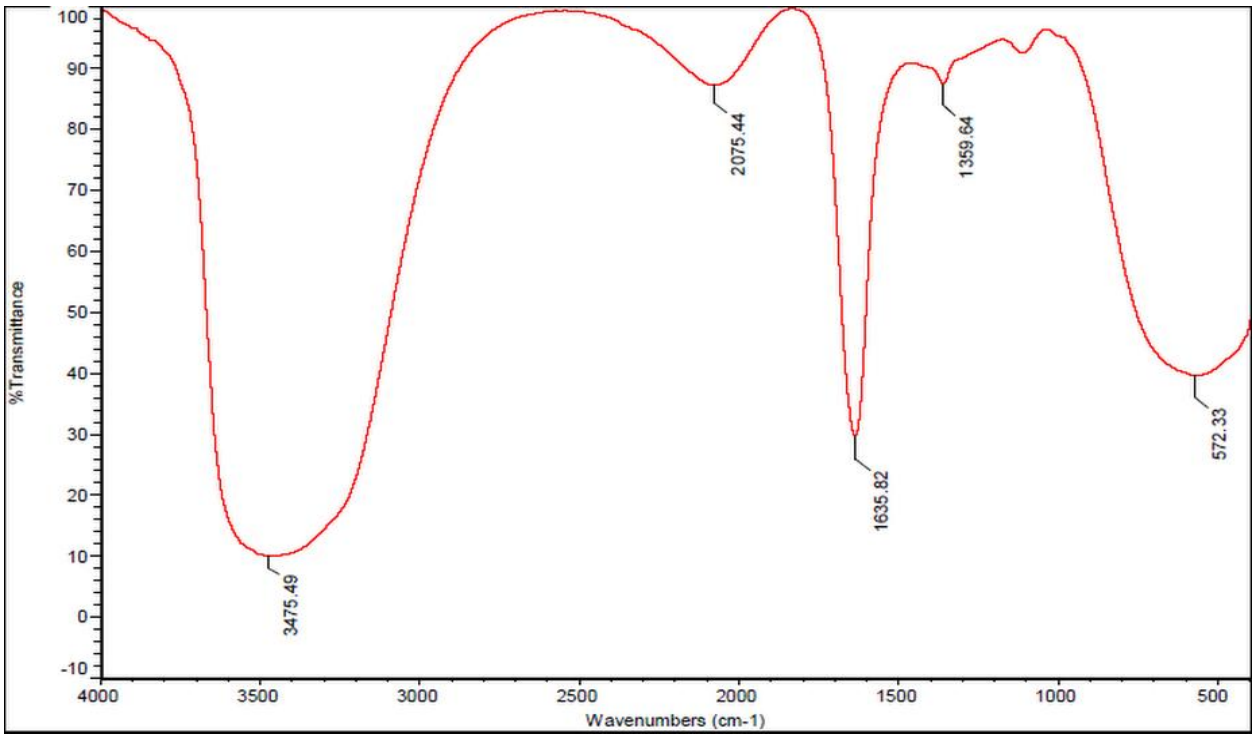


Figure 3.25: Wavelength range 4000 - 400 cm^{-1}.

potentially as ligands are also present in tuibur, so we could not assign any particular band below 700 cm^{-1} for different type coordination complexes that may be present in tuibur solution. In addition, for aromatic compounds the 'breathing' mode vibrational frequencies are also falling under the same region, these bands may also be indicative of the presence of xenobiotic compounds with aromatic rings. Additional detailed studies using Raman spectroscopy methods are required to ascertain the nature of species exhibiting vibrational frequencies in this region.

A very broad band at 3475.49 cm^{-1} (Figure 3.25) and 3445.25 cm^{-1} (intense broad band) (Figure 3.24) may be indicative of the presence of H-bonded labile proton attached with electronegative oxygen (O-H) or nitrogen (N-H) such as phenols, or hydroquinone (with two O-H groups). Alternatively, the strong intensity band with medium line width also could be indicative of the presence of secondary amine with free N-H. Although, the vibrational bands arising from C-H attached to aromatic rings are expected around 3000-3150 cm^{-1}, the observed intense broad band centered at ~ 3470 cm^{-1} for a hydrogen bonded polar species is masking the region, for tuibur sample, we could not observe these aromatic proton vibrational bands. Furthermore, a weak band centered at ~ 1100 cm^{-1} suggests the presence of C-O bond such as present in alcohols/phenols or carboxylic acids.

A weak band that occurs at 2075 cm^{-1} the presence an organic compound with -C≡N bond or -C=N- bond that may be indicative the presence of an aromatic nitrogen containing compounds such as pyridine type compounds that exhibits –C=N- bonding. The observed sharp intense band at 1635.99 cm^{-1} (Figure 3.23) along with an intense band at 1635.82 cm^{-1} (single band) (Figure 3.25) indicates the presence of alkene (cis) C=C stretching band or the presence of a compound with aromatic ring. Weak absorption bands at 1362.33 cm^{-1} (Figure 3.23) and

1359.64 cm^{-1} (single band) (Figure 3.25) also indicates C-N stretching in an aromatic compound such as pyridine or pyrrolidine. Thus, aromatic ring containing nitrogen may be present in the tuibur sample.

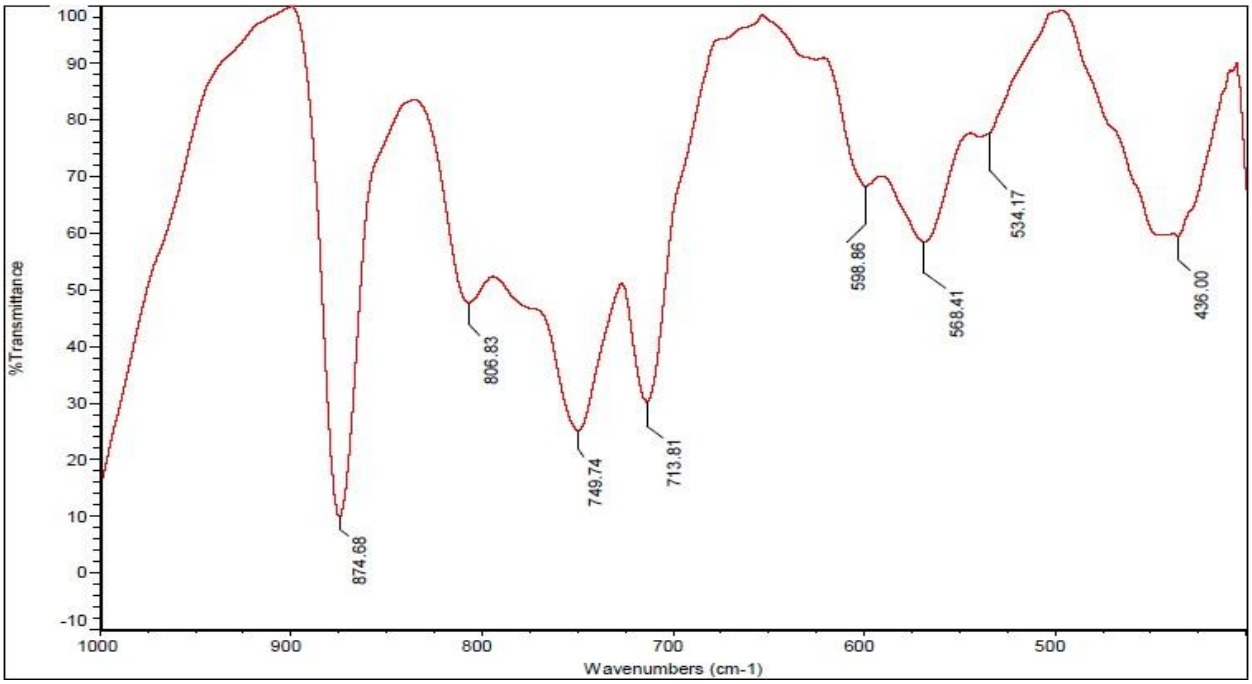


Figure 3.26: FTIR of tar in the long wavelength region.

In the long wavelength region (900 – 600 cm^{-1}), there were many peaks observed for the tar sample as displayed in figure 3.26. A sharp and intense peak distinctly observed at 875 cm^{-1} is attributed to the para-substituted aromatic compound(s). In addition, vibrational bands were observed at 806, 780, 750 and 714 cm^{-1}, which is indicative of the pattern of substitution in the aromatic ring, corresponding to para-, ortho-, and meta-substituted aromatic compounds, respectively as meta-substituted aromatic compounds generally exhibit split bands in the low-frequency region[Stuart, 2004; Silverstein *et al.*, 2005].

CHAPTER 4:

Genotoxic effects of tuibur – mtDNA Study

CHAPTER 4

GENOTOXIC EFFECTS OF TUIBUR – mtDNA STUDY

4.1. Extraction of DNA

As very limited hospital based case and control related studies are available on the putative ill effects of the prolonged consumption of tuibur, it is the most opportune time to carry out a detailed characterization of tuibur, identify the various inorganic as well as organic chemical constituents present in it and their potentially adverse effects on human health. Moreover, it is prudent to evaluate the mutagenic and carcinogenic potency of tuibur by measuring the type, the frequency and the extent of polymorphism(s) in mtDNA from a pool of donor samples, within the tribal population from the state of Mizoram, classified according to their life style habits, *viz.*, consumption of tuibur, smoking status, etc. Using the information from the single nucleotide polymorphisms (SNPs) and restriction fragment patterns, it may be possible to predict the genetic predispositions leading to the development of cancer, for early diagnosis and optimal medical treatment. Thus, it is of significant interest to investigate the toxicity of *tuibur* and other tobacco product(s) on genomic level. The essential purpose of the current study is to identify and understand the genetic etiology of the tribal population besides the evaluation of the mutagenic effects of tuibur and other tobacco products in inducing mtDNA polymorphism(s) among the Mizo population. It may be also possible to characterize the immiscible as well as denser distillate and its effect on human health as it is occasionally used as insecticide for household gardens.

The present study was undertaken to elicit the correlation between the non-coding D-loop gene polymorphism(s) and stomach cancer risk considering various dietary habits, tobacco and

alcohol habits for the first time in the high risk state of Mizoram in the northeastern region of India. In order to identify and evaluate the genetic abnormalities, the collection of satisfactory amount of good quality DNA that can be obtained from different sample systems of the body is a major, yet, cumbersome task. The most common method employed in a wide variety of genetic population/survey studies is to obtain nDNA from blood. But this approach could be tedious in obtaining samples from study subjects. Other alternative sources of DNA isolation include buccal cell, hair with follicle, urine, etc., which are relatively easier than the 'prickly' blood collection.

DNA isolation from cells collected with the use of buccal swabs has many advantages such as cost effective processing, long-term archiving, suitability of self-collection besides relatively more comfortable for the patient to provide the sample specimen. Buccal swabs also offer a simple method of obtaining cells that are yielding sufficient quantity of relatively pure DNA for the polymerase chain reactions (PCR) in which only ng (nanogram) quantities of DNA are needed. Extraction of DNA was done by following the modified protocol of Ghatak *et al.*, 2013.

The integrity of genomic DNA was assessed by resolving DNA extracts on a 0.8% agarose gel by electrophoresis (Bio-Rad), followed by visualization with ethidium bromide staining (Figure 4.1). Each DNA sample was graded according to the electrophoretic migration of DNA in comparison to a known molecular weight marker ladder (Fisher Scientific, Fermentas). Storage of the extracted DNA for one month at −20 °C did not alter the quality as well as PCR performance of DNA obtained from buccal swabs and blood [Ghatak *et al.,* 2013].

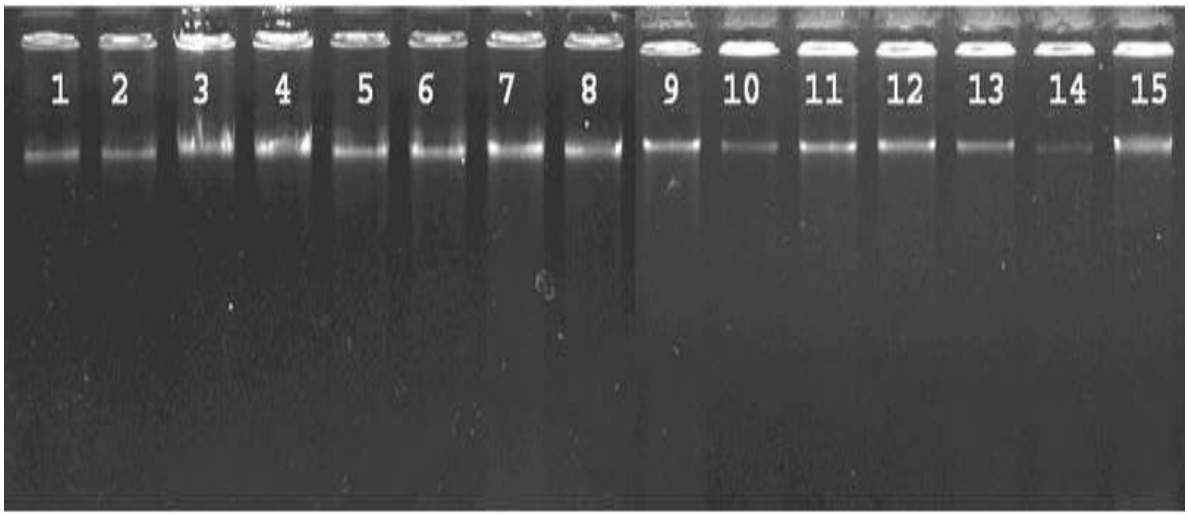


Figure 4.1: Genomic DNA extracted from buccal swab and blood samples.

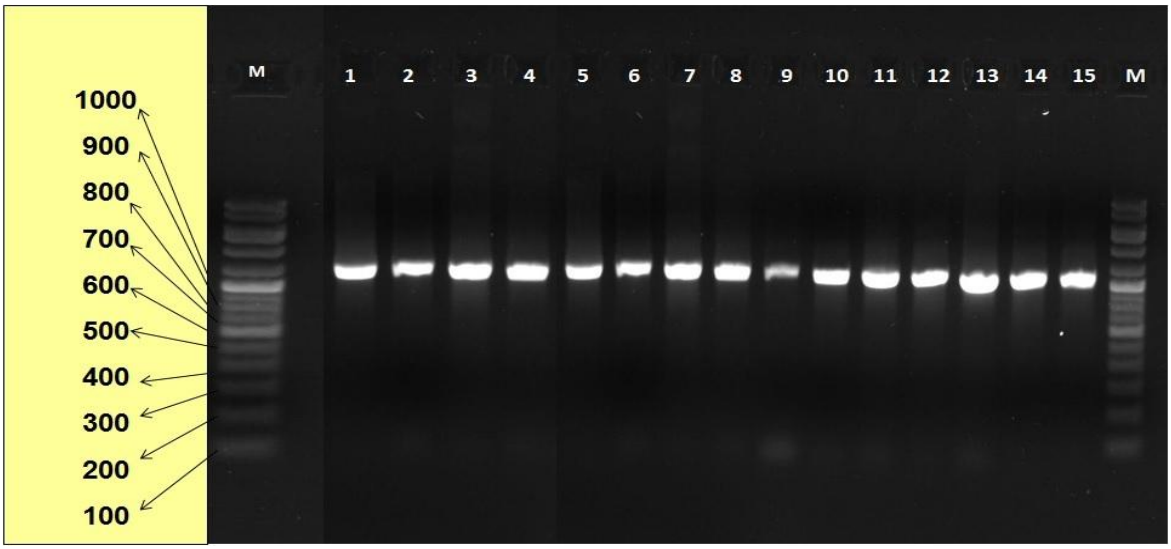


Figure 4.2: Amplified mitochondrial control region (~1050 bp of D-loop) from isolated genomic DNA from buccal swab and blood samples.

4.2. PCR amplification of mtDNA D-loop region

The extracted DNA was amplified for the target region (D-Loop region) by the appropriate primer such as HMt-F (forward primer) and HMt-R (reverse primer) [Salas *et al.*, 2001]. PCR products for D-loop region of mtDNA on an agarose gel showed a fragment of about 1050 bp (Figure 4.2).

4.3. Restricion digestion of the mtDNA

Restriction fragment length polymorphism (RFLP) of mtDNA D-loop region was performed to check the contamination in the isolated DNA. PCR products were digested with *AluI*, *HaeIII*, *MspI* and *KpnI* (Fermentas, Thermo Scientific). PCR-RFLP techniques were used to reveal polymorphism of mtDNA D-loop region in Mizoram. Digestion of amplified fragments of mtDNA D-loop region by *AluI, HaeIII*, *MspI* and *KpnI* restriction endonucleases along with digested band pattern (Figure 4.3, 4.4, 4.5, and 4.6, respectively) and their rate of frequency are listed in figure 4.7(B).

The results showed 8 restriction patterns for *AluI* enzyme (with 5 heteroplasmy from tuibur consumer and 2 heteroplasmy from cancer patient samples) and 7 restriction patterns for *HaeIII* enzyme (with 3 heteroplasmy from tuibur consumer and 2 heteroplasmy from cancer patient sample) within the study population. The *MspI* enzyme also showed two restriction patterns with 1 heteroplasmy variation in case of tuibur consumer, while four restriction patterns were shown for *KpnI* enzyme. In total, four restriction enzymes displayed 21 different polymorphic patterns in the study population of Mizo tribe.

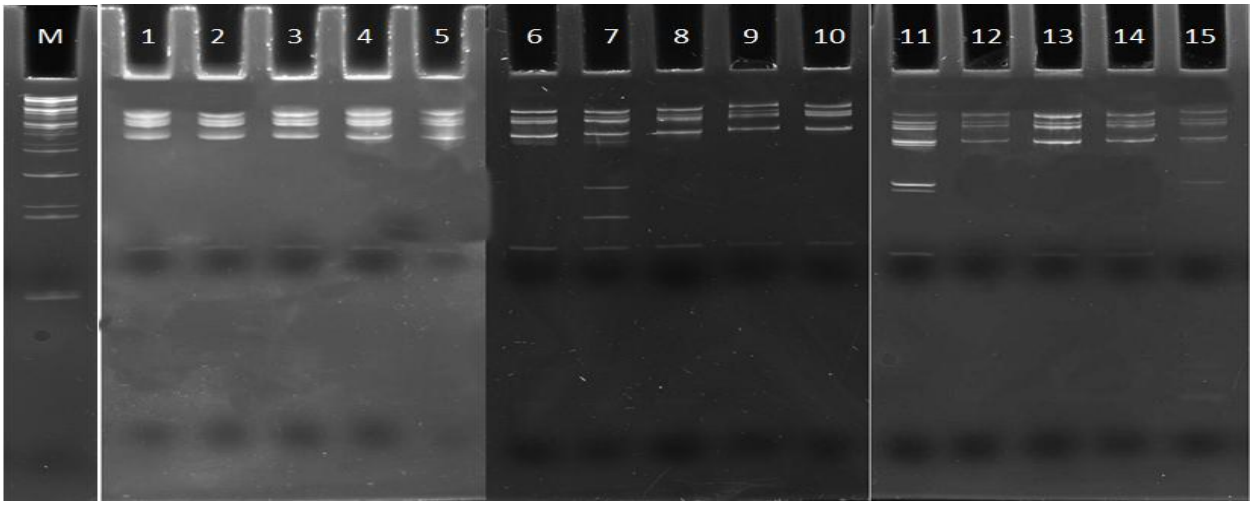


Figure 4.3: RFLP digestion pattern using *AluI* restriction enzyme. 1 – 5: Healthy (normal) samples; 6 – 10: Tuibur consumer samples; 11 – 15: Stomach cancer samples.

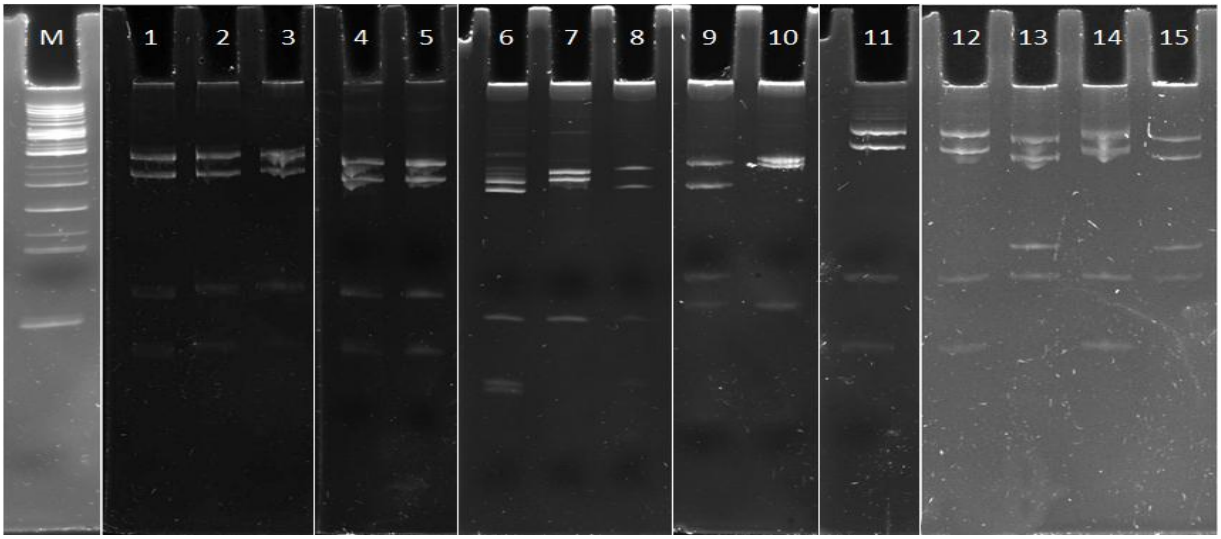


Figure 4.4: RFLP digestion pattern using *HaeIII* restriction enzyme. 1 – 5: Healthy (normal) samples; 6 – 10: Tuibur consumer samples; 11 – 15: Stomach cancer samples.

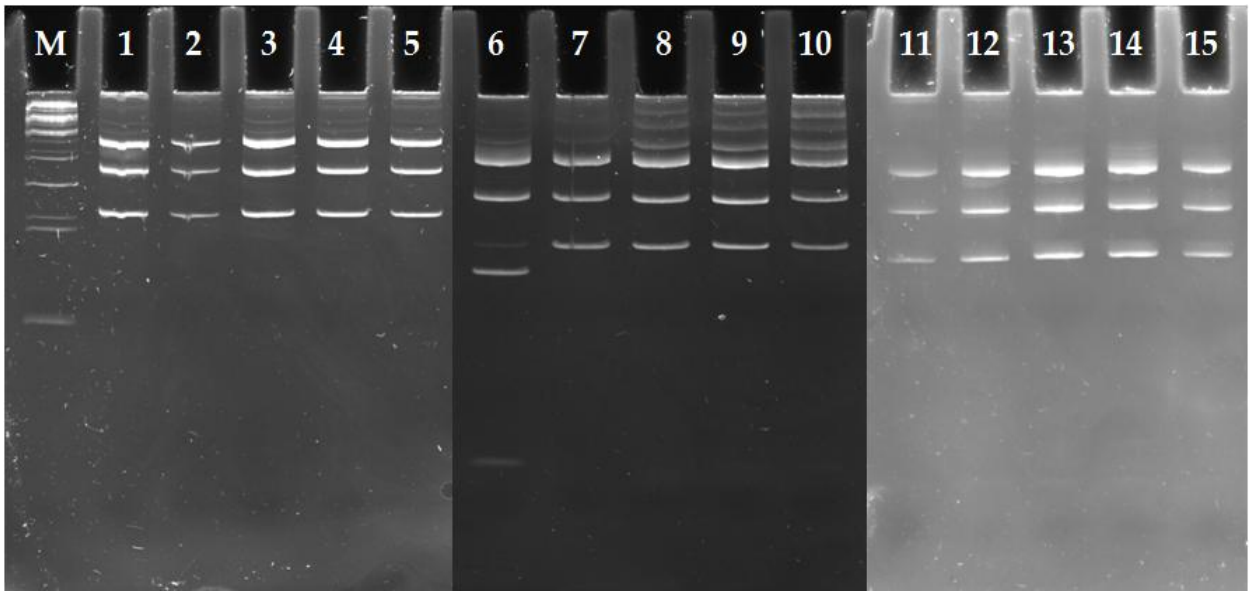


Figure 4.5: RFLP digestion pattern using *MspI* restriction enzyme. 1 – 5: Healthy (normal) samples; 6 – 10: Tuibur consumer samples; 11 – 15: Stomach cancer samples.

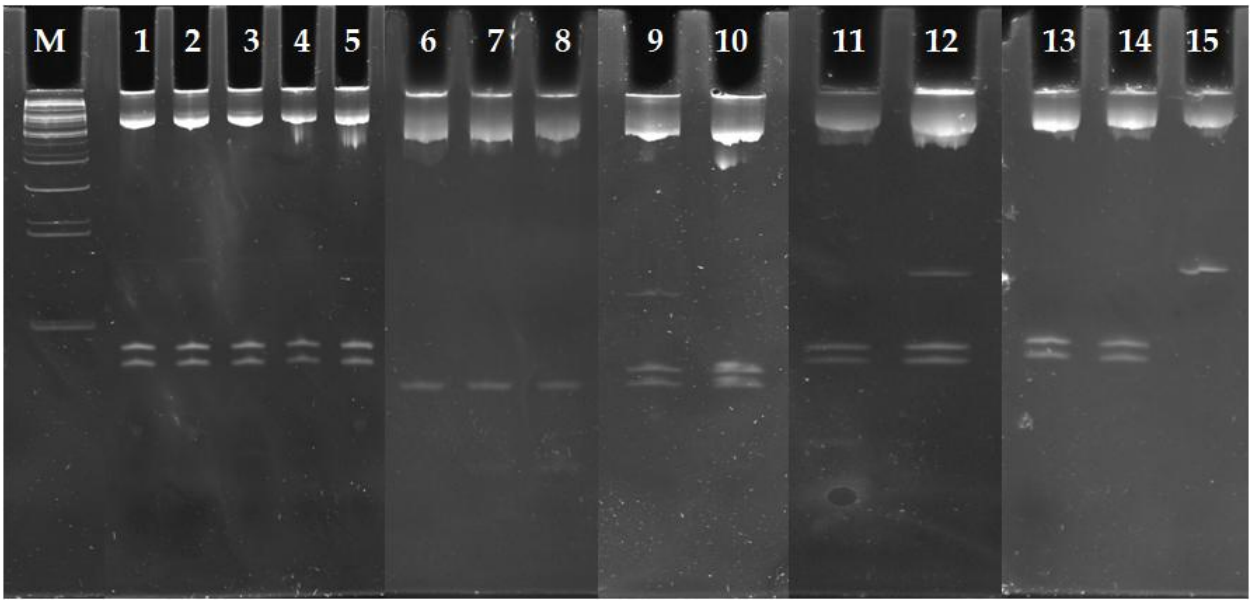


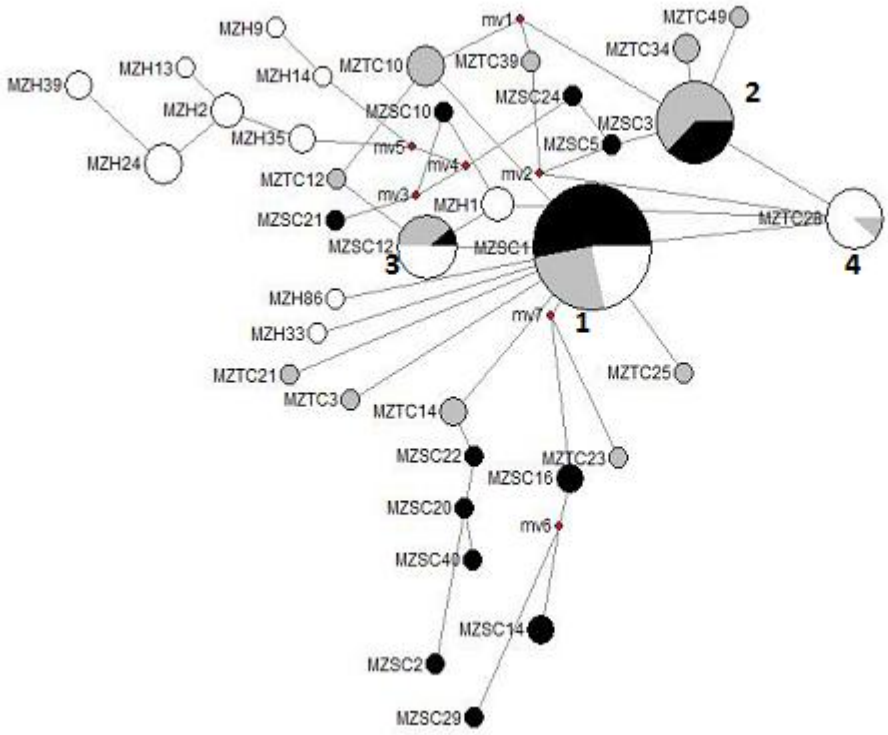
Figure 4.6: RFLP digestion pattern using *KpnI* restriction enzyme. 1 – 5: Healthy (normal) samples; 6 – 10: Tuibur consumer samples; 11 – 15: Stomach cancer samples.

4.4. Phylogenetic relationship of the RFLP product

Buccal swab samples of control and tuibur consumers along with blood samples of stomach cancer patient samples were analyzed by PCR. The network analysis showed frequency distribution of motifs in the population based on the PCR-RFLP product. In figure 4.7 (B), 37.5 % of stomach cancer patient and 62.5 % of tuibur consumers are clustering together. Figure 4.7(A) and 4.8 showed the phylogenetic network and Neighbor-joining tree depicting the genetic affinities among three different sample groups, respectively. Tuibur consumers and stomach cancer patient sequences have shown close relationship and hence clustered together. Buccal swab samples of control and tuibur consumers along with blood samples of stomach cancer patient samples were also analyzed, in the present study, for sequence variation in the mtDNA D-loop region.

4.5. Sequence analysis of the PCR product

Among the stomach cancer and tuibur consumer samples, it has been found that the type of mutations are mostly transition base substitution (Figure 4.9) with 55.55% T>C, C>T, A>G and G>A base substitution in stomach cancer and 92.86% T>C, C>T, A>G and G>A base substitution in tuibur consumer. The new sequences were submitted in the EBI repository database and the accession numbers are given in Table 4.1 along with demographic factors, diet, and lifestyle and haplotype information. A total of 27 mtDNA D-loop sequence variations (Table 4.2) at 27 distinct nucleotide positions were found in 10 samples.



Group		Colour
Healthy Samples		White
Tuibur Consumer Samples		Gray
Cancer Patient Samples		Black
Type	Sample Name	Rate of Frequency
1	MZSC(20)	52.63 %
	MZTC(10)	26.31 %
	MZH(8)	21.05 %
2	MZSC(5)	37.5 %
	MZTC(10)	62.5 %
3	MZSC(1)	10 %
	MZTC(4)	40 %
	MZH(5)	50 %
4	MZTC(1)	11.11 %
	MZH(8)	88.88 %

(A) (B)

Figure 4.7: Phylogenetic network illustrating the genetic affinities among three different sample groups (A) and different sample frequency (B).

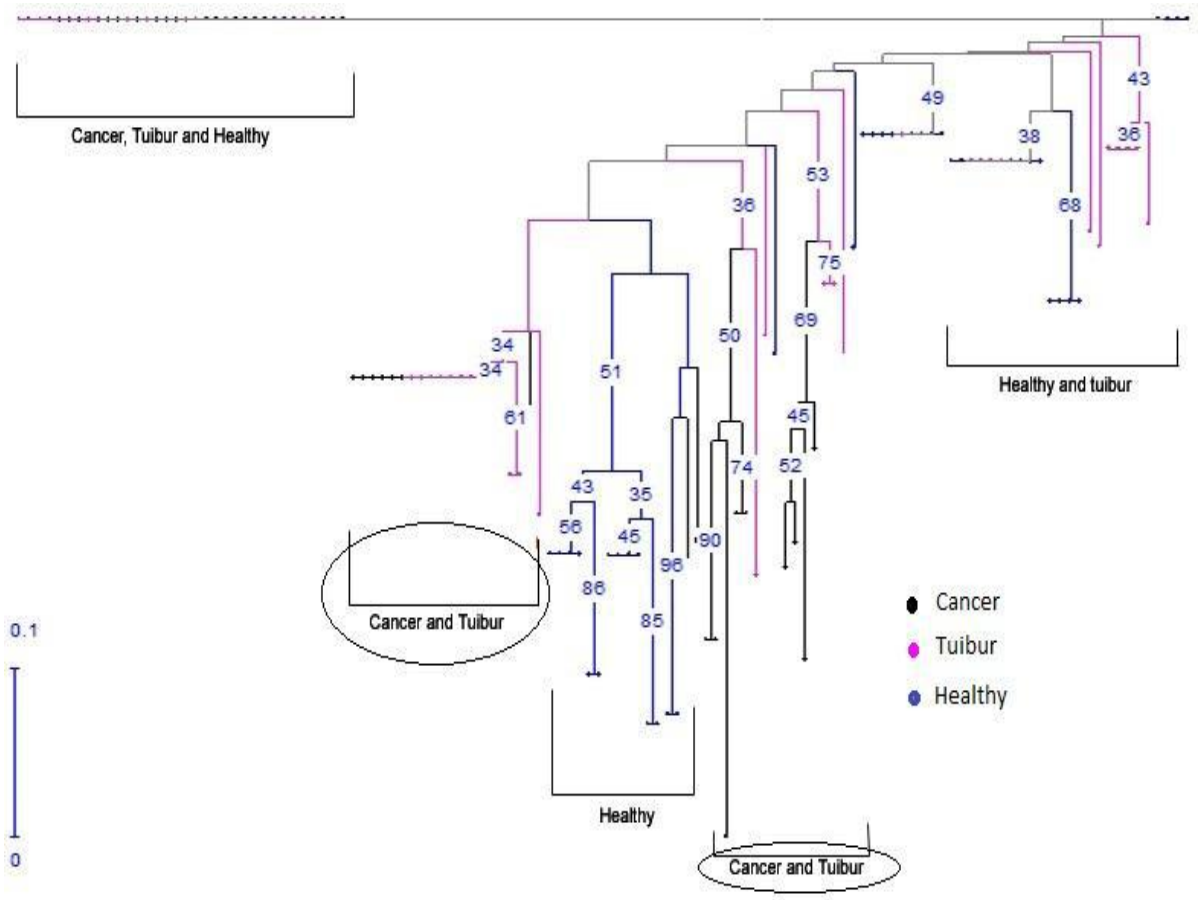


Figure 4.8: Neighbor-joining tree depicting the genetic affinities among three different sample groups.

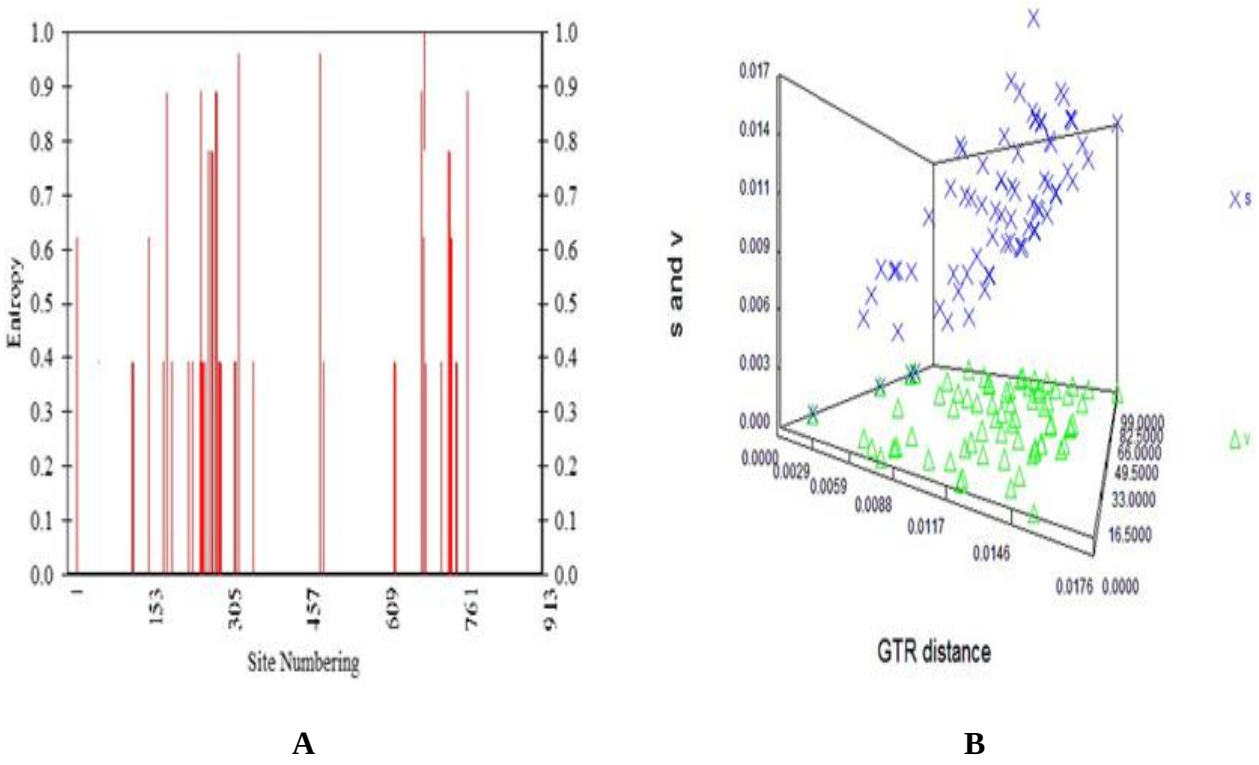


Figure 4.9: (A) Plot of variable substitution rate over site, (B) 3-D plot of transition and transversion versus divergence.

Table 4.1: Demographic, haplogroup and lifestyle information of the samples.

Sample Name	Haplo group	Sample mutation frequency	Meat	Smoked Meat	Smoked Vegetable	Fat	Saum	Extra Salt	Pickle	Tuibur	Cigarette	Zozial	Biri	Oral Snuff	Kuhva	Alcohol	EBI accession number
MZSC 1	***M33b***	**7**	*++	*+++	+++	+++	++	*-	+	-	+++	-	-	-	++	-	LN 558427
MZSC 3	***A14***	**4**	++	*+	+	++	++	-	+	++	+++	+++	-	++	++	-	LN 558428
MZSC 10	***C7b***	**3**	++	+	+	++	++	-	-	-	-	-	-	-	-	++	LN 558430
MZSC 11	***M9a 1b1***	**3**	++	+++	+++	++	+	-	-	-	-	-	++	++	++	-	LN 558431
MZSC 24	***U2b***	**2**	++	-	-	++	++	-	-	+++	-	-	-	++	-	-	LN 558432
MZSC 31	***A14***	**4**	++	+	+	++	++	+	-	-	-	+++	-	-	-	++	LN 558433
MZSC 33	***A14***	**4**	++	+	+	+++	+++	++	+	+++	-	-	-	++	-	-	LN 558434
MZTC 1	***R9***	**8**	++	-	-	+++	++	-	-	+++	-	-	-	-	-	-	LN 558435
MZTC 24	***A1***	**3**	++	+	+	++	++	-	++	+++	-	-	-	-	++	-	LN 558436
MZTC 40	***D4e 1a2***	**2**	++	-	-	+	-	-	-	+++	-	-	-	-	-	-	LN 558437

*- represents non-consumer; *+ represents light consumer; *++ represents medium consumer; *+++ represents heavy consumer.

Table 4.2: Variations in D-loop region of tuibur consumer and stomach cancer samples.

(Sample information contains the sample number. Sc- Stomach cancer, T – Tuibur.)

Sample Information	Sample Frequency	Mutation position and type
Sc10, 24	18.1818	16051A>G
T 40	9.0909	16092T>C
		94G>A
		214A>G
Sc 1	9.0909	16093T>C
		16266C>T
		16324T>C
		16355C>T
		16391G>A
		*292A>AA…
T1	9.0909	16218C>T
		16289A>G
		16293A>G
		16526G>A
		183A>G
		184G>A
		185G>A
Sc 11	9.0909	16234C>T
		153A>G
Sc3,31, 33,T24	36.3636	16290C>T
		16319G>A
		235A>G
Sc10	9.0909	16298T>C
		16327C>T
Sc 11, 24	18.1818	150C>T
Sc 3, 31, 33	27.2727	151C>T
Sc1, T1	18.1818	*316C>CC…
***292A>AA… and *316C>CC… are novel mutation.**		

Table 4.2 also showed sequence variation in the mtDNA D-loop region of the two different groups (tuibur consumer and stomach cancer patient samples), sample frequency, nucleotide position, mutant type and the name of mutation compared with the healthy (control) group. ***It is important to note that 292A>AA... and 316C>CC... are novel base insertions (novel microsatellite instability) reported herein for the first time as they have not been reported earlier in the mitochondrial database.*** 292A>AA... position of MT-HV II region is a locus for the mtTF1 binding site Y. Due to the microsatellite instability at this position, the binding of mtTF1 may be altered. 316C>CC... insertion is present at the conserved sequence block II in MT-HV II region of the mitochondrial control region. Due to the presence of microsatellite instability at this position, the function of the mitochondrial control region may also be altered.

The DNA sequence analysis showed that a correlation between tuibur consumer and stomach cancer patient mtDNA control region (HVR1 and HVR2) genomics. *Three mutations, viz, 16290C>T, 16319G>A and 235A>G, are present in mostly tuibur consumer as well as stomach cancer specimen and hence we can conclude that* ***due to the mutagenic effects of tuibur, these three potent mutations might have occurred in tuibur consumer samples.*** **292A>AA… mutation is a potential pathogenic mutation** for stomach cancer patient due to the alteration of TF1 binding site Y, while *316C>CC… mutation is also exhibited by both tuibur consumer and stomach cancer patient samples, indicative of the potent pathogenic mutation among both groups.*

The non-coding displacement (D)-loop, especially a mononucleotide repeat (poly-C) between 303 and 315 nucleotides (D310), has been recently identified as a frequent hotspot for mutations in human neoplasia, including stomach cancer [Xu *et al.*, 2012]. The observed frequency of mutations, in the present study population, indicates a medium correlation with the

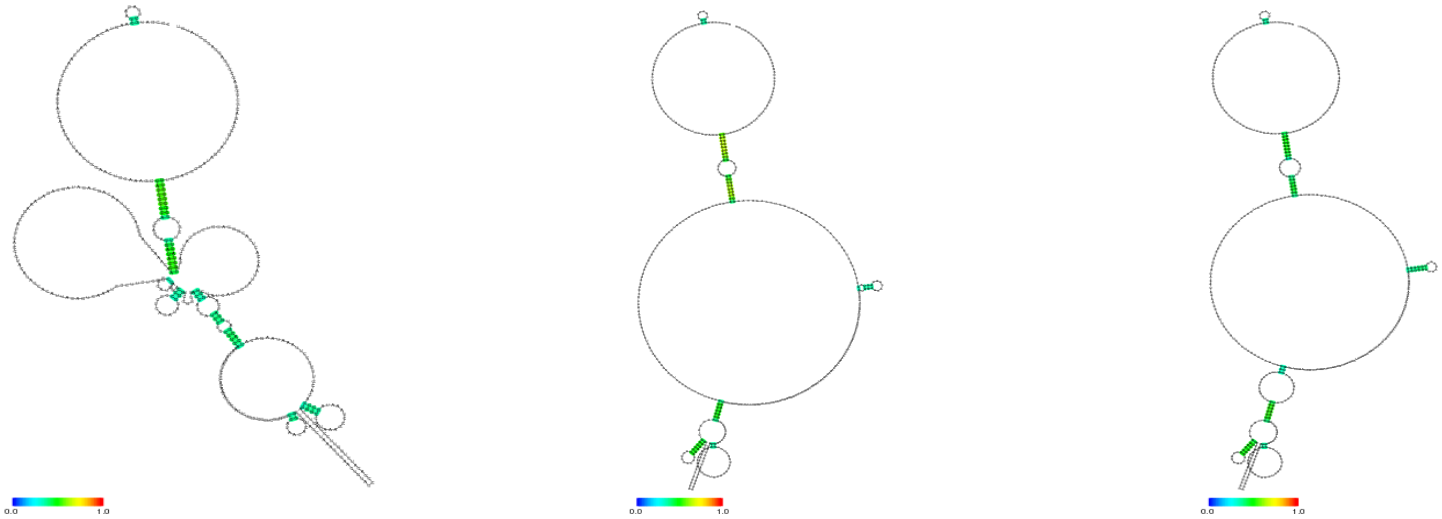
relative mutation rates in HV1. The sites, at which mutation(s) are identified, in the present study, appeared to be predisposed towards mutation. The D-loop control region sites such as 309C>T, 310T>C and 16223C>T are observed in virtually every stomach patient specimen analyzed as it demonstrates near confluence in this cohort. This is perhaps due to a biological propensity to rapid mutation at this region. As such, this unique attribute may be considered as a potential biomarker for stomach cancer provided this behavior is consistent in transforming to blood. Functional studies are further needed to elucidate the biological significance of these mutant alleles in the tumorigenesis process of various tissues. Studies on other genes [Cleveland *et al.*, 2006] have suggested that the number of CA repeats in the promoter region is contrariwise correlated with transcription activity, with five-fold decrease in the activity depending on the number of repeats.

Unique properties of mtDNA D-loop that makes it a very valuable tool for both evolutionary and human identification studies include the high copy number, cytoplasmic inheritance, and rapid rate of evolution. Based on the RFLP and sequencing data, tuibur consumer and stomach cancer patient samples at mitochondrial control region, it has been found that there were different clusters containing tuibur consumer and stomach cancer together. The PCR-RFLP results showed a significant level of heteroplasmy and polymorphic patterns at the D-loop region of mtDNA within Mizoram population among the following groups: tuibur consumer, healthy (control) volunteer and stomach cancer patient. In addition, few of the polymorphisms presented (*vide infra*) are reported for the first time, providing a strong evidence to gain a better understanding of the phylogenetic relationship of mtDNA sequences from the major human ethnic groups. ***The phylogenetic analysis revealed a correlation between tuibur consumers and stomach cancer subjects, indicative of the susceptibility of the tuibur***

consumers for stomach cancer. The phylogenetic study also commensurate with PCR-RFLP analysis, so we can employ the simple and robust PCR-RFLP technique as a potential tool for an early detection of biomarkers in the study of tobacco specific carcinogens induced stomach cancer. Based on the sequencing analysis, there were four different clusters observed leading to the convergence of tuibur consumer and stomach cancer patient samples. The mutations observed are 16290C>T, 16319G>A and 235A>G with maximum frequency of 36.37% which are transition base substitutions and 316C>CC…which is a novel transition base insertion.

4.6. D-Loop secondary structure determination

The search for the secondary structures of DNA also uncovered numerous stable inverted perfect or imperfect repeats that constantly turn into stable stem-loop structures when the DNA is stable and stranded for healthy control. For tuibur consumers and stomach cancer patients, the D-Loop secondary structures are similar and relatively unrelated in comparison with the healthy control specimen based on the major loop (Figure 4.10). These repeats are mainly grouped in the confirmation rearrangements (CR) extremities, following the mini-satellite and near the L strand 3'region of DNA. Inverted repeats may be closely arranged in tandem manner forming hairpin structures with complementarity parts, such as 5'ATGTATATAGTACAT3', 5'ATAAAAAATTTTTT AT3', 5'GCTTTGTCAGGACCAAA CAACAAAGC3', or separated by up to one hundred bases like 5'TTAAAAATTAACT AAAATAAAA3'… 5'TTTTATTAAGTTATTTAA3'. Other repeats, even imperfect ones, systematically form stem-loop structures using the online software MFOLD (RNA Secondary Structure).



A B C

Figure 4.10: Secondary structures of Mitochondrial control region.

(A. Healthy control, B. Tuibur Consumer, C. Stomach Cancer.)

It is worthwhile to mention here that all conserved segments depicted in fragments a-c form hairpin-like structures, *viz*, these regions have mirror like sequences (e.g. inverted tandem repeats) but with the possible exception of healthy Control's CR, none of the CR (Stomach Cancer and Tuibur consumer) form stable secondary structures. Finally, two TCCC motifs exist in our sequence CR, but none of them are linked to the putative cloverleaf secondary structures.

Taken together, confirmation rearrangements (CRs) of stomach cancer samples could not be divided into distinct conserved or variable domains, while tandem repeats of poly C stretch and conserved structural elements in the mid-region were observed. We have now observed that the G+C-rich region of stomach cancer and healthy (control) population, while a poly C stretch is present which may be involved in transcriptional control or may be the site for initiation of replication that is consistent with an earlier observation [Cha *et al.,* 2007]. For tuibur consumer and stomach cancer samples, stem-loop secondary structures plus flanking sequences were completely identical and the stem-loop was formed by a perfect match of 17 nucleotide pairs. Phylogenetic analyses have also indicated that samples comprised of few of the tuibur consumers besides few of the stomach cancer patients formed a monophyletic group.

4.7. Statistical Analysis – Epidemiological Data

The information on 77 stomach cancer patients about their diet and lifestyle were obtained using standard questionnaire. One-way ANOVA was performed using SPSS statistical package (V 16.0) for testing the statistical significance to check whether demographic, food habit, tobacco habit and haplotyping are significantly related with the number of D-loop mutation(s) by a binary data. The P-value <0.05 indicates that there is no significance difference among the variables as determined by the one-way ANOVA test within patient samples with different parameters.

Table 4.3: Statistical Analysis (One-way ANOVA).

Factors	Significant Value
Smoked Meat	0.003
Smoked Vegetable	0.065
Fat	0.071
Saum	0.389
Extra Salt	0.147
Pickle	0.736
Tuibur	0.032
Cigarette	0.073
Zozial	0.008
Bidi	0.467
Oral Snuff	0.306
Kuhva	0.250
Alcohol	0.163

Among the dietary factors, a P-value of 0.003 for smoked meat indicates that there is significance relationship between smoked meat consumption and stomach cancer. Tuibur also shows 0.032 p-values less than 0.05; it indicates that there is significance relationship between tuibur consumption and stomach cancer. Zozial (indigenous 'hand-rolled' cigarette of Mizo household) has P-value of 0.008 which is less than the statistical table value (0.05) it also indicate that there is significant relationship between Zozial smoking and stomach cancer.

The state of Mizoram being a high risk/incidence region for stomach cancer in India for more than a decade [Rao and Ganesh.,1998; Phukan *et al.,* 2004], few hospital based case-control type epidemiological studies have been carried out earlier to ascertain potential risk factors capable of inducing stomach cancer. A matched case-control study had been carried out

at the Aizawl Civil hospital to investigate influence of the use of various kinds of tobacco products on the incidence of stomach cancer. It has been pointed out that the consumption of *tuibur* may be one of the important risk factors for the high prevalence of stomach cancer among the populace in Mizoram [Phukan *et al.,* 2005] which is corroborates with the present study. Putative toxic effects of tuibur were also studied using modified version of *Allium* test [Mahanta *et al.,* 1998]. Microscopic features have exhibited the reduction of mitotic index, formation of micronuclei, lagging chromosome and c-mitosis in the root tip cells treated with different concentrations of *tuibur*. Moreover, a recent study showed that tuibur consumers have three times higher risk of stomach cancer than tuibur non-consumers. Persons who smoked tobacco and / or consumed tuibur had an increased risk of acquiring stomach cancer [Malakar *et al.,* 2012].

Xenobiotic species such as 2-aminonaphthalene, 4-aminobiphenyl, tobacco specific nitrosamines (TSNAs) which are thought to be responsible for many cancers [Hecht, 1999], along with the presence of nicotine, nornicotine and myosmine in tuibur solution was identified in the present. As tuibur is not scientifically well characterized and only very limited health literature is available on the adverse effects of prolonged consumption of tuibur, the evaluation of potentially deleterious effects of tuibur solution on human health will be the focus of future research.

CHAPTER 5:

Summary and Conclusion

CHAPTER 5

SUMMARY AND CONCLUSION

In Mizoram, there is prevalent consumption of tuibur among smokers as well as non-smokers. Habitual consumption of tuibur among the populace of Mizoram that use tuibur orally several times (5-30 times) in a day eventually becomes addicted to this nicotine rich tobacco smoke saturated aqueous solution (tuibur). The present study is the first major scientific endeavour in elucidating the cytotoxic effects of tuibur by the exploration of xenobiotic constituents that may be present in tuibur and attempting to establish a correlation between the consumption of tuibur and its stimulated carcinogenic effects on stomach among the populace of Mizoram.

The present study represents the first step towards a much needed systematic investigation into the carcinogenic/mutagenic properties of tuibur solution. Tuibur solution is commercially made from tobacco available in the local market, mostly sourced across the international border from Myanmar which is still processed indigenously based on the tribal tradition, unlike well established commercial tobacco processing by cigarette manufacturers. Tuibur is made by passing tobacco smoke, generated by smoldering tobacco, through water until the preparation turns cognac in color and has a pungent ammoniacal nicotine smell. Indigenously developed steel containers/pipelines are employed for the production of tuibur applying rudimentary scientific principle(s). As the preparation of tuibur, on a cottage industrial level, is completely different from the controlled laboratory preparation of aqueous extract of cigarette

smoke, the nature of oxidants, and other pro-oxidant chemical constituents present in tuibur *is anticipated to be different kind* and hence its mode of action.

The feedstock, for the production of tuibur, is generated using the tobacco stalk ash (spent tobacco stalk), and it is anticipated that water would 'leach' out all water soluble ionic species and polar organic species of spent tobacco stalk. Eventually, an additional load of gas phase polar species and ionic species that are being generated during the combustion of tobacco stalk would also be dissolved in the feedstock solution. During the production of tuibur, aspiration of tobacco stalk is facilitating the pyrolysis of tobacco stalk, *i.e*., a rudimentary form of mimicking smoker's inhalation-exhalation and hence, it is expected that the tobacco smoke (generated at a temperature range of 400-500 °C) passing through feedstock would have the properties similar to side-stream smoke, rather than the main-stream smoke inhaled by smokers.

Cigarette smoke has been known as a complex mixture containing long-lived radicals, including p-benzosemiquinone that induces oxidative damage. Similar radical species are also anticipated in tuibur solution as the pH of the feedstock is in the range of 8 – 9.8 which is more or less maintained by the tuibur solution. Though, it is expected that most of the inorganic toxic metal ions would be precipitated out of the alkaline pH of tuibur, yet trace amount of the presence of heavy metallic species and other inorganic species may be retained in the tuibur solution.

ICP method is the most suited method that can be applied for the identification and quantification of the metal ions present in tuibur solutions at trace levels. Tuibur solutions of different concentrations collected from various sites of Mizoram were characterized and they

were quantitatively determined for various metal ion concentrations and the results were analyzed and compared with the updated certified reference material [US EPA, 2012]. The ICP-AES and ICP-MS data of tuibur solution shows that there is a wide distribution of concentrations of metals in tuibur solution depending on the manufacturing location. Measurements made on tuibur samples collected from different regions of Mizoram highlighted low levels of metals Ca, Mg, Cr, Mn, Ni, Cu, Fe, and Zn below the permissible limits.

On the other hand, measurements made on samples taken from different areas highlighted extremely high concentration of toxic inorganic species such as As, Cd and Pb above permissible levels and these three metals are toxic even at trace quantity. Furthermore, they are potent carcinogens, designated as Group I carcinogens [IARC, 2004]. The present study provides a reliable data on the concentration levels of various metal ions in commonly consumed tuibur solution. Hence, the results of this study would be helpful for biochemists, toxicologists and environmental chemists to evaluate the health effects of tuibur and their potential implications towards overdosing and/or bioaccumulation of toxic metal species among the tuibur consumers. Quantity of these trace elements in tobacco depends upon plant variety, water, type of soil, soil pH, applied fertilizers & pesticides besides the environment [Musharraf *et al*., 2012].

In commercially available tuibur solutions, there is trace amount of water insoluble tar components that exist as tiny droplets that always interact with tuibur solution. EPR spectrum of the putative paramagnetic species present in the tar (water immiscible dense layer) that was extracted into acetonitrile solution shows a sharp signal centered 3369.6 Gauss, with the g-value of 2.0018 and having a line width of 80 Gauss, without any discernable hyperfine features

indicative of the presence of free radicals in tar which may be arising from an organic system with extensive delocalization of the unpaired electron over the molecular structure.

The EPR spectral feature exhibited by the chloroform soluble fraction of the tar component of tuibur resembles very similar to the spectral feature shown by the EPR spectrum of tar that was extracted into acetonitrile solution, although the spectral intensity much higher than the acetonitrile soluble fraction and hence with better accuracy for the determination of the g-value (the centre magnetic field).The g-value of the putative paramagnetic species present in the tar that was extracted into chloroform solution is calculated as 2.0016 and 2.0013. The experimentally observed g-value indicates the presence of an organic free radical species and the g-value may be indicative of the admixture of excited state spin levels with the ground state levels. This observation also indicates that the putative organic radical present in the tar portion of tuibur (immiscible layer) is presumably an aromatic radical species that would commensurate with the stability of the observed organic free radical species as the polarity of acetonitrile is less than the polarity of chloroform and the solubility of the aromatic radical species is probably less in acetonitrile in comparison to the solubility of this species in choloroform.

The EPR spectrum of feedstock solution exhibited a broad spectral feature spread over 500 Gauss lacking any hyperfine splitting and it is centered at 3200 Gauss. The observed g-value is 2.1602, indicative the presence of a paramagnetic 3d transition metal ion species with half-filled d-orbitals.

Mass spectral analysis of various fractions arising from the LC-MS study on tuibur solution has indicated the presence of a wide range of heterocyclic species. As revealed by the

HPLC analysis (with APCI method), the prominent base ion (the most intense peak) as well as molecular ion peak belongs to nicotine with an m/z= 163.2. The major polar alkaloid component of tuibur is nicotine. It is a pharmacologically active and addictive alkaloid component of most of the smoke and smokeless tobacco products. When subjected to LC-MS/MS analysis with soft ESI method, the presence of other alkaloidal species, nornicotine along with the pyrolysis product of nicotine, myosmine in addition to the fragmented daughter ion of nicotine with m/z = 133 have been observed.

The most important findings of the present LC-MC study using mass spectral analysis is the detection of tobacco specific nitrosamines, N'- nitrosonornicotine (NNN), 4-methylnitrosamino-1-(3-pyridyl)-1-butanone (NNK) and its metabolite 4-(methylnitrosamino)-4-(3-pyridyl)-butanal (NNAL), which are classified as highly carcinogenic species. NNN and NNK are the most potent tobacco-specific nitrosamines in smokeless tobacco products & cigarette smoke [Hecht, 1999; 2002]. In contrast to nicotine, NNK and NNN may also induce cancer cell growth as it has been demonstrated that NNK can stimulate the growth of pulmonary adenocarcinoma through β-AR followed by COX-2 over expression. The FT-IR analysis of whole tuibur solution has indicated the presence of aromatic heterocyclic species with H-bonding.

In vitro studies using the *Allium* root test of tuibur provided supporting evidence for the carcinogenic nature of tuibur [Mahanta *et al.,* 1998]. The present study revealed that there is a strong association between mitochondrial control region (D-loop) polymorphism and tuibur consumption leading to stomach cancer risk in Mizo population. To our knowledge, the present work is the first experimental study that has provided the types of mtDNA D-loop mutations

culminating in the development of stomach cancer with respect to the carcinogenetic effects of tuibur. In conclusion, prolonged tuibur consumption and its manifestedmitochondrial gene alterations are believed to provide an alternative measure of stomach cancer risk in Mizoram population. The mtDNA analysis that probes D-loop alterations might help to identify individuals with tobacco consumption in general, and consumption of tuibur in particular, who are specifically at high risk for stomach cancer.

According to the findings reported here, the variations at the D-loop region of human mtDNA as detected by the number of different RFLP morphs may be useful for the detection of maternal inheritance (germline) as well as tobacco specific carcinogens containing tuibur solution induced DNA alterations (somatic), which may be further extended to human evolutionany studies. However, our findings must be validated with further functional studies involving large number of samples to invoke mtDNA as a potential biomarker for the early detection of stomach cancer in relation to lifestyle habits such as cigarette smoking, consumption of tuibur, etc.

In view of the presence of a wide range of chemical constituents in tuibur, as determined in the present study, it may have multiple, highly diverse adverse effects on human health. It is not unexpected that multiple xenobiotic chemicals present in tobacco smoke derived tuibur solution can contribute to any single adverse health effect. Hence, more detailed study, in the near future, using *in vivo* and *in vitro* models, is needed to be performed in order to ascertain the various mechanisms of cytotoxic, mutagenic and carcinogenic potential of tuibur.

It is also important to note that the habit of tuibur consumption has the potential to initiate and sustain tobacco dependence. It is expected that the toxic metals present in tuibur solution may elicit multiple adverse effects on human health as they are considered as cumulative metabolic poisons (bio-accumulation). Likewise, the other toxic chemical compounds found in tuibur solution may also adversely affect human health.

The populaces of Mizoram and Manipur are least knowledgeable with respect to the health risks associated with tuibur consumption, while tuibur sold in Mizoram mostly do not display any health warnings. In Mizoram and Manipur, surprisingly both illiterate as well as erudite are willing to believe that tuibur is a safe alternative nicotine delivery medium to cigarette smoking. However, there is no experimental evidence to support such presumption. In fact, tuibur consumption increases the risk of oral, pharyngeal, breast, stomach and uterine cancers [Phukan *et al*., 2004; Malakar *et al*., 2012]. Nevertheless, misconceptions about the benefits of using tuibur/hidakpu are widespread. In present study, the determination of toxic heavy metals and toxic chemical constituents in tuibur defies the misconception among the populace that tuibur consumption is innocuous. At present, sales of tuibur are mostly without health warnings and appropriate regulatory measures concerning tuibur sales are necessary for the benefit of public health. It is suggested that commercially sold tuibur in Mizoram and Manipur need to be labelled with appropriate warnings of potential toxicity risks due to the heavy metal content.

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List of Publications

(A) Journals:

1. Lalmuanpuii R., Ghatak S., Pautu J.L., Lallawmzuali D., Muthukumaran R.B., Kumar S.N., (2015). Mutation profiling in mitochondrial D-loop associated with stomach cancer and tobacco consumers. *J. Clin. Med. Genom*. **3(1) 1000122**: 1-5.

2. Muthukumaran R.B., Lalmuanpuii R., (2015). Ligand field analysis of cytochrome C-PPD adduct. *Science and Technology J*. **3(11):**61-66. ISSN: 2321-3388.

(B) Conferences/Symposiums:

1. Rebecca Lalmuanpuii, Muthukumaran R. Isolation of Xenobiotics of tuibur: An HPLC study. *Proceedings of One day National Seminar cum Training Progam on Green & Environmental Chemistry*, 30th March, 2011, Department of Chemistry, Mizoram University, Aizawl, Mizoram, India.

2. Rebecca Lalmuanpuii, N. Mohondas Singh. Comparative oscillator strengths of Praseodymium and Praseodymium mixed with L-Alanine in different solvents using 4f-4f transition Spectra as probe. *Proceedings of National Seminar on Environment, Biodiversity, Veda and Traditional Systems*, 10-12 April, 2012, Department of Zoology, Mizoram University, Aizawl, Mizoram, India.

3. Rebecca Lalmuanpuii, Souvik Ghatak, Khawlhring Lalsangmawia, Nachimuthu Senthilkumar, Jeremy L. Pautu, Doris Lallawmzuali, Rajendra Bose Muthukumaran. Phylogenetic relationship and gastric cancer related polymorphism among Mizoram, Northeast Indian Populations based on the Mitochondrial D-loop. *Proceedings of Internatinal Conference on Global Opportunity for Latest Developments in Chemistry and Technology-2014, (GOLD-CT-2014)*, 06-08 February, 2014, School of Chemical Sciences, North Maharashtra University, Jalgoan, India.

4. Rebecca Lalmuanpuii, Souvik Ghatak, Khawlhring Lalsangmawia, Nachimuthu Senthilkumar. Phylogenetic relationship and Stomach cancer related polymorphism among Mizoram, Northeast Indian Populations based on the Mitochondrial D-loop. *Proceedings of National Conference on Advances in Cancer Genomics*, 30-31 May, 2014, Mizoram State Cancer Institute, Aizawl, Mizoram and Department of Biotechnology, Mizoram University, Aizawl, Mizoram, India.

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Research Article Open Access

Mutation Profiling in Mitochondrial D-Loop Associated with Stomach Cancer and Tobacco Consumers

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Abstract

Within North East India, the people of Mizoram are mongoloid in origin and Cancer is a major disease condition among this tribal populace. A peculiar habit of consuming "*tuibur*" (tobacco smoke–infused aqueous solution) has been practiced in Mizoram. Blood and oral swab samples were collected from stomach cancer patients, tuibur consumers and healthy people. DNA was extracted followed by PCR amplification of the D-loop region of mtDNA. Restriction enzyme digestion of 1050 bp of the hyper variable control region of mtDNA was performed in order to gain an insight into the phylogenetic relationship of populace of Mizoram besides the genetic variations among tuibur consumers. The phylogram based on restriction enzyme analysis (*AluI, HaeIII*, *MspI* and *KpnI*) of the D-loop region subsumed within same mtDNA haplogroups and the markers resulted in a similar clustering of the population. The polymorphic samples were sequenced, analyzed and compared with the MITOMAP database. In the hypervariable control region, 292A>AA and 316C>CC are novel microsatellites instability as they have been reported for the first time as it has not been found in the mitochondrial database. 292A>AA position of MT-HV II region is a locus for the mtTF1 binding site Y. Due to the microsatellite instability of this position, the binding of mtTF1 may be altered. 316C>CC is present at the conserved sequence block II in MT-HV II region of human mitochondrial control region. The present study revealed a variety of mtDNA D-loop region mutations and polymorphisms among tuibur consumer besides stomach cancer patients of Mizoram, some of which might be involved in the development of carcinogenesis in stomach for the tuibur consumer.

Keywords: Mitochondrial DNA; Tuibur; Mizo- mongoloid; Polymorphisms; Phylogram

Introduction

The epidemiologic investigations have implicated various putative risk factors to the incidence of stomach cancer, albeit the etiology still remains unclear. However, it has been possible to identify certain risk factors that may contribute significantly to the cause of cancer leading to death. Most important among them are lifestyle habits, *viz*, tobacco consumption (in diverse forms) and diet. These two factors are especially important in the context of prevalence of various kinds of cancer [1]. While it is clear that diverse genetic pathways exist for the development and progression of various cancers, the emerging evidence strongly suggests that the pathogenesis of cancer is complex and multifactorial, ranging from the environment and dietary habits to genetic and molecular alterations [2].

Mitochondrial DNA is relatively more vulnerable to oxidative damage and it also exhibits higher mutation rate than nuclear DNA (nDNA) as a manifestation of the absence of protective histone proteins, limited DNA repair mechanisms as well as a high rate of Reactive Oxygen Species (ROS) generation due to oxidative phosphorylation [3]. The displacement loop (D-loop) is the only non-coding region (nucleotide position (np) 16024-576=1124 bp) of mitochondrial genome and is known to accumulate mutations at higher frequency than other regions [4]. It is a hot spot for mtDNA alterations comprising of two hyper variable regions (HVR) which are HVR1: nucleotide position 16024–16383 and HVR2: nucleotide position 57–333. In addition, D-loop contains crucial elements for replication and transcription of mtDNA [5]. Hence, sequence alterations in D-loop region may contribute to impaired replication and/or transcription of mitochondrial genes which may impede the overall mitochondrial function and exacerbates cellular ROS generation. Accumulation of D-loop alterations has been implicated in several complex human diseases [5-7]. D-loop region of the mtDNA is the most potent accumulation site for many of these mutations as numerous polymorphisms have also been reported in this region as it does not contain protective histones, exhibits high oxidative stress associated with deficient DNA repair mechanisms [8]. D-loop is the only non-coding mtDNA region which possesses crucial elements for replication and transcription. Thus, the sequence alterations of this region may contribute to altered replication or transcription properties [9,10].

Stomach cancer is the second leading cause of cancer death worldwide. In India, incidence of stomach cancer is the highest in Aizawl district of Mizoram with an age-adjusted rate (AAR) of 55.4 (male) and 24.4 (female) per 10^5 populations [11]. In fact, the incidence is comparable and in some cases higher than Japan, Korea, Chile and Costa Rica where these cancers were traditionally considered to be high [1]. The people of Mizoram are culturally and ethnically distinct from the other tribes and communities of India. A peculiar habit of consumption of "*tuibur*" (tobacco smoke–infused aqueous solution) has been observed in Mizoram. Therefore, correlation of consumption of tobacco with the incidence of stomach cancer in Mizoram cannot be

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ruled out [12]. The present study was undertaken to elicit the D-Loop gene polymorphism in stomach cancer patients and correlating with tobacco consumption in the 'liquid form' as tuibur.

Materials and Methods

Sampling

Blood samples were collected from 40 stomach cancer patients from Mizoram State Cancer Institute (Mizoram, India). Buccal swab samples were collected from 40 tuibur consumers and 40 non-tuibur consumers (without record of any disease). All volunteers were fully informed about the study and participated with their full consent. Detailed information on demographic factors, dietary habits, previous disease history, physical activity, tobacco habit, alcohol consumption, weight, and family history were collected from the study subjects using a standardized structured questionnaire. The age distribution of the three groups (tuibur consumer, control, and cancer patients) is ranging from 19 to 95, 19 to 74, and 40 to 78 years, respectively. The undertaken protocol were reviewed and approved by the Institutional Review Board of all institutes involved in the study. All sample were immediately stored under appropriate condition until further use.

DNA extraction and PCR amplification

DNA was extracted from the samples by following the modified protocol of Ghatak et al. (2013) [13]. The extracted DNA was dissolved in 1XTE buffer (pH 8.0) and stored at -20°C until used. DNA extracts was amplified for the mtDNA D-loop region by using primers HMt-F (5'CACCATTAGCACCCAAAGCT-3') and HMt-R (5'-CTGTTAAAAGTGCATACCGCCA-3') as described by Salas et al. (2001) [14] for the HVI region. PCR (vapo.protect; Eppendorf) was carried out in 25 µl total reaction volumes (containing 100 ng template DNA, 0.2 pM of each primer, 1X PCR buffer, 1.5 mM $MgCl_2$, 200 mM dNTPs, 1 unit Taq DNA polymerase). The reaction mixture was heated to 94°C for 5 min, followed by 40 cycles, each consisting of 1 min denaturation at 94°C, 1 min annealing at 63°C, 1.5 min extension at 72°C, and a final 10-min extension at 72°C. The PCR amplification products (10 µl) were subjected to electrophoresis (Bio-Rad) on 1.2% agarose gel in 1X Tris-acetate-EDTA buffer at 80 V for 30 min and stained with ethidium bromide (Himedia) and images were obtained in gel documentation (G-Box; Syngene, UK) system.

Restriction digestion of the PCR product

Restriction fragment-length polymorphism (RFLP) of the D-loop region was performed to check the polymorphism pattern. PCR products were digested with *Alu*I, *Hae*III, *Msp*I and *Kpn*I (Fermentas, Thermo Scientific) in a total volume of 10 µl (2 µl PCR products, 1 µl enzyme buffers, 1 U enzymes) and placed in the incubator at 37°C for 4 h. The restriction products were analyzed by electrophoresis (Bio-Rad) on a 10% polyacrylamide gel and the molecular weight of restricted fragments was analyzed by gel documentation system (G-Box; Syngene) after ethidium bromide (Himedia) staining [15].

Sequence and statistical analysis

PCR products (selected on the basis of polymorphism pattern) were sequenced from both the directions to ensure reading accuracy. The sequences are submitted in the EBI repository database (EBI Accession No. LN558427 - LN558437). Sequences and chromatograms obtained were examined using chromas software version 2.13 and aligned by BLAST (http:// www.ncbi.nlm.nih.gov/blast). Sequences were aligned using CLUSTAL W and mutations were noted by using MEGA software ver 5.0. All sequences were compared with the latest version of Revised Cambridge Reference Sequence (rCRS) of the human mitochondrial DNA (NC_012920) and subsequently analyzed for the variation in sequences using Mito Tool Programming. The results of DNA sequence analysis were compared with the published Cambridge Sequence using Mutation Surveyor version 1.4 DNA mutation analysis software (Softgenetics, State College, PA). Sequence differences between tuibur consumer, stomach cancer and healthy blood samples were recorded as mtDNA polymorphisms. Each polymorphism was then verified against the Mitomap database (http://www.mitomap.org/) and further classified as novel or reported, depending on whether or not it is recorded in the database. Genetic relationship analyses, based on the RFLP pattern, of stomach cancer, control and tuibur consumers were performed using NETWORK 4.6.1.2. All mtDNA sequences were aligned by Clustal W program according to the rCRS. MEGA 5.05 program was used to calculate the distance matrix [16]. Frequencies of D-loop polymorphism among the various groups were tested for Hardy–Weinberg equilibrium by a chi-square (χ^2) test with one degree of freedom (df). The polymorphisms within each group were estimated using odds ratios (ORs) and 95% confidence intervals (CIs). Logistic regression analyses were performed to compute the influence of tuibur consumption on stomach cancer risk. For all tests, a two-sided P-value<0.05 was considered statistically significant.

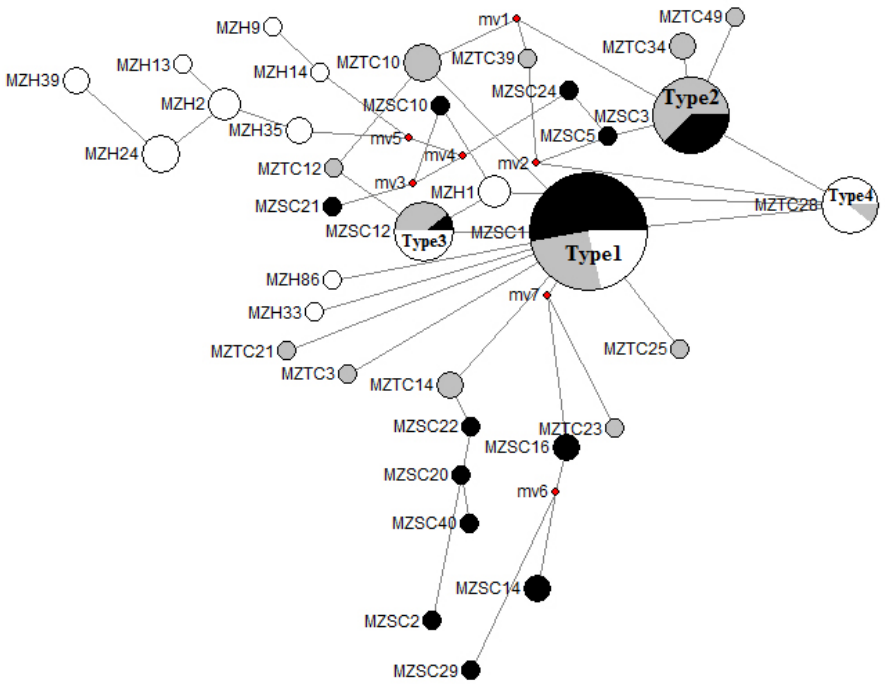
D-Loop secondary structure determination

Secondary structure of the mitochondrial control region was inferred from the D-loop sequences [17]. The application predicts the structure based on the free energy parameters and the enthalpies were measured at 37°C. However, they are assumed to be constant within the range of temperatures that might occur *in vivo* or in the laboratory. This enables the server to extrapolate free energies to other temperatures and to fold at these temperatures.

Results

PCR products for mtDNA D-loop region on agarose gel showed a fragment of about 1050 bp. Digestion of amplified fragments of mtDNA D-loop region by *HaeIII*, *AluI*, *MspI* and *KpnI* restriction endonucleases along with their rate of frequency are listed in Figure 1. Eight restriction patterns for *AluI* enzyme (with 5 heteroplasmy from tuibur and 2 heteroplasmy from cancer) and seven restriction patterns for *HaeIII* enzyme (with 3 heteroplasmy from tuibur consumer tuibur and 2 heteroplasmy from cancer) in Mizoram population were observed. The four chosen restriction enzymes exhibited 21 different polymorphic patterns. Moreover, *MspI* enzyme also showed two restriction patterns with 1 heteroplasmy variation in case of tuibur consumer, while four restriction patterns were revealed for *KpnI* enzyme. The network analysis showed frequency distribution of motifs in the population based on the PCR-RFLP product. Figure 1 also depicts that 37.5 % of stomach cancer patient and 62.5 % of tuibur consumers are clustering together.

A total of 27 mtDNA D-loop sequence variations were observed in 25% of samples. In Table 1 shows the sequence variation of the mtDNA D-loop region of the two different groups (tuibur consumer and stomach cancer patient samples), sample frequency, nucleotide position, mutant type and the name of mutation in comparison with the healthy (control) group. Among the stomach cancer and tuibur consumer samples, it has been found that the type of mutations are mostly transition base substitution with 55.55% T>C, C>T, A>G and G>A base substitution in stomach cancer and 92.86% T>C, C>T, A>G and G>A base substitution in tuibur consumer.



Group		Colour
Healthy Samples		White
Tuibur Consumer Samples		Gray
Cancer Patient Samples		Black
Type	Sample Name	Rate of Frequency
1	MZSC(20)	52.63 %
	MZTC(10)	26.31 %
	MZH(8)	21.05 %
2	MZSC(6)	37.5 %
	MZTC(10)	62.5 %
3	MZSC(1)	10 %
	MZTC(4)	40 %
	MZH(5)	50 %
4	MZTC(1)	11.11 %
	MZH(8)	88.88 %

Figure 1: Phylogenetic network illustrating the genetic affinities among three different sample groups.

Sample Name	Sample Frequency (%)	Mutation position and type
Sc 10, 24	18.18	16051A>G
T 40	9.10	16092T>C
		94G>A
		214A>G
Sc 1	9.10	16093T>C
		16266C>T
		16324T>C
		16355C>T
		16391G>A
		*292A>AA
T 1	9.10	16218C>T
		16289A>G
		16293A>G
		16526G>A
		183A>G
		184G>A
		185G>A
Sc 11	9.10	16234C>T
		153A>G
Sc 3,31, 33 T 24	36.36	16290C>T
		16319G>A
		235A>G
Sc 10	9.10	16298T>C
		16327C>T
Sc 11, 24	18.20	150C>T
Sc 3, 31, 33	27.30	151C>T
Sc 1 T 1	18.20	*316C>CC

Sc- Stomach cancer, T – Tuibur

***292A>AA and *316C>CC are novel mutations**

Table 1: Variations in D-loop region of stomach cancer and tuibur consumers.

The DNA sequence analysis elicited a correlation between tuibur consumer and stomach cancer patient mtDNA control region (HVR1 and HVR2) genomics. Three mutations, *viz.*, 16290C>T, 16319G>A and 235A>G were observed in mostly tuibur consumers along with stomach cancer samples and hence we can conclude that these three potent mutations might have occurred in tuibur consumer samples as the manifestations of mutagenic effects of tuibur. 292A>AA mutation is a potential pathogenic mutation for stomach cancer patient due to the alteration of TF1 binding site Y, while 316C>CC mutation is also exhibited by both tuibur consumers and stomach cancer patient samples, indicative of the potent pathogenic mutation among both groups.

Unique properties of mtDNA D-loop that makes it a very valuable tool for both evolutionary and human identification studies include the high copy number, cytoplasmic inheritance, and rapid rate of evolution. The tuibur consumers and stomach cancer samples were found to cluster together showing close similarity (Figure 1). Bivariate analysis of the three groups (stomach cancer, tuibur consumer and healthy control) revealed that the mutation frequency due to consumption of tuibur (OR: 2.33; 95% CI: 0.717–7.58; P<0.0003) was found to be a major risk factor for the development of gastric cancer.

The observed transition base substitutions were 16290C>T, 16319G>A and 235A>G with maximum frequency of 36.37%, besides a novel transition base insertion (316C>CC) was also found. For tuibur consumers and stomach cancer patients, the D-Loop secondary structures were similar and relatively unrelated from the healthy control specimen based on the major loop (Figure 2). These repeats are mainly grouped in the Control Region (CR) extremities, following the mini-satellite and near the L strand 3′ region. Inverted repeats may be tandemly or closely arranged forming hairpin structures by use of complementarity the parts, such as 5'ATGTACGGTAAATGGCTTT3', 5'CCAAAAGATAAAATTTGAAA3', 5'GGGGTGGCTT TGGAGTT3',

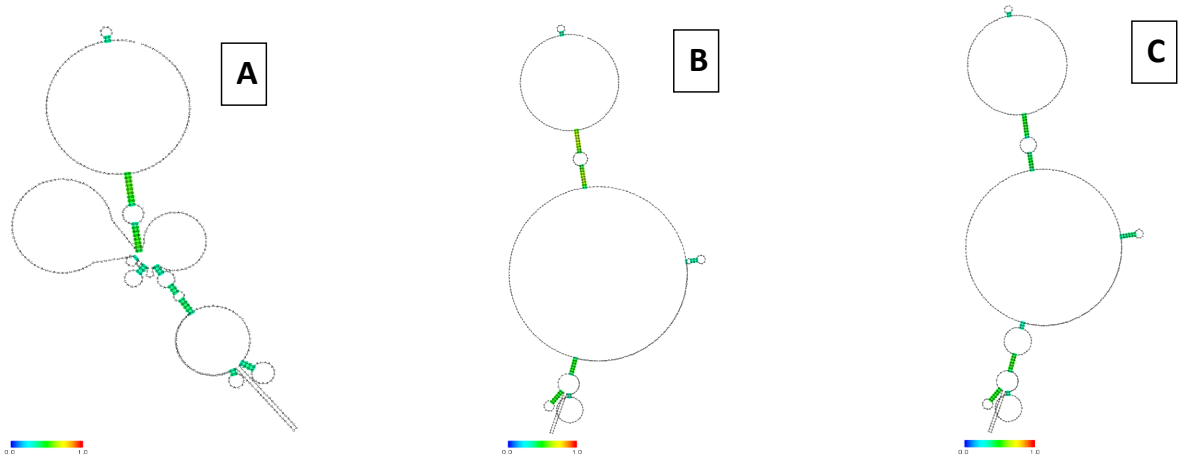


Figure 2: Secondary structures of Mitochondrial control region. (A) Healthy control, (B) Tuibur Consumer, (C) Stomach Cancer.

or separated by up to one hundred bases like 5'GGGGTGACTGTTAAAA GTG3'… 5'TTTTATGTACTACAGGT3'. Other repeats, even imperfect ones, systematically form stem-loop structures using MFOLD software.

Discussion

Tuibur consumers and stomach cancer patient sequences have shown close relationship and hence clustered together. Buccal swab samples of healthy control and tuibur consumers along with blood samples of stomach cancer patient samples were also analyzed, in the present study, for the identification of sequence variation in the mtDNA D-loop region. Although, the investigation of all sequence data have exhibited characteristic variations, yet it provides the essential information for defining the macrohaplogroups such as D, A, M and R. Haplogroups—A, M were common in the population, in all type of samples (cancer, tuibur and normal) in this population. All the healthy samples could be placed in macrohaplogroups—M or N and in the case of stomach cancer and tuibur samples it was R, D and A, interestingly, the present data suggest novel haplogroups—A, D and R.

PCR-RFLP analysis is a simple and robust technique that can be used as a potential tool for early detection of biomarkers in the study of tobacco specific carcinogens induced stomach cancer [15]. The PCR-RFLP results have shown a significant level of heteroplasmy and polymorphic patterns at the D-loop region of mtDNA in Mizo population among the following groups: tuibur consumer, healthy (control) volunteer and stomach cancer patient. It is important to note that 292A>AA and 316C>CC are novel base insertions (novel microsatellite instability) reported herein for the first time, as they have not been reported earlier in the mitochondrial database. 292A>AA position of MT-HV II region is a locus for the mtTF1 binding site Y and the microsatellite instability of this position is indicative of the impaired binding of mtTF1. 316C>CC is present at the conserved sequence block II in MT-HV II region of human mitochondrial control region. Due to the presence of microsatellite instability at this position, the function of the mitochondrial control region may also be altered.

The mutation sites identified, in the present study, appeared to be predisposed towards mutation. The D-loop control region sites such as 309C>T, 310T>C and 16223C>T are observed in almost every stomach patient specimen analyzed as it demonstrates near confluence in this small cohort. The non-coding displacement (D)-loop, especially a mononucleotide repeat (poly-C) between 303 and 315 nucleotides (D310), has been recently identified as a frequent hotspot for mutations in human neoplastic, including stomach cancer [18]. As such, this unique attribute may be considered as a potential biomarker for stomach cancer provided this behavior is consistent in transforming to blood. Functional studies are further needed to elucidate the biological significance of these mutant alleles in the tumorigenesis process of various tissues.

The search for the secondary structures also uncovered numerous stable inverted perfect or imperfect repeats that constantly turn into stable stem-loop structures when the DNA is stable and stranded for healthy control. It is worthwhile to note that all conserved segments depicted in fragments a-c form hairpin-like structures indicative of mirror like sequences for these conserved regions (e.g., inverted tandem repeats), but with the possible exception of healthy Control's CR, none of the CR (Stomach Cancer and Tuibur consumer) form stable secondary structures. Finally, two TCCC motifs exist in our sequence CR, but none of them are linked to the putative cloverleaf secondary structures. Taken together, confirmation rearrangements (CRs) of stomach cancer could not be divided into distinct conserved or variable domains, while tandem repeats of poly C stretch and conserved structural elements in the mid-region were observed. In the present study, we have observed that the G+C-rich region of stomach cancer and healthy (control) population besides a poly-C stretch is present which may be involved in transcriptional control or may be the site for initiation of replication that is consistent with the study reported by others [19]. For tuibur consumers and stomach cancer samples, the stem-loop secondary structures plus flanking sequences were completely identical and the stem was formed by a perfect match of 17 nucleotide pairs.

The state of Mizoram being one of the high risk/incidence regions of stomach cancer in India [20], few epidemiological studies have been carried out earlier to find out various potential risk factors for stomach cancer. It has been pointed out that the consumption of tuibur may be one of the important risk factors for the high prevalence of stomach cancer among the populace in Mizoram [12] which is consistent with the present study. Putative toxic effects of tuibur were also studied using modified version of Allium test [21-23]. Oxidative damage caused by smoking has also been shown to inhibit mitochondrial enzyme activity in platelets and cause mitochondrial dysfunction in alveolar macrophages [21]. In addition, an increase in mtDNA content and decline in mitochondrial function also occurs in response to DNA-

damaging agents, including tobacco related product [22]. Moreover, a recent study showed that tuibur consumers have three times higher risk of stomach cancer than tuibur non-consumers [20,24]. Xenobiotic species such as 2-aminonaphthalene, 4-aminobiphenyl, tobacco specific nitrosamines (TSNAs) which are thought to be responsible for many cancers [25], along with nicotine, nornicotine and myosmine might be also present in tuibur solution. As tuibur is not scientifically well characterized and only very limited health literature is available on the adverse effects of prolonged consumption of tuibur, the evaluation of potentially deleterious effects of tuibur solution on human health will be the focus of future research.

To our knowledge, the present work is an experimental study that has provided the types of mtDNA D-loop mutations in the development of stomach cancer with respect to the putative carcinogenetic effects of the tobacco smoke infused water (tuibur). The mtDNA analysis presented, in this study, has demonstrated that D-loop alterations may be helpful to identify individuals with tobacco consumption in general, and consumption of tuibur in particular, who are specifically at high risk for stomach cancer.

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Conflict of Interest

The authors report no conflict of interests. The authors alone are responsible for the content and writing of the paper.

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Ligand Field Analysis of Cytochrome C-PPD Adduct

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Abstract—Release of cytochrome c, from mitochondria, into cytosol is irreversible as it plays an essential role in mitochondria-dependent apoptosis. The redox state of cytochrome c would be important as the redox state change at the central metal ion elicit conformational changes that may be critical for cytochrome c to be an effective proapoptotic factor. Due to the interaction of PPD, a major metabolite of oltipraz, the change in the redox status of more than 90% of proapoptotic factor cytochrome c along with a small population (less than 10%) of cytochrome c in the altered coordination sphere with smaller g-anisotropy was observed by EPR spectroscopy (Velayutham et al., 2007). The determined g-values are indicative of a change in heme environment with the distal endogenous protein ligand displaced by an exogenous ligand with strong ligand field that exhibits moderate distortions which is manifested as smaller g-anisotropy. Using Taylor's formalism, the ligand field correlation analysis for the cytochrome c-PPD adduct was carried out and it was found that cytochrome c-PPD adduct was clustered in the P-domain exhibiting a larger tetragonality than native cytochrome c indicative of less electron density at the iron center in heme for cytochrome c-PPD adduct than cytochrome c.

Keywords: Cytochrome c, Low-Spin Heme, Tetragonality, Rhombicity

INTRODUCTION

Heme proteins are a special group of proteins containing iron porphyrin (heme) prosthetic group either covalently or noncovalently bound to the protein framework [1]. The heme prosthetic group is one of the most versatile and well characterized metal cofactor in biological systems, displaying a wide range of functions varying from electron transport to biochemical catalysis [1, 2]. C-type cytochromes are globular heme proteins widespread among almost all organisms, essentially involved in the electron transfer process [2]. The closest contact of prosthetic group (heme moiety) with the protein is facilitated by the axial ligands of metal ion, while for c-type hemes, further contact is through the thioether linkages generated by reaction of thiol groups of cysteine residues of protein backbone with vinyl groups of the heme moiety. Cytochrome c has been primarily known for its function in the mitochondrion as an electron transfer protein as it is loosely bound to the mitochondrial inner memebrane where it shuttles the electron between cytochrome c reductase (complex III) and cytochrome c oxidase (complex IV) during the oxidative phosphorylation process leading to the reduction of molecular oxygen to water with the consequent synthesis of ATP by ATP synthease (Complex V).

As the relatively planar core structure of all hemes is about identical, the functional properties of iron are tuned, to a large extent, with the modulation of electron density at the iron site, by the axial ligand(s). The proximal axial ligand of iron plays an important role in controlling the redox chemistry and the function of the various classes of hemoproteins. In c-type cytochromes, the proximal axial ligand of the heme iron atom is always a histidine. Either a histidine residue or a methionine residue occupies the sixth distal axial ligand position of the heme iron atom. When the sixth coordination is completed by the sulphur of distal methionine, c-type heme exhibits a characteristic weak absorption maximum in the visible region at 695 nm [3]. The structural characterization and variations in the redox state of central metal ion in heme proteins can be followed using a combination of electronic UV-visible absorption, magnetic circular dichroism (MCD) and electron paramagnetic resonance (EPR) spectroscopies [4].

EPR spectroscopy essentially provides important information on the spin state of ferric heme iron, besides the nature and orientation of the axial ligands of iron center [4, 5, 6]. During various functions of heme proteins, ligands are frequently removed or replaced in order to facilitate a wide array of bioactivities [1, 2]. EPR could provide important spectral data confirming the displacement of the distal axial

ligand to the heme iron and proportionate populations of such species as EPR spectroscopy is very sensitive to subtle variations in the coordination sphere, especially at the axial ligands of the redox metal center. Experimentally observed g-values in an EPR spectrum essentially describe the symmetry of metal coordination site and energy splittings of the nearest excited states of the d-electron system [6, 7]. In heme proteins, displacement of the endogenous distal protein ligand by an exogenous strong field ligand such as cyanide broadens the EPR signal of the resulting low-spin complex and as a consequence an increase in g-anisotropy is observed, where as heme proteins with imidazole as the sixth ligand have exhibited sharp EPR signals with smaller g-anisotropy [5, 7].

Apoptosis is a genetically regulated "programmed" cell death program, which is essential for multicellular organisms. It is necessary for the tissue homeostasis by mediating elimination of unwanted cells which can be removed for the benefit of the organism [8]. When exposed to various cellular stresses, cytochrome c is rapidly released from mitochondria into the cytosol for the activation of the cell death protease, caspase-3 in addition to interrupting electron-transport chain linkage leading to the prevention of oxidative phosphorylation process, promoting free-radical production besides depriving the production of ATP in the cell [8, 9].

Release of cytochrome c into cytosol is irreversible as it plays a major role in mitochondria-dependent apoptosis [10]. Peroxidation of mitochondrial cardiolipin by the oxidized cytochrome c perturbs the mitochondrial outer membrane permeabilization (MOMP), thereby promoting the release of cytochrome c and/or AIF into the cytosol [10, 11]. Experimental evidence also demonstrated that significantly decreased amounts of cytochrome c in the mitochondrial membrane meanwhile increased levels of cytochrome c at the cytoplasm is observed when HCG-27 (human gastric cancer) cells were treated with various doses of capsaicin [12]. For initiating the apoptosis process, the oxidized form of cytoplasmic cytochrome c (Fe^{3+}) can evoke caspase activation by binding with the protein Apaf1 leading to assembly of the heptameric apoptosome, while the reduced form (Fe^{2+}) cannot. Thus, the redox state of cytochrome c would be important as the redox state change at the central metal ion elicit conformational changes that may be critical for cytochrome c binding to Apaf-1 and procaspase-9 [13, 14].

Glutathione (GSH) is one of the major cellular antioxidants, a tripeptide with thiol functional group, in cells. In association with glutathione-stransferase (GST) and glutathione peroxidase (GPx), it plays central role in the protection against deleterious free radicals. Depletion of glutathione levels occurs during apoptosis and the decrease in the GSH levels due to various other reasons can also enhance the induction of apoptosis, in certain biological systems. While cancer chemoprotective agents, l,2-dithiole-3-thiones such as oltipraz would elicit elevated expression of the phase 2 protective enzymes along with increased levels of non-enzymatic glutathione, GSH, which are employed in the detoxification of carcinogens such as aflatoxins.

During its metabolism in humans, oltipraz generates a major metabolite, pyrrolopyrazinethione (PPD) which has similar strong phase 2 enzyme inducing capacity as oltipraz itself [15]. In an earlier study, due to the interaction of PPD, the change in the redox status of more than 90% of proapoptotic factor cytochrome c along with a small population (less than 10%) of cytochrome c in the altered coordination sphere with smaller g-anisotropy was observed by employing UV-visible and low temperature EPR methods [16]. This account amplifies and extends our earlier study on cytochrome c-PPD adduct and thus we are interested in carrying out the ligand field correlation analysis of the g-tensor that was observed in the previous study [16] using Taylor's formalism [17] with the following objectives: to establish the symmetry of coordination geometry of metal site along with the evaluation of the extent of energy splittings of nearest excited states of the d-electron system with the ground state and to gain basic insight into the corresponding electronic structure of metal site.

METHODOLOGY

A right-handed Cartesian coordinate axis system with the origin at Fe(III) center of the heme is considered for the present study as it is known as "improper" according to the definition of Taylor [17] as similar improper axis system was implemented by Blumberg and Peisach for the correlation analysis [18, 19]. As a result, for few proteins with larger g-anisotropy, v/Δ ratios of greater than 2/3 may be found. According to the improper axis system, the three experimentally measured g-tensor parameters are designated for the ligand field correlation as $g_{max} = g_y$, $g_{mid} = g_x$, and $g_{min} = g_z$. If the heme prosthetic group of a metalloprotein along the proximal and distal axial ligands exhibits axial g-anisotropy owing to the compression of axial ligand field, the d-orbitals along z-axis perpendicular to the porphyrin plane, *viz*., d_{xz} and d_{yz} would be equally destabilized by the amount Δ (tetragonal symmetry component) compared

with the orbital d_{xy} in the t_{2g} manifold. Further loss in the degeneracy of t_{2g} sub-manifold, *i.e.*, the separation of d_{xz} and d_{yz} orbitals attributed to the ligand field distortions along the x and y axes in the prophyrin plane, is quantified by the parameter, v (Fig. 1).

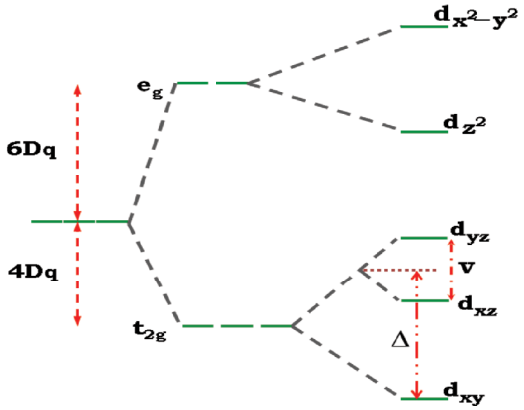

Fig. 1: Energy Level Diagram of the d-orbitals for Low-spin Ferric ion in Heme Proteins. Axial (Δ) and Rhombic (v) Ligand Field Distortions are also Indicated

Knowledge of the experimentally determined three g values enables the calculation of the two ligand field parameters, axial or tetragonal and rhombic splitting, Δ and v, respectively, in units of the spin-orbit coupling constant (λ). Since the variation of λ among various heme proteins is negligible, both the ligand field distortion parameters can be derived from the experimental EPR spectra. These ligand field parameters describe the axial and rhombic splittings of the one-electron orbitals depicted in Figure 1. A plot of the rhombicity (v/Δ) versus the tetragonal (Δ/λ) field for proteins containing heme iron displays data points for hemes of the similar ligand field strength and/or same ligation state fall within a region that can be clustered on the correlation diagram [18, 19]. From the observed g-values of low spin ferric iron, the strength of tetragonal and rhombic symmetry components of the crystal field (Δ and v, respectively) as ratios to the spin-orbit coupling energy, λ, can be determined. For the calculation of ligand field parameters, Δ and v, the equations obtained by Palmer based on Taylor formalism was employed [7]. Thus ligand field parameters v and Δ can be described by the energies of the one-electron orbital energies and also correlated with the determined g-anisotropy as follows:

$$\frac{v}{\lambda} = \frac{E_y}{\lambda} - \frac{E_x}{\lambda} = \frac{g_x}{g_z + g_y} + \frac{g_y}{g_z - g_x} \quad (1)$$

$$\frac{\Delta}{\lambda} = \frac{E_y}{\lambda} - \frac{E_x}{\lambda} - \frac{v}{2\lambda} = \frac{g_x}{g_z + g_y} + \frac{g_z}{g_y - g_x} - \frac{v}{2\lambda} \quad (2)$$

RESULTS AND DISCUSSION

If all six ligands surrounding the transition metal ion were equivalent, due to eletrostatic repulsion interaction, a splitting of the 3d orbitals into two sub-manifolds, labeled e_g and t_{2g} under O_h symmetry, would occur. Thus, the ground state of low-spin ferric heme is a Kramers doublet arising from a t_{2g} configuration with the unpaired electron occupying the d_{xy}, d_{xz} and d_{yz} orbitals. The relative energies of the t_{2g} and e_g orbitals of the central metal ion are modulated by both the axial ligands along with the porphyrin ring for the heme model complexes and heme proteins [7]. When the magnitude of electrostatic field due to axial ligands is different from the equatorial ligand(s) leading to a lower symmetry field, the lower sub-manifold of ferric ion is further split, the remaining orbital degeneracy of t_{2g} sub-manifold is completely lost (Fig. 1). Then, five d electrons of the low-spin ferric ion occupy the three lowest energy levels, leaving an unpaired electron in the d_{yz} orbital.

The distal ligand in the low-spin form of cytochrome c is displaced readily by the metabolite of oltipraz, PPD, as the change in coordination sphere at the metal site is clearly reflected in the low-spin EPR spectral features. The symmetry of ferric iron is perturbed in heme as the ligand at the sixth coordination position varies, causing distortions in the ligand-field strength which is manifested as the modulations of the experimentally observed rhombic g-values of low-spin heme. The addition of an equivalent of PPD to cytochrome c solution was sufficient to abolish the cytochrome spectrum completely with concomitant shifts of the EPR g-values, suggesting ligand exchange of distal methionine by dithiolthiolene moiety in PPD, which is further corroborated by the disappearance of 695 nm band in the visible region of the optical spectrum of cytochrome c-PPD adduct and confirmed that major metabolite of oltipraz, PPD is able to react with heme centre with the reduction of ferric iron to ferrous iron [16]. However, a small population of cytochrome c in its ferric low-spin form was detected by the low temperature EPR spectroscopy implying the presence of PPD occupying the distal coordination position of the heme iron site. The determined *g*-values are indicative of a change in heme environment with the distal endogenous protein ligand displaced by an exogenous ligand with strong ligand field that exhibits moderate distortions which is manifested as smaller g-anisotropy.

Using the ligand field correlation diagram, the spectral properties of EPR detectable low-spin Fe(III) species (cytochrome c-PPD adduct) can be compared with those observed in other heme protein(s) and model heme

compounds with similar ligands and coordination geometry to gain an insight into the identity of the axial ligands, their potential orientation with respect to the heme plane along with the Fe-N bond direction, and the electron density distribution at the metal center. In present study, we have attempted the ligand field correlation analysis for the cytochrome c-PPD adduct which displayed smaller g-anisotropy than cytochrome c that may be attributed to the modulation of the electronic environment of porphyrin and/or metal ion due to the binding of PPD to the metal ion in cytochrome c. For heme proteins exhibiting relatively smaller g-anisotropy, the ground electronic state is with a $(d_{xy})^2(d_{xz}d_{yz})^3$ electronic distribution [6, 7].

Estimated ligand field parameters, Δ and v, the tetragonal and rhombic splittings (Fig. 1) in units of the spin–orbit coupling parameter, λ, using Taylor's formalism, besides the measured g-values are shown in Table 1. We have generated a ligand field correlation diagram (rhombicity (v/Δ) vs tetragonality (Δ/λ)) similar to that of originally developed by Peisach and Blumberg [18] as it is also known as Peisach's "truth" diagram. In Peisach's correlation diagram, when hemoglobin and myoglobin species subjected to under various experimental conditions, their measured g-values clustered as C, B, H, O, and P groups. As demonstrated by Blumberg and Peisach [18, 19], the measured g-values of protein species falling within the O-region are only scattered owing to the experimental conditions but not due to the nature of protein environment. While the protein species under B-group where both the axial ligands are of imidazole containing histidines in which the ligating imino-nitrogen is bound with hydrogen whereas in H-group, the ligated imino-nitrogen is without the proton indicating that the central metal ion has relatively less electron density. The group of protein species falling under P-group has a set of axial ligands similar to that of cytochrome P450, while protein species of C-group have similar ligand environment of cytochrome c.

The ligand field analysis using the experimentally measured g-values for various protein species chosen (with different set of axial ligands) along with cytochrome-PPD adduct (Table-1) is displayed in Figure 2. Similar to the Peisach's "truth" diagram, we also observed the estimated ligand field parameters are clustering and falling into the following domains *viz*., C, B, O and P. The estimated ligand field parameters of horse heart cytochrome c were falling into the c-domain, whereas the mutant of cytochrome c (M→C), the imidazole adduct of cytochrome P450 along with the thiol adduct of ferric myoglobin are having similar N/S axial ligand environment can be found in the O-domain. The substitution of distal methionine (thioether side-chain) with cysteine (thiol side-chain) facilitates a larger tetragonality besides it also reduces the rhombicity. According to Blumberg and Peisach [19], rhombicity is purely a geometric term, while tetragonality represents the alteration of electron density at the ferric iron center of heme. Furthermore, displacement of water ligand which is occupying the sixth coordination site (axial) by thioether group of methionine (Table 1) in cytochrome P450 causes an increase in the rhombicity with a modest shift towards left side of tetragonality (i.e., a decrease in tetragonality).

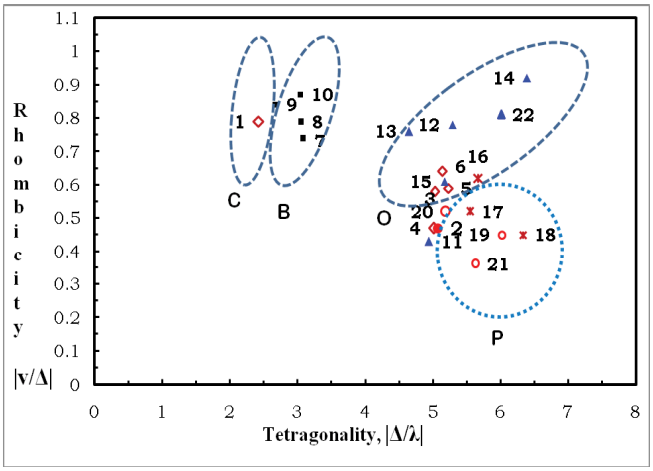

Fig. 2: Ligand Field Correlation Diagram for Various Low-spin Ferri-heme Proteins. The Figure has been Modified from that of Blumberg and Peisach [18, 19] to include Data on Cytochrome c-PPD Adduct Besides the Omission of H-domain. All Data Points are based on three g-values of Heme Proteins and the Label of Data Points is Indicated as in the Order of Table 1. Domain Classification (C, B, O and P) is based on the Ligand Field Parameter Calculations using the Measured g-values of Proteins. (÷)–Heme Proteins with N/S Axial Ligation; (o)–Heme Proteins with N/N Axial Ligation; (<)–Heme Proteins with N/O Axial Ligation; (▲)–Heme Proteins with S/S Axial Ligation ; (*)–Heme Proteins with S/O Axial Ligation and (•)–Cytochrome c-PPD Adduct

Interestingly, cytochrome c-PPD adduct along with SoxA protein, a c-type heme protein, found in *R. Sulfidophilum* [20], with thiolate/imidazole coordination is situated in the P-domain. In addition, these two cases, the estimated ligand field parameters exhibited lower rhombicity, (i.e., more axial) while cytochrome c-PPD adduct also exhibited larger tetragonality (i.e., shifted more towards right side) in comparison with cytochrome c (C-domain) indicating less π–electron density at the iron center for cytochrome c-PPD adduct. Similar observations were made by Blumberg and Peisach for myoglobin and sulfmyoglobin, where sulfmyoglobin was exhibiting a larger tetragonality compared with myoglobin [19].

Table 1: Experimentally Measured G-tensor values along with the Calculated Rhombicity (V/Δ), Tetragonal (Δ/λ) and Rhombic (V/λ) Splittings for Various Proteins with their Axial Ligands

Sl. No.	Protein/Variant	Ligation	g_{max}	g_{mid}	g_{min}	v/λ	$\lvert\Delta/\lambda\rvert$	$\lvert v/\Delta\rvert$	Ref.
1	Cyt c horse heart	His/met	3.071	2.234	1.257	1.91	2.42	0.79	16
2	Cyt c (horse heart)+ PPD	His/S^- (?)	2.539	2.308	1.87	2.35	5.01	0.47	16
3	Cyt c (m →c)	His/Cys	2.56	2.27	1.85	2.90	5.03	0.58	20
4	Cyt c SoxA	His/Cys	2.53	2.30	1.87	2.40	5.07	0.47	20
5	$P450_{cam}$-im	Cys/im	2.56	2.27	1.87	3.11	5.23	0.59	20
6	Metmb+HS^-	His/HS^-	2.56	2.24	1.84	3.29	5.14	0.64	21
7	Metmb-im	His/im	2.90	2.26	1.52	2.28	3.08	0.74	20
8	Cyt c+im	His/im	2.96	2.26	1.51	2.39	3.05	0.79	21
9	Cyt b_5 (housefly)	His/His	3.07	2.22	1.35	2.24	2.67	0.84	22
10	Cyt b_5 (barley)	His/His	3.02	2.22	1.48	2.64	3.04	0.87	22
11	c.chloroplast Hb	His/Tyr	2.52	2.31	1.86	2.1	4.94	0.43	23
12	LegHb+phenol	His/phenol	2.65	2.24	1.86	4.14	5.29	0.78	21
13	Catalase+im	Tyr/im	2.67	2.25	1.78	3.52	4.65	0.76	24
14	Metmb-OH^-	His/OH^-	2.59	2.17	1.88	5.87	6.39	0.92	21
15	LegHb- OH^-	His/ OH^-	2.54	2.24	1.84	3.15	5.17	0.61	21
16	P450-met	Cys/met	2.53	2.25	1.89	3.51	5.67	0.62	25
17	P450-dimethylsulp.	Cys/RS	2.50	2.27	1.89	2.86	5.55	0.52	25
18	P450-dimethyldisulp.	Cys/RSS^-	2.42	2.25	1.92	2.83	6.33	0.45	25
19	P450	Cys/OH_2	2.45	2.26	1.91	2.71	6.02	0.45	20
20	T. elongates cyt c_{550}	Cys/Tyr (?)	2.52	2.27	1.86	2.71	5.19	0.52	26
21	nNOS	Cys/ OH_2	2.43	2.28	1.89	2.01	5.63	0.36	20
22	wt Chlorite dismutase	His/OH^- (?)	2.56	2.19	1.87	4.83	6.00	0.81	27

The modulation of electron density at the ferric iron center with low-spin configuration, due to the variation of axial ligands, was also corroborated by Zoppellaro *et al.* [28] by performing ^{1}H-NMR and EPR experiments for a series of ferric low-spin cytochrome *c* mutants derived from *Pseudomonas aeruginosa* cytochrome c_{551} and *Nitrosomonas europaea* cytochrome c_{552}. Therefore, the lower g-anisotropy of cytochrome c-PPD adduct in comparison with native cytochrome c suggests that PPD, a major metabolite of the anticancer drug, oltipraz, may be acting as a π-acceptor.

CONCLUSION

In conclusion, the less π–electron density at the iron center for cytochrome c-PPD adduct, in comparison with cytochrome c, may be the manifestation of the nature of the axial ligands (proximal and distal) besides their orientation and position leading to the observation of lower rhombicity (i.e., more axial) along with larger tetragonality in the low temperature EPR spectrum of cytochrome c-PPD adduct.

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