# PHYSICO-CHEMICAL CHARACTERIZATION AND THE EVALUATION OF OXIDATIVE POTENTIAL OF *ZOZIAL*, THE INDIGENOUS HAND-ROLLED CIGARETTE OF MIZORAM

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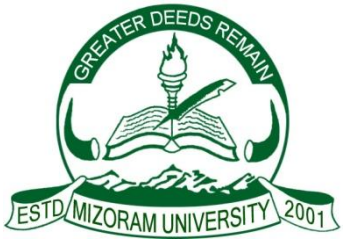


**DEPARTMENT OF CHEMISTRY**
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# PHYSICO-CHEMICAL CHARACTERIZATION AND THE EVALUATION OF OXIDATIVE POTENTIAL OF *ZOZIAL*, THE INDIGENOUS HAND-ROLLED CIGARETTE OF MIZORAM

**BY**

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Submitted

In partial fulfillment of the requirement of the Degree of Doctor of Philosophy in Chemistry of Mizoram University, Aizawl.

# MIZORAM UNIVERSITY

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## Thesis Certificate

This is hereby certified that the research work for the dissertation entitled "***Physico-Chemical Characterization and the Evaluation of Oxidative Potential of Zozial, the Indigenous Hand-rolled Cigarette of Mizoram***" submitted by ***Mr. K. Lalrammawia*** to Mizoram University, Tanhril, Aizawl, for the award of the Degree of Doctor of Philosophy in Chemistry is *bona fide* record of the research work carried by under my supervision. The contents of this dissertation, in full or in parts, have not been submitted to any other Institute or University for the award of any degree or diploma.

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I, **K.Lalrammawia**, 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.

This is being submitted to the Mizoram University for the degree of Doctor of Philosophy in Chemistry.

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**DEDICATION**

*To all the **SMOKERS** who have **QUITTED,** you all took a brilliant move.*

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(K.LALRAMMAWIA)

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## ABBREVIATIONS

| | |
|---|---|
| 8-OHdG | 8-hydroxy-2' –deoxyguanosine |
| AAS | Atomic absorption spectroscopy |
| ACT | Aqueous cigarette tar |
| AFS | Atomic Fluorescence Spectrometry |
| APCI | Atmospheric Pressure Chemical Ionization |
| AT | Average exposure time |
| ATSDR | Agency for Toxic Substances and Disease Registry |
| BSA | Bovine Serum Albumin |
| BW | Body weight |
| CAT | Catalase |
| CCD | Charge-coupled device |
| CID | Charge-injection device |
| CNS | Central nervous system |
| COPD | Chronic obstructive pulmonary disease |
| CORESTA | Cooperation Centre for Scientific Research Relative to Tobacco |
| CR | Carcinogenic Risk |
| CS | Cigarette smoke |
| CSC | Cigarette smoke condensate |
| CSF | Cancer Slope Factor |
| DDT | Dichlorodiphenyltrichloroethane |
| DMA | Dimethylarsonic acid |
| DNA | Deoxyribonucleic acid |
| ED | Exposure duration |
| EDI | Estimated Daily Intake |
| EFr | Exposure frequency |
| EFSA | European Food Safety Authority |
| ELISA | Enzyme-linked immunosorbent assay |
| EOM | Epithelial oral mucosa |
| EPR | Electron paramagnetic resonance |

| | |
|---|---|
| ESI | Electron-spray Ionization |
| ETS | Environmental tobacco smoke |
| ETV | Electrothermic vaporization |
| FAPAS | Food Analysis Performance Assessment Scheme |
| FCV | Flue Cured Virginia |
| FDA | Food and Drug Administration |
| FIR | Food ingestion rate |
| FISH | Fluorescent In Situ Hybridization |
| FTC | Federal trade conditions |
| GABA | γ-aminobutyric acid |
| GATS | Global Adult Tobacco Survey |
| GC | Gas Chromatography |
| GPX | Glutathione peroxidase |
| GRIN | Germplasm Resources Information Network |
| GSH | Glutathione |
| GSSG | Glutathione disulfide |
| HI | Hazard Index |
| HPLC | High-Performance Liquid Chromatography |
| HQ | Hazard Quotient |
| IARC | International Agency for Research on Cancer |
| ICP-AES | Inductively coupled plasma atomic emission spectroscopy |
| ICP-MS | Inductively coupled plasma mass spectroscopy |
| ICP-OES | Inductively coupled plasma optical emission spectroscopy |
| INAA | Instrumental neutron activation analysis |
| IRZ | Initial radiation zone |
| ISIRI | Institute of Standards and Industrial Research of Iran |
| *iso*-NNAL | 4-(methylnitrosamino)-4-(3-pyridyl) butanoic acid |
| *iso*-NNAL | 4-(methylnitrosamino)-4-(3-pyridyl)-l-butanol |
| IUPAC | International Union of Pure and Applied Chemistry |
| IUPHAR | International Union of Basic and Clinical Pharmacology |
| JECFA | Joint FAO/WHO Expert Committee on Food Additives |

| | |
|---|---|
| LA-ICP-OES | Laser ablation Inductively coupled plasma optical emission spectroscopy |
| LBC | Liquid-based cytology |
| LC | Liquid chromatography |
| LDL | Low-density lipoprotein |
| MAO | Monoamine oxidase |
| MC | Mean concentration |
| MDA | Malondialdehye |
| MMA | Monomethylarsonic acid |
| MN | Micronucleus |
| MS | Mass Spectrometry |
| NAB | *N*′-nitrosoanabasine |
| nAChR | Nicotinic acetylcholine receptors |
| NAT | *N*′-nitrosoanatabine |
| NAZ | Normal analytical zone |
| NCBI | National Center for Biotechnology Information |
| NEI | North-East India |
| NNA | 4-(methylnitrosamino)-4-(3-pyridyl) butanal |
| NNAL | Nicotine-derived nitrosaminoaldehyde |
| NNK | Nicotine-derived nitrosaminoketone |
| NNN | N'-nitrosonornicotine |
| NRCC | National Research Council of Canada |
| OS | Oxidative stress |
| PAH | Polycyclic aromatic hydrocarbons |
| PBCR | Population Based Cancer Registries |
| PHZ | Preheating zone |
| pLGIC | Pentameric ligand-gated ion channels |
| PMT | Photo-multiplier tube |
| PWS | Prader-Willi syndrome |
| RfD | Reference dose |
| RNS | Reactive nitrogen species |
| ROS | Reactive oxygen species |

| | |
|---|---|
| SOD | Superoxide dismutase |
| TDI | Tolerable Daily Intake |
| TEA | Thermal energy analyzer |
| THQ | Target Hazard Quotient |
| TMB | Tetramethylbenzidine |
| TSNA | Tobacco-specific nitrosamines |
| US EPA | United States Environmental Protection Agency |
| UV-VIS | Ultraviolet-visible Spectroscopy |
| WHO | World Health Organization |
| WST | Water-soluble tetrazolium salt |
| XO | Xanthine oxidase |
| XRF | X-ray fluorescence spectroscopy |
| β –AR | β-adrenergic receptors |

## DISCLAIMER

The findings of this Research Study in any way are **NOT PROMOTING** smoking. The findings also are **NOT PROMOTING** any smoking tobacco product over other tobacco product.

# CHAPTER - 1

## INTRODUCTION AND REVIEW OF LITERATURE

# CHAPTER - 1

# INTRODUCTION AND REVIEW OF LITERATURE

## 1.1 Background and Review of Literature

A plant, who's Global Market value accounted for $663.76 billion in 2019 (Market Watch, 2019), whereas on the other hand leaving ~ 7 million deaths per year, Tobacco is indeed a "Valuable Assassin". Belonging to a *Nicotiana* of the Solanaceae (nightshade) family, the genus consists of 72 species according to the Germplasm Resources Information Network (GRIN) taxonomy for plants. This cash crop was known to originate in the American continent and was exclusively consumed by the Native Americans. It was introduced to the rest of the world during the 15$^{th}$ and 16$^{th}$ century by the European explorers.

Tobacco cultivation has started around 8000 years ago. Now, tobacco is an agricultural plant which is multi-purpose in terms of its usage, cultivated in all continents. In India, tobacco was introduced around 16$^{th}$ century by the Portuguese. The first cultivation was carried on at Botanical Garden Sidburah, Calcutta (now Kolkata) in the year 1787. The Government of India established the Tobacco Board under the Tobacco Board Act 1975 (which came into existence on 1$^{st}$ January, 1976) to take full responsibility on tobacco related issues such as cultivation, production, marketing and control (Prasad, 2007). Tobacco industry since then has been growing in India with the introduction and collaboration with several international companies. India has been among the top largest producer of tobacco in the world next to China and Brazil.

The availability of different kinds of tobacco plants globally depends on the nature and conditions of its cultivation. Tobacco has been one of the most important commercial crops grown in India. 45.7 million People are either directly or indirectly involved in tobacco industry. It contributed ₹22,737.07 crore (2016-17) as excise duty and ₹ 5,539.94 crore as foreign exchanges to the National treasury during 2017-

18. India produced different varieties of tobacco with a majority being FCV (Flue Cured Virginia) and exported to over 114 countries globally. These varieties of tobacco including FCV vary in their physical and chemical attributes (Tobacco Board India, 2018). Andhra Pradesh, Karnataka, Gujarat, Maharashtra, Bihar and Tamil Nadu are the main tobacco-producing states (WHO, 2004).

Aside from the common uses of tobacco in products such as cigarettes, pipes, snuffs, cigars, chewing tobacco, etc., (Al-Ibrahim *et al*., 1990), there are several unique and traditional methods by which tobacco is consumed around the world by the populations of different cultures and regions. Smoking alone leads to > 8 million deaths annually (WHO, 2019). The current global incidence of smoking in men is 25%, where 3 Asian countries - China, India, and Indonesia contributes to half of that population (Yang *et al*., 2019). A volatile alkaloid, nicotine, which is a colorless compound, is present in tobacco. Nicotine is responsible for stimulation of the brain functions, increasing blood pressure, constriction of peripheral blood vessels and affects the heart rate. The top causes of death on a global scale, according to WHO, includes cases such as heart diseases, stroke, chronic pulmonary diseases, lower respiratory infection, trachea, bronchus, lung cancer, diabetes, tuberculosis are all known to be associated with tobacco. Tobacco use reflects negatively on the global economy, with an estimated ~ US$ 1.4 trillion annually, that includes higher health-care costs, lost productivity, fire damage, environmental damage and destructive farming practices (WHO, 2020).

On the contrary, the yin and yang of tobacco plant must also be taken into consideration. Tobacco has also been known to serve as traditional medicine all over the world. The medicinal value of tobacco was first observed by Columbus in the New World (Charlton, 2004). Tobacco serves as a good natural pest control and insect repellant as the smell is quickly rejected by these organisms. Tobacco has also been known to have some medicinal values. This “holy herb” was known to express curative properties in treatment of a wide range of disorders. It was used as a painkiller for problems such as earache, toothache and treatment of inflammation and soreness. In Mizoram, juices extracted from tobacco is also used for treatment of

pig's skin disease (similar to leucoderma) (Bhardwaj and Gakhar, 2004). Smoking was enunciated 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).

**Classification information**

Domain: Eukarya

Kingdom: Plantae

Phylum: Magnoliophyta

Class: Magnoliopsida

Order: Solanales

Family: Solanaceae

Genus: *Nicotiana*

Species: *Nicotiana tabacum*

**Figure 1.1**: *Nicotiana tabacum*.

The global prime method of consuming tobacco is smoking by means of cigarettes, pipes, and cigars (Al-Ibrahim *et al*., 1990). In commercial cigarettes, while processing tobacco leaves are frequently mixed with few 'chemical' additives and subsequently marketed for consumption (Wingand, 2006). During smoking, the glowing tip of the lighted cigarette burning with temperature reaching ~ 900°C (1600-1800°F) generates a hot mixture of gases and several microscopic particulate matter entering the smoker's lungs. The resulting active substances initiate chemical reactions in nerve endings enhancing the heart rate, memory, alertness (Parrot and Winder, 1989), and reaction time (Parkin *et al*., 1998). The International Agency for Research on Cancer (IARC) has identified cigarette smoking as the major causative factor of cancer, prominently, at organ sites than any other human carcinogens (IARC, 2004). Cigarette smoke is known to induce apoptosis in cells when exposed at increased concentrations (Ramage *et al*., 2006; Ishii *et al*., 2001; Hoshino *et al*., 2001). Smoking is known to cause inflammatory diseases such as COPD (Chronic obstructive pulmonary disease) and asthma and further foster cellular 'functional' alterations leading to tumorigenesis (Koczulla *et al*., 2010).

Worldwide, among the smokers, men are more liable to smoking related morbidity and mortality as compared to women (Guindon and Boisclair, 2003), though at younger age there is little or less difference between gender (WHO, 2001; Satcher, 2001). The rate of addiction of smoking cigarettes is higher in the population of developing countries than those of developed countries (WHO/WPRO, 2002). Many smokers begin their habit at an early stage as at these stages, smoking provides satisfying ambiance. Quitter experiences withdrawal symptoms such as anxiety, stress and an intense craving for nicotine since they are habituated to nicotine for an extended period of time. It is a well established fact that nicotine is effectively addictive and it is the prime reason behind tobacco addiction (Benowitz, 1988). Nicotine constitute approximately 0.6-3.0 % (30 mg/g) of the dry weight of the tobacco plant (Hoffmann and Hoffmann, 2012).

Nicotine is frequently exploited naturally occurring stimulant and a cholinergic receptor stimulating alkaloid found in plants belonging to the nightshade

family. Distilled nicotine inhaled from cigarette smoke enters the lung and gets rapidly absorbed by the pulmonary veins. The arterial circulation then quickly transfers the nicotine to the brain where it binds to nicotine cholinergic receptors opening a channel that permits the entry of sodium or calcium (Dajas *et al*., 2004). These cations stimulate the activation of voltage-dependent calcium channels further allowing the entry of more calcium (Laviolette and Vander, 2004; Benowitz, 1990; 2009) which leads to the release of neurotransmitters (Dajas *et al*., 2004; Wonnacott, 1997). The other constituents of cigarette smoke also add to nicotine addiction. Monoamine oxidases, enzymes located in catecholaminergic and other neurons, facilitate the metabolism of dopamine, norepinephrine, and serotonin. The addictiveness of smoking is also known to depend on the inhibition of monoamine oxidase (MAO) reducing the metabolism of dopamine (Fowler *et al*., 2003; Lewis *et al*., 2007).

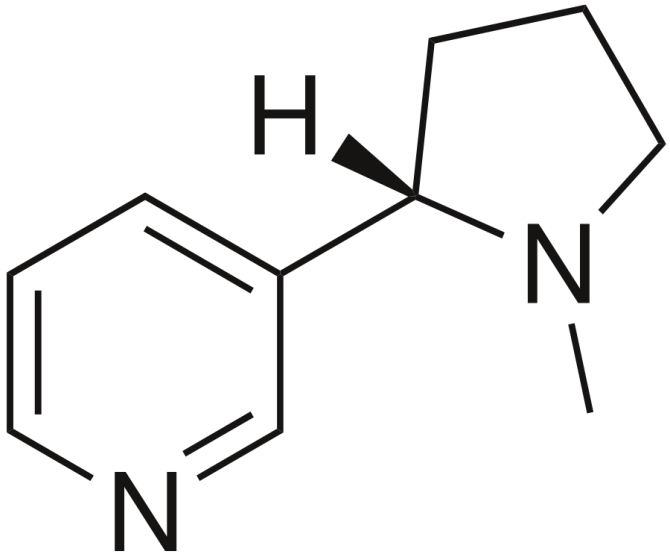



**Figure 1.2**: Molecular structure of Nicotine.

Nicotine is a strong parasympathomimetic alkaloid present in plants belonging to the nightshade family (Solanaceae) and considered a milder stimulant drug (IUPHAR, 2014). It is produced in the roots and accumulates in the leaves. Nicotine is found specifically in the leaves of *Nicotiana tabacum* and *Nicotiana rustica* and also in *Duboisia hopwoodii* and *Asclepias syriaca* (Metcalf, 2007). According to the International Union of Pure and Applied Chemistry (IUPAC) nomenclature, nicotine is known as 3-(1-methyl-2-pyrrolidinyl) pyridine. The molecular formula of nicotine is $C_{10}H_{14}N_2$ and is a bicyclic compound consists a pyridine cycle and a pyrrolidine cycle (Figure 1.2). Nicotine belongs to a class of organic compounds known as pyrrolidinylpyridines (a ring system consisting of pyrrolidine ring linked to pyridine ring). It is strong basic colorless liquid compound which may also exist in solid form at a very low temperature. It is hygroscopic and miscible with water at temperature below 60°C and highly soluble in alcohol, chloroform, ether, petroleum ether, kerosene and oils (NCBI, 2020). Due to its asymmetry, it is optically active and exists in two enantiomeric forms. Nicotine is levorotatory in its natural form [(–)-nicotine] which is physiologically more active than its dextrorotatory form [(+)-nicotine]. (–)-nicotine is also more toxic than (+)-nicotine (Gause, 1941). The salts of (+)-nicotine are usually dextrorotatory (Henry, 1949).

Nicotine is by far one of the most addictive and psychoactive agents. The mechanism of its effect on the Central nervous system (CNS) is through interaction with nicotinic acetylcholine receptors (nAChRs). The interaction with these pentameric ligand-gated ion channels (pLGICs) initiates a rotation in the receptor leading to the opening of the entire cation channel (Unwin, 2003). Neuronal nAChRs are diversified, with at least six α (α2–α7) and three β (β2–β4) nAChR subgroups in mammalian neurons. The assembly of such homomeric (e.g. α7 nAChR) or heteromeric (e.g. α4β2 nAChR) receptors results in the production of several nAChR subtypes of nAChR which differs in their pharmacological and biophysical aspects, such as nicotine sensitivity and rate of desensitization (Le Novèjre *et al.*, 2002). The relative frequency of α4β2 nAChR subtype in the brain and the high affinity of nicotine towards α4β2 nAChR subtype receptors have led to the assumption that it

might be a major target mediating the psychoactive properties of nicotine. Upregulation of α4β2 nAChR binding sites in the brain was observed in rodents due to regular exposure to nicotine (Gentry and Lukas, 2002), which is a common observation on postmortem results of brains of human smokers (Benwell *et al*., 1988; Breese *et al*., 1997). Intense increase in the prefrontal cortex, thalamus, and visual system followed with the activation of corticobasal ganglia-thalamic brain circuits due to the effect of nicotine has been observed (Brody, 2006). Stimulation of central nAChRs by nicotine causes the release of dopamine in the mesolimbic area, the corpus striatum, and the frontal cortex by stimulating the central nAChRs. 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).

Apart from dopamine, other neurotransmitters such as norepinephrine, acetylcholine, serotonin, γ-aminobutyric acid (GABA), glutamate and endorphins are also released as a result of nicotine stimulation. Despite the direct release of neurotransmitters, significantly, most of the nicotine-mediated release of neurotransmitters occurs via modulation by presynaptic nAChRs (Wonnacott, 1997). Dopamine release is facilitated by nicotine-mediated augmentation of glutamate release and, with long term treatment, by the inhibition of GABA release (Mansvelder, 2002). Studies in animals on the development of brain suggest that nicotine can induce permanent alterations leading to addiction, increasing the risk of dependence when smoking begins at an early age (Bonnie *et al*., 1994). On exposure to nicotine, the changes in brain of adolescent rats are greater than those of adult rats. Adolescent rats experiencing nicotine exposure have higher rates of nicotine self-administration, which is in accordance with the perception that early exposure to nicotine increases the intensity of addiction (Placzek *et al*., 2009; Dwyer *et al*., 2009).

To satiate the nicotine addiction by the ‘addicts’, a wide variety of smoking and smokeless products are consumed all over the world. In India, the main use of tobacco is for smoking or chewing purposes regardless of age, sex and socio-

economic background either for pleasure or for ritual purpose, or for self medication or out of habit due to addiction (Verma *et al*., 2010). In India, the frequency of tobacco consumption has been reported to be considerably higher in the Northeast India (NEI) compared with the rest of India (GATS, 2017). Situated in the Northeastern corner of India, Mizoram is located between 92°16‟ to 93°26‟ E longitude and 21°56‟ to 24°31‟ N latitude, bordering Myanmar in the east and Bangladesh in the west (Phukan *et al*., 2005). With a land area covering 21,081 sq. km it has a population of 1,091,014 (2011 census). The Mizo people, believed to have their ancestral origin in China (Thawnga, 1986), are culturally and ethnically distinct from other communities of India (Phukan *et al*., 2005). The study on prevalence of tobacco use suggests that 58.7% of Mizo population is engaged in tobacco consumption (GATS, 2017). Latest Population Based Cancer Registries (PBCR, 2014) data of Mizoram indicated that gastric cancer accounts for 18.5% in males and 11.3% in females and that of Lung cancer is 14.1% in males and 15.6% in females. Healthcare professionals have called for increased advocacy for stringent tobacco control and regulation.

Tobacco use, including exposure to passive smoking has also been implicated worldwide as a causal or contributory agent in an ever-expanding list of cancers. The majority of the population in Mizoram consumes one or the other form(s) of a wide range of smoke and/or smokeless products of tobacco (Phukan *et al*., 2005). ‘*Meizial’* is the local name for branded cigarettes of India and Myanmar, ‘*zozial’* is a local hand-made cigarette available in Mizoram, it is hand-rolled and without cellulose acetate type filter that is normally found in the machine made, commercially produced, branded cigarettes. The unique feature of zozial when compared to factory-made cigarettes is the indigenous way by which it is processed. The tobacco is cultivated, cured and processed under controlled environment for producing the automated machine made (factory) commercial cigarettes. On the other hand, for making zozial, the matured tobacco leaves are, in general, plucked, dried under direct sunlight for a short while, subsequently squeezed manually by using feet and is then sun-cured. Tobacco curing is also known as color curing as the main purpose of curing is to change the color of the leaves by reducing

their chlorophyll content. The left over tobacco on the feet of the person were also added to leafy components following the removal of petiole and the main vein on the tobacco leaves. The hand rolled zozial also does not have any filter to remove the particulate matter from the mainstream smoke and the left over tobacco on the feet of the person were also added when rolled as it is said to improve the flavor of the cigarette. Many organic and inorganic chemicals in the volatile gaseous and particulate phases of tobacco smoke appear to contribute to its toxicity. Due to the absence of the putative 'protective' filter in *zozial*, concentration of long-lived organic radical species in the particulate phase, known as 'tar' (water insoluble species which are relatively non-polar), would be significantly high compared with commercial cigarette smoke.

**Figure 1.3**: Vaihlo fun – a bag of ready to smoke tobacco along with rolling paper.

Tobacco, in various forms, has played an important role in Mizo society for ages. The Mizo people grew their own tobacco to meet their needs. Looking back at the history of Mizo culture, sharing of tobacco in the form of zozial, tuibur, etc were looked upon as a sign of approval and favoritism within the community. It has been deeply rooted and has been a normal practice in the Mizo culture that a group of young men would court a lady at night at her house, during which, if the lady offers her hand rolled '*zozial'* that is knotted with her hair, then, that is a sign that she is interested on that particular man. The color of the string being used to knot the zozial would also play an important role in describing the quality of the zozial. A bag of tobacco along with rolling paper called 'vaihlo fun' (Figure 1.3) is a 'must carry' item for Mizo farm workers. There is a common saying in Mizo society that forgetting your lunch pack can be excused, but forgetting your tobacco pack is unforgivable. They would even go a long way back to get their forgotten tobacco pack while they hardly would go back for their lunch pack! Tobacco plants were cultivated for various reasons, other than being consumed; majority of the Mizo population being farmers, the practice of cultivating tobacco plant along their field was a good natural pesticide for their crops. As medication, tobacco leaves were crushed and the juices were used as first aid measures for fresh cuts, treatments of boils and even as mosquito and leech repellant.

Programmes concerning awareness on tobacco and its products initiated in Mizoram only on the year 1985 but implementation were not on its best. Smoking at public places and gatherings were still a common sight till the early 2000s. It is pertinent to mention here that zozial, made on a cottage industrial scale and sold in Mizoram does not have any health warnings concerning its consumption till date. Zozial smoking is considered to putatively pose a major health risk due to the potential synergistic effects of various xenobiotic compounds that are potentially hazardous to ingest and it is imperative to carry out a detailed scientific study on the oxidative/carcinogenic effects of heavy metal species in the presence of various tobacco-specific carcinogens.

More akin to the poor man 'cigarette' i.e., bidis, zozial has rather low combustibility and un-perforated nature of its rolling paper in addition to the absence of cellulose acetate filter. It requires more frequent, recurrent and deeper puffs to keep it lit which may be consequently grueling on the smoker's lungs than commercial cigarette smokers (Kamboj, 2008). Bidis are reported to produce equal or higher levels of nicotine, tar, and other toxic chemicals (Gupta and Asma, 2008). The specific association between bidi smoking and lung cancer has been analyzed and reported (Notani *et al*., 1977; Jindal *et al*., 1982; Malhotra *et al*., 1986; Prasad *et al*., 1998; Gupta *et al*., 2001; Gajalakshmi *et al*., 2003). The indigenous manner of processing zozial differs from other hand-rolled cigarettes that still contain additives and other noxious chemicals (FDA, 2018). Particulate matter generated by tobacco smoke has been reported to alter the iron equilibrium in lungs (Ghio *et al*., 2008). The presence of polycyclic aromatic hydrocarbons (PAHs) in cigarette smoke has been reported (Selikth, 1980) and PAHs are proven Group-I carcinogens (Hoffmann *et al*., 1976).

Other tobacco products such as *tuibur* (an aqueous, smoked infused alkaline tobacco product) and *sahdah* (a moist snus containing raw tobacco mixed with slaked lime) are also a common practice in Mizoram. Consumption of *betel quid* (Areca nut along with betel leaf and a small quantity of slaked lime paste) has been classified as carcinogenic (IARC, 2004) and is a regular practice along with smoking. Another common tobacco product known as *zarda paan* (mixture of dried and boiled tobacco leaves, limes, areca nut, additives, spices and tannins) has also been reported to contribute to development of oral cancer (Shah *et al*., 2012). Apart from unique tobacco consumptions, the NEI population also has a few distinctive diet compositions as compared to other parts of India. In Mizoram, consumption of sa-um, a fermented pig fat (indigenously fermented using bottle gourd) is a common side dish (added to traditional vegetable soup or mixed with chilley flakes as chutney). Smoked meat and smoked vegetables (cured by smoking using firewood) are also common food items.

In order to meet the demand due to increasing global population, farmers apply chemical fertilizers to soils to improve the quality and yield of crops annually.

Pesticides herbicides, insecticides, etc are also sprayed on crops to manage plant, animal and insect pests. These chemical species may eventually enter the crop system by uptake of certain quantities by roots from the soil or through the stems and foliar deposition in case of spraying process. Post-harvest processing of crops also results in deposition of externally applied chemicals. Some of these chemicals are noxious even at low volume of consumption to the human consumers. Correlations between some of these chemicals and several diseases in man have been established (Aminu, 1997; Lindsay and Hugh, 2001). Previous studies have shown that over 4000 chemicals including toxic metals are present in cigarettes, many of which are egregiously well established carcinogens (Sawyer *et al*., 1990; Hoffmann and Hoffmann, 1997). Chemicals present in cigarettes and cigarette smoke are known to develop not only cancer but also other critical health effects (Hoffmann and Hoffmann, 1997).

The detrimental effects of DDT using animal model indicated that DDT induced infertility (Jonsson *et al*., 1976), a decrease in the number of implanted ova (Lundberg, 1974), intrauterine growth subnormality (Fabro *et al*., 1984), cancer (Cabral *et al*., 1982), neurologic developmental disorders (Eriksson *et al*., 1990), and mortality (Clement and Okey, 1974). Heavy elemental species such as copper, arsenic, nickel, cadmium, zinc and lead, and other chemicals like nicotine, tar, ammonia, acetone, butane, formaldehyde, sulphuric acid, freon, geramic acid, and methoprene can also cause similar reproductive functional disruptions. Few among these chemical species are augmented as additives in cigarette tobacco matter by the manufacturer (Challa and Kalla, 1995).

Cigarette smoke is a highly complex aerosol that consists of a broad range of chemical species dispersed in both the gas and the particulate phases. The particulate phase contains dense microscopic droplets that condense into particles (semivolatile and non-volatile tobacco constituents without water and nicotine) with size ranging from 0.1 – 1.0 μm that normally gets trapped in the filter of cigarette. The gas phase of tobacco smoke is oxidizing, while the tar phase is reducing (Halliwell and Poulsen, 2006). The water soluble oxidants of cigarette smoke can rapidly reach

both the systemic circulation and thus they can directly promote vascular oxidative stress (Csiszar *et al.*, 2008), leading to various smoke-related degenerative diseases.

The tobacco employed for the consumption as *zozial* is grown exclusively within the state of Mizoram in 'organic' manner, *viz.*, without the application of fertilizers and pesticides. Among the various species of tobacco, *Nicotiana tabacum* species are grown and used for producing *zozial*. Tobacco plants are known to uptake and transport essential and nonessential elements predominantly as ions from the soil through the roots, subsequently sequester the nonessential elemental species predominantly in the leaves (Lougon-Moulin *et al*., 2004). Cigarette smoke is known to contain essential ionic species such as $Na^+$, $K^+$, $Ca^{2+}$, $Mg^{2+}$ (in molar concentrations) and $Fe^{2+}$ (in millimolar concentration). Nonessential heavy metals like Cadmium ($Cd^{2+}$), Lead ($Pb^{2+}$), Mercury (Hg) and Chromium (Cr) along with the metalloid species Arsenic ($AsO_3^-$, $AsO_5^-$) are also known to be present in tobacco. Heavy metals are generally defined as metals that have a relatively high density compared to water and are naturally occurring elements that are found throughout the earth's crust (Fergusson, 1990). Most heavy metals are known to be cancer-inducing agents (Tchounwou *et al*., 2012). Heavy metals also include metalloids, such as arsenic, that are capable of inducing toxicity even at low exposure level (Duffus, 2002). Metal concentrations in tobacco have been found to be influenced by factors such as soil type, soil pH, plant genotype, stalk position, and soil and leaf residues resulting from the application of pesticides/fungicides containing metals and from soil amendments, including fertilizers and municipal sludge (Karaivazoglou *et al*., 2007). Soil pH is generally the most important factor determining plant uptake of trace metals, whose mobility is most often greater under acidic conditions (Gondola and Kadar, 1994; Golia *et al*., 2007). Therefore, heavy-metal contamination is likely to be a bigger concern in acidic soils. Depending on the soil properties, the chemical forms of metals in soil can vary and influence plant uptake (Lehoczky and Kiss, 2002).

Despite the natural availability of heavy metals throughout the earth's crust, majority of environmental contamination and the concomitant human exposure are

the outcome of several anthropogenic activities, domestic and agricultural utilization of metals and metal-containing compounds (He *et al*., 2005; Goyer, 2001; Herawati *et al*., 2000; Shallari *et al*., 1998). Corrosion of metals, atmospheric deposition, soil erosion of metal ions, leaching of heavy metals, sediment resuspension, and metal evaporation from water resources to soil and groundwater can also contributes to environmental contamination (Nriagu, 1989).

Cadmium and mercury interacts with DNA as they mimic zinc, while arsenic and chromium generates reactive oxygen species (ROS) as they mimic intermediates in the catabolism of carbohydrates. Arsenic interacts with glucose transport thereby generates reactive oxygen species; Chromium is poisonous when present in high concentrations. Several scientific reports have suggested that metal-induced oxidative stress can be partially responsible for the toxicity of these metals (Ercal *et al*., 2001). Reports on sources of heavy metal pollution includes industrial sources such as refineries, coal power plants, petroleum combustion, nuclear power stations, plastics, textiles, microelectronics, wood preservation, and paper-processing plants (Arruti *et al*., 2010; Strater *et al*., 2010; Pacyna, 1996) and natural phenomena including weathering and volcanic eruptions (Fergusson, 1990; Bradl, 2002; He *et al*., 2005; Shallari *et al*., 1998; Nriagu, 1989). Essential elements such as cobalt (Co), copper (Cu), chromium (Cr), iron (Fe), magnesium (Mg), manganese (Mn), molybdenum (Mo), nickel (Ni), selenium (Se), and zinc (Zn) at appropriate concentration levels are required for proper physiological functioning, whereas shortage supply of which results in variety of syndromes (WHO, 1996).

Effects on exposure to heavy metals have been documented on the biological system such as cell membrane, mitochondrial, lysosome, endoplasmic reticulum, nuclei, and certain enzymes entailed in metabolism, detoxification, and damage repair (Wang and Shi, 2001). Interactions of metal ions with cell components such as DNA and nuclear proteins have been reported, leading to DNA damage and conformational changes which may further results in modulation of cell-cycle, carcinogenesis, or apoptosis (Chang *et al*., 1996; Wang and Shi, 2001; Beyersmann and Hartwig, 2008). Generation of reactive oxygen species (ROS) and oxidative

stress are the main characteristics of the toxicity and carcinogenicity of metals such as arsenic (Yedjou and Tchounwou, 2006, 2007; Tchounwou *et al*., 2004), cadmium (Tchounwou *et al*., 2001), chromium (Patlolla *et al*., 2009), lead (Yedjou and Tchounwou, 2008; Tchounwou *et al*., 2004), and mercury (Sutton and Tchounwou, 2007; Sutton *et al*., 2002). Due of their high degree of toxicity, these elements are all systemic toxicants that are known to induce multiple organ impairment, even at lower levels of exposure. These metals have been classified as both "known" or "probable" human carcinogens by the United States Environmental Protection Agency (USEPA) and the International Agency for Research on Cancer (IARC).

Arsenic exposure takes place through the oral track (ingestion), inhalation, dermal contact, and to some extent the parenteral route (ATSDR, 2000; Tchounwou *et al*., 1999; NRCC, 1978). Arsenic is normally methylated and eliminated from the body through urine. Cigarette smoking curtails methylation capacity of arsenic and concomitant elimination of arsenic from the body. Cigarette smoking also can act synergistically with arsenic exposure to cause DNA damage in lungs (Hays *et al.*, 2006). In addition, As and Cd also have exhibited non-cancer toxicities towards the cardiovascular and renal systems (Dorne *et al.*, 2011). The expression of genes associated with DNA repair was attenuated and the expression of genes involved in the cellular response to oxidative stress increased due to arsenite (Hamadeh *et al.*, 2002). The growing body of evidence has shown that arsenic shares many properties with tumor promoters by inducing intracellular signal transduction, activating transcription factors, and changing the expression of genes that are involved in promoting cell growth, proliferation, and malignant transformation (Schoen *et al*., 2004). The inorganic arsenic exist in two major forms namely the trivalent arsenite and the pentavalent arsenate while organic arsenic forms are the methylated metabolites such as monomethylarsonic acid (MMA), dimethylarsonic acid (DMA), and trimethylarsine oxide (Tchounwou *et al*., 2012). Arsenic toxicity of human has been mostly associated with exposure to inorganic arsenic. The toxicity of inorganic trivalent arsenite [As(III)] is 2–10 times more than that of pentavalent arsenate [As(V)] (Goyer, 2001). On proteins, As(III) can deactivate over 200 enzymes by binding to thiol or sulfhydryl groups which is likely the mechanism responsible for

the prevalence of the effect of arsenic on different organ systems. Due to electronic distribution similarities, As(V) can replace phosphate, which is essentially required in many biochemical pathways (Goyer, 2001; Hughes, 2002). A potential carcinogenic character of arsenic by inducing DNA hypomethylation facilitating abnormal gene expression has also been reported (Zhao *et al*., 1997).

Lead plays a significant role in tobacco toxicity. Lead exposure mainly occurs through inhalation of lead-contaminated dust particles or aerosols and ingestion of lead-contaminated food, water, and paints (ATSDR, 1999, 1992). The nervous system is the most susceptible target of lead poisoning. Lead is capable of causing serious effects on the brain and bone mineral density. The blood brain barrier of children and infants is relatively impermeable to lead nevertheless they are at high risk of accumulating lead in the brain and central nervous system which may cause impaired development of hematologic, skeletal and nervous systems, are well characterized (Amodio-Cocchieri *et al*., 1996; Kaul *et al*., 1999;Landrigan, 1991; Litvak *et al*., 1998; USEPA, 2002). Lead is known to have competence to mimic the action of calcium to interact with protein thereby exerting its toxic effects (ATSDR, 1999). When activities of antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase in erythrocytes were evaluated, it was found that the antioxidant enzyme levels of workers exposed to lead are remarkably higher than that in non-exposed workers (Bechara *et al*., 1993). Lead-induced toxicity and apoptosis in human cancer cells have suggested that lead may have engaged certain cellular and molecular processes involving induced cell death and oxidative stress (Yedjou and Tchounwou, 2008; Yedjou *et al*., 2006), transcriptional activation of stress genes (Tchounwou *et al*., 2004), DNA damage (Yedjou and Tchounwou, 2008), externalization of phosphatidylserine, and activation of caspase 3 (Yedjou *et al*., 2010).

A great number of people have become victims of environmental tobacco smoke (ETS) as they participate passively, especially in Mizoram. Smoking of tobacco is an important source of polluted air exposure for smokers (Scherer and Barkemeyer, 2002). Investigation concluded a significant level of Cadmium (Cd)

found in cigarette smokers blood compared with nonsmokers (EL-Agha *et al.*, 2002). Studies have shown that, each year about 3000 non-smoking adults may be dying due to lung cancer as a result of inhaling the second-hand smoke from cigarette smokers (Hynes, 2007). Cadmium is a widely distributed heavy metal with an average concentration of about 0.1 mg/kg and is frequently used for various industrial activities (Tchounwou *et al*., 2012). Though the main source of cadmium exposure being inhalation (cigarette smoke) and food ingestion (Tchounwou *et al*., 2012), other sources like emissions from industrial activities (IARC, 1993) and airborne cadmium inhalations (Mascagni *et al*., 2003) have also been documented. Cadmium has been speculated to cause cell damage mainly through ROS generation (Stohs and Bagchi, 1995), causing damage on single-stranded DNA thereby obstructing the synthesis of nucleic acids and proteins (Mitra, 1984). Various stress response systems such as heat shock, oxidative stress, stringent response, cold shock, and SOS protein expressions were observed in studies using 2D-gel electrophoresis on response to cadmium exposure (Blom *et al*., 1992; Ferianc *et al*., 1998; Coogan *et al*., 1992). *In vitro* studies on cadmium exposure indicated that at concentrations ranging from 0.1 to 10 mM, cadmium induces cytotoxic effects and free radical-dependent DNA damage (Tsuzuki *et al*., 1994; Mukherjee *et al*., 2002).

Mercury is a heavy metal that naturally occurs in three forms – elemental ($Hg^0$), organic and inorganic ($Hg^{1+}$& $Hg^{2+}$), each with different toxicity (Clarkson *et al*., 2003). It is a prevalent environmental toxicant and pollutant capable of inducing severe tissue alteration along with a wide range of adverse health effects (Sarkar, 2005). The toxicity of mercury as oxidative stress has been demonstrated in the form of sulfhydryl reactivity (Valko *et al*., 2005) where the reaction with cystine residues of proteins with mercury which concomitantly causes the depletion of the cellular antioxidants which serves as a major form of cellular defense (Valko *et al*., 2006). Exposure and engagement with mercury compounds induced the generation of oxidative damage by the accumulation of ROS which were typically eliminated by cellular antioxidants under normal conditions (Tchounwou *et al*., 2012). Inorganic mercury interferes with the oxidative phosphorylation and electron transport at the ubiquinone–cytochrome $b_5$ step (Palmeira and Madeira, 1997) and disrupts the flow

of electrons to molecular oxygen which may results in enhancement of ROS generation (Lund *et al*., 1991) and concomitantly it may lead to the genetic mutations.

Chromium is a naturally occurring transition element which exists in a diverse oxidation states ranging from divalent [Cr(II)] to hexavalent [Cr(VI)] compounds, of which exposure to high concentration levels of trivalent [Cr(III)] and hexavalent [Cr(VI)] compounds elicit significant biological toxicity (Dayan and Paine, 2001; Eastmond *et al*., 2008; Katz and Salem, 1993). The respiratory tract is the main target of inhalation exposure to chromium compounds in humans. Acute exposure to Cr(VI) compounds may develop asthma and other signs of respiratory distress (Wilbur *et al*., 2012). Study showed that exposure to high concentrations of Cr(III) may lead to cell damage (Eastmond *et al*., 2008). Cr(VI) produces reactive hydroxyl radicals in the bloodstream which elicited blood cell damage due to oxidation and the concomitant functional degradation of the liver and kidney (Shi and Dalal, 1993; Hamilton and Wetterhahn, 1986; Dartsch *et al*., 1998). Reduction of Cr(VI) to Cr(V) have been reported to fluster the normal cellular processes by binding to DNA (Eastmond *et al*., 2008; Hamilton and Wetterhahn, 1986; Aiyar *et al*., 1991). Study on carcinogenic mechanism of chromium by molecular pathway analysis showed that chromium and chromium compounds mainly induce apoptosis, oxidative stress and DNA damage (Kim *et al*., 2015).

Nickel (Ni) is among the essential elements that are required by many microorganisms, plants, animals (Cempel and Nikel, 2006; Tyagi *et al*., 2013; Ling and Leach, 1979), and at low concentrations, by humans too (Wen and Brune, 2002). At high concentration, exposure to Ni is toxic for both humans and animals (Wataha *et al*., 2002). Ni exposure generally occurs through ingestion of contaminated water and food (Cempel and Nikel, 2006; Haber *et al*., 2000) and plants being the primary source of Ni for humans (Ragsdale, 1998). Ni exposure has been reported to cause neurotoxicity, hepatotoxicity, nephrotoxicity, gene toxicity, reproductive toxicity, and elevated risk of cancer (Oller *et al*., 1997; Xu *et al*., 2015; Gathwan *et al*., 2012; Scutariu et and Ciupilan, 2007; Chen *et al*., 2010; Goodman *et al*., 2009; Forgacs *et*

*al*., 2012; Duman and Ozturk, 2010; Kasprzak *et al*., 2003; Spears *et al*., 1986). Bones, kidneys, lungs, liver, and heart are the primary organs of accumulation of Ni (Spears *et al*., 1986; Das and Dasgupta, 2000; Ragsdale, 1998). Lung and nasal cancer incidence due to Ni exposure has been reported (Das *et al*., 2008; Shi, 1994). International Agency for Research on Cancer (IARC) has classified all Ni compounds (except metallic Ni) as carcinogens. (IARC, 1990).

*Nicotiana tabacum* necessitate various essential elements for growth and survival. These elements enter the plant system from the soil, flows through the roots, through the stem, and ultimately get absorbed in the leaves which are the main destination for all elements. Metals and metalloids constitute 12 % of the 98 of most hazardous components of smoke. Cr (VI) is proven carcinogen (Skwarzec et al., 2001) and its elicit significant biological toxicity has been conferred (Eastmond et al., 2008). Co may cause respiratory problems, Cu may cause distress in the lung and immune system, Mn have consequence relating to the nervous system, and Ni may result in persistent active inflammation and lung fibrosis (Talhout et al., 2011). At high concentration, exposure to Ni is toxic for both humans and animals (Wataha et al., 2002). Elevated concentration of Cu and Zn may results in metabolic disorder with potential fatality (Stojanovic et al. 2004; Zhang et al. 2005).

With the development of several sophisticated instruments, there are various analytical techniques available for evaluating the elemental profile of various lifestyle products. Instrumental neutron activation analysis (INAA) provides simultaneous multi-elemental determination with high accuracy; low detection limits, and is rather free from array of interferences. When combined with atomic spectrometric methods, these quantitative measurements can provide crucial information on the elemental contents in various life style products (Kumara *et al*., 2005; Monged, 2016; Zinicovcaia *et al*., 2019, 2020a,b).

INAA is used in determination of the concentrations of trace and major elements from an array of matrices. Samples subjected to neutron flux produce radioactive nuclides and as these nuclides decay, they emit gamma rays whose energies are distinctive for each nuclide. The intensity of these emitted gamma rays

is compared against a standard which gives a quantitative measure of the concentrations of various nuclides. A gamma-ray spectrometer consists of a detector (with high voltage power supply), pre-amplifier, spectroscopy amplifier, analog-to-digital converter, multi-channel analyzer, and an output device. A sample is introduced to the detector (Ge in the case of gamma-ray analysis). The detector is maintained at cryogenic temperatures (liquid nitrogen, temperature = 77K) in order to reduce thermal noise. The pre-amplifier attached directly to the detector amplifies the signal produced. The signal produced by the amplifier is then converted from an analog to a digital signal by the converter and are stored (multi-channel analyzer). In recent gamma-ray spectroscopy systems, the high-voltage power supply, spectroscopy amplifier, analog-to-digital converter, and multi-channel analyzer are combined into a single module. While liquid samples can be analyzed by INAA, solids are the matrix of choice for this technique. Virtually any material can be analyzed and limitations are largely due to the chemistry of the matrix.

Several investigations have been carried out on determination of heavy metal concentration in tobacco leaves (Golia *et al*., 2003; Zaprjanova and Boshinova, 2004). Pyle *et al*. (1996) have reported a significant difference in the determination of cadmium concentration of 50 soil samples by four techniques (ICP - AES, AAS, PSA and XRF). On analyzing experimental results from Food Analysis Performance Assessment Scheme (FAPAS) during the period 1991 – 2000, Rose *et al*. (2001) showed that the percentage range of 34 to 100 were significant which depends on the material, sample digestion and detection process. The reports of these authors suggested that ICP-hyphenated techniques give better result compared to AAS. Similar conclusion was arrived at comparing the dissolution techniques for multi-elemental analysis of some reference plant materials by Poykio *et al*. (2000). Based on these authors, microwave dissolution, coupled with ICP-OES or ICP-MS determination is a very rapid and accurate method for analyzing elemental concentrations in plant specimens.

ICP-OES (Inductively coupled plasma - optical emission spectrometry) is a destructive analytical technique in which the elemental composition of (mostly

liquid-dissolved) samples can be resolute using plasma and a spectrometer. ICP-OES employs inductively coupled plasma to generate the excited atoms and ions which emit the electromagnetic radiation at particular wavelengths as a measure of orbital energies which is the characteristic feature of an element. The sample is subjected to temperatures high enough to cause not only dissociation of sample into their constituent atoms but also cause significant amounts of collisional excitation (and ionization) of the sample atoms to take place. Once the atoms or ions are in their excited states, they can decay to lower states through thermal or radiative (emission) energy transitions. The plasma is a high temperature source (6000 – 10000 K) of ionised source gas (often argon) and is sustained and maintained by inductive coupling from cooled electrical coils at radiofrequencies. The intensity of the emitted wavelengths is measured and used to determine the concentrations of the elements precisely within the sample. The high temperature excitation sources can populate a large number of different energy levels for several different elements at the same time which in turn can then emit their characteristic radiation at nearly the same time. This provides the resilience to choose from several different elements simultaneously.

Optical Emission Spectroscopy (OES) provides the much needed higher sensitivity and selectivity than other atomic spectral methods. The wavelength of the atomic spectral line gives the identity of the element while the intensity of the emitted light is proportional to the concentration of atoms of the element present in the sample. The sensitivity and selectivity of OES method can be considerably enhanced by the high-temperature generating atomization method, *viz*., Inductively Coupled Plasma (ICP) method. Advantages of ICP-AES are excellent limit of detection and linear dynamic range, multi-element capability, low chemical interference and a stable and reproducible signal. The intensity of spectral line can be directly correlated with the concentration of various elements present in the sample (Lokeshappa *et al.*, 2012). When compared with other spectral methods, *viz.* Atomic Absorption Spectroscopy (AAS), Atomic Fluorescence Spectrometry (AFS), Ultraviolet-visible Spectroscopy (UV-VIS), etc., the sample requirement is very less as OES can simultaneously measure many elemental ions in one measurement.

Cigarette smoke (CS), implicated as an etiological factor for the development of many forms of cancers, is a complex mixture containing more than 7000 different constituents, including reactive oxygen and nitrogen species (ROS and RNS) (Rodgman and Perfetti, 2009) whose production further mediated through inflammatory processes that may eventually exacerbate as a result of the direct exposure to smoke. Oxidative damage induced by CS is caused by a variety of water soluble oxidants that may undergo circulation through the body fluids manifested as the sustained oxidative damage in various organ systems. CS has been known to be a complex mixture containing long-lived radicals, including p-benzosemiquinone that induces oxidative damage. Exposure of guinea pigs to cigarette smoke has resulted in progressive protein damage, inflammation, apoptosis and lung injury. While, p-Benzosemiquinone has also mimicked cigarette smoke in causing protein modification and apoptosis *in vitro* and in A549 cells *ex vivo* as well as apoptosis and lung damage *in vivo* (Banerjee *et al.*, 2008).

Cigarette smoking can be divided into two phases, the tar phase and the gas phase. Both phases contain high concentrations of ROS, including nitric oxide (NO), peroxynitrite, peroxynitrate, and free radicals of organic compounds. NO is known to act as the nitrosation agent of tobacco alkaloids leading to the formation of tobacco-specific nitrosamines (TSNAs) (Hoffmann and Hoffmann, 2001). The strongest carcinogens are polycyclic aromatic hydrocarbons (PAHs), *N*-nitrosamines, and aromatic amines. These compounds occur in small quantities, typically about 5–200 ng per cigarette. The other prevalent carcinogens in CS are aldehydes and other volatile organic compounds such as benzene and butadiene. These compounds are found in quantities of 10–1000 µg per cigarette. In relation to human lung cancer, arguably the most important carcinogens are PAHs such as benzo[*a*]pyrene and the tobaccospecific nitrosamines, N'-nitrosonornicotine (NNN), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, also known as nicotine-derived nitrosaminoketone (NNK) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol, i.e., nicotine-derived nitrosaminoaldehyde (NNAL) (Hecht, 1999; Hoffmann *et al.*, 2001).

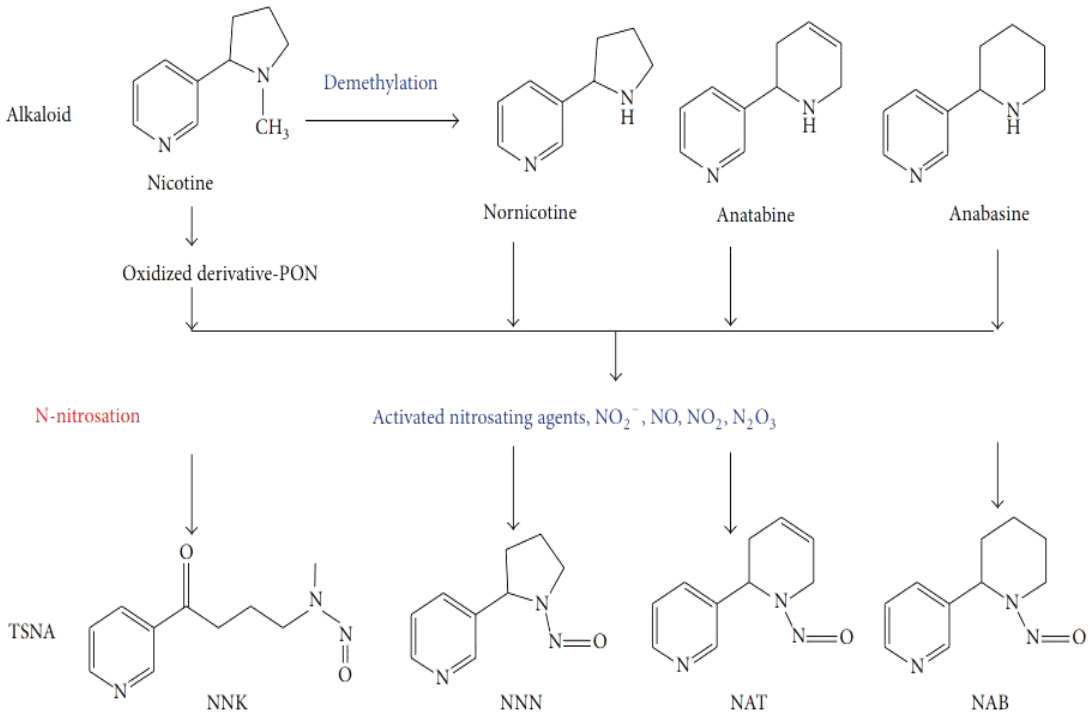



**Figure 1.4**: Formation of the major TSNAs found in cured tobacco leaves (Wang *et al*., 2017).

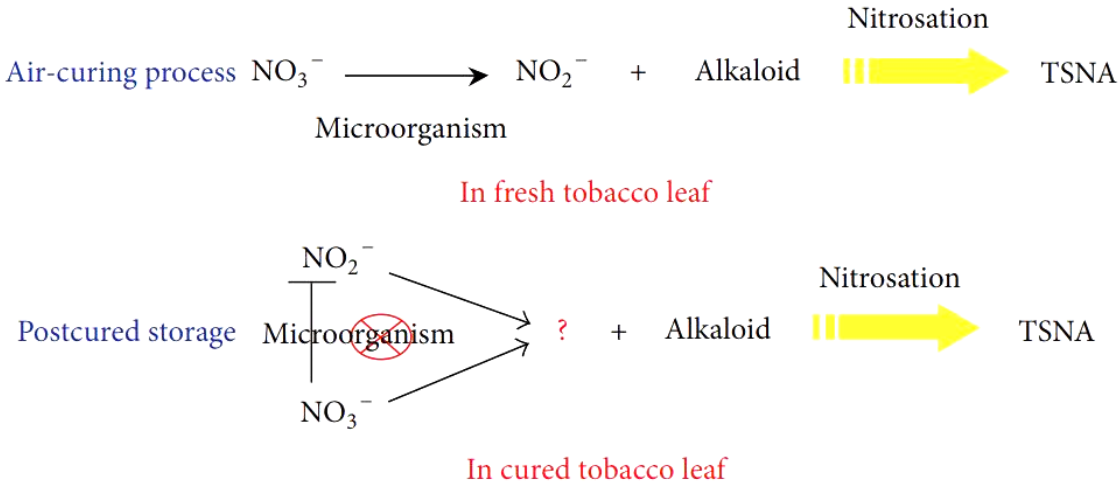



**Figure 1.5**: Activated nitrosating agents participate in nitrosation of alkaloid to form TSNAs during different processes of air-cured tobacco production (Wang *et al*., 2017).

Tobacco smoke comprises of a complex series of potent carcinogens, including tobacco-specific nitrosamines (TSNAs). TSNA characteristically develops in tobacco during the post-harvest stage, which later gets transferred as semi-volatile constituent into the mainstream smoke when a cigarette is lighted and consumed. TSNAs are generated through endogenous nitrosation of tobacco alkaloids during the curing and storage process of tobacco leaves (Figure 1.4) (Schnur *et al*., 1972; Shi and Zhang, 2004; Bush *et al*., 2001; Djordjevic *et al*., 1989; Stepanov *et al*., 2011). Levels of TSNAs were reported to increase substantially in the leaf lamina and midrib during the fourth to seventh weeks of air-curing (Cui, 1998). At this time, microbial activity reduced nitrate to nitrite, which in turn, is involved in the nitrosation reactions with the naturally existing alkaloids at this air-curing stage, then leading to the formation of TSNAs (Figure 1.5) (Burton *et al*., 1994). Nitrate and nitrite are reported to be the major contributors in the formation of TSNAs during warm temperature storage of tobacco (Wang *et al*., 2017). Tobacco grown with the augmentation of fertilizers tends to accumulate more nitrate than tobacco grown in an 'organic' manner (Wu *et al*., 2020).

Nitrosamines have also been identified as components of food, beverages, air, cosmetics, and industrial environments; accordingly, these chemicals have been a comprehensive topic of research and review for several years (IARC, 1978; Loeppky and Michejda, 1984; Tricker, 1997; Magee, 1982). It is generally accepted that nitrosamine exposure is on a daily basis on human; despite that, the accurate exposure level and the impact corresponding to its exposure remains ambiguous (Tricker, 1997). Induced carcinogenesis promoted by NNK and NNN has been well established where the mechanism involves DNA adduct formations and mutations along with promotion of tumor proliferation through receptor-mediated effects facilitated by associated receptors, such as nicotinic acetylcholine receptor (nAChR) and β-adrenergic receptors (β-AR) (Hecht, 2002; Falter *et al*., 1994).

Consumption of tobacco is a major and chronic source of exposure to nitrosamines in general and tobacco-specific nitrosamines in particular. Tobacco-specific nitrosamines (TSNA) are a class of nitrosamines putatively tobacco specific,

and have been reported to be present in a number of tobacco-related products (Hoffmann *et al*., 1980; Adams *et al*., 1984; IARC 1985; Hoffmann *et al*., 1991; Tricker *et al*., 1991; Hoffmann and Hoffmann, 1997; Hoffmann *et al*., 1997; Hoffmann and Hoffmann, 1998; Hecht, 1999). Documented TSNA comprises of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone [NNK], 4-(methylnitrosamino)-4-(3-pyridyl) butanal [NNA], *N′*-nitrosonornicotine [NNN], *N′*-nitrosoanabasine [NAB], *N′*-nitrosoanatabine [NAT], 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol [NNAL], 4-(methylnitrosamino)-4-(3-pyridyl)-l-butanol [*iso*-NNAL], and 4-(methylnitrosamino)-4-(3-pyridyl) butanoic acid [*iso*-NNAC] (Hecht and Tricker, 1999). *iso*-NNAL and *iso*-NNAC hardly occur in mainstream cigarette smoke (Hecht and Tricker, 1999). NNK, NNN, and NNAL are mutagenic *in vitro* and carcinogenic on animal model study (Boyland *et al*., 1964; Hoffmann *et al.,* 1984). International Agency for Research on Cancer (IARC) also classified NNN and NNK as Group 1 human carcinogen (IARC, 2004). NAT and NAB exhibit constrained mutagenic and carcinogenic potential in laboratory animals during *in vitro* testing (Hoffmann *et al*., 1984; Padma *et al*., 1989). NNK and NNN has been well documented to induce several malignancies, including lung, esophageal, liver, pancreatic and nasal cavity (Chang *et al*., 2010; Chang *et al*., 2011; Huang *et al*., 2011; Raja *et al*., 2016; Nilsson, 2011; Hecht, 2003; Tang *et al*., 2001). Cases of lung tumors are observed for NNK in all tested species irrespective of site of its induction, and its carcinogenicity in rats is notably strong (Hecht, 1998).

Organic and freshly harvested tobaccos are basically free of TSNA (Green and Rodgman, 1996; Parsons *et al*., 1986). During the post-harvest processing (e.g., curing) nicotine and minor tobacco alkaloids can be converted to TSNAs through nitrosation (Ramage *et al*., 2006; Ishii *et al*., 2001; Hoshino *et al*., 2001). Several studies have been carried out to understand putatively the mechanism of TSNA formation (Peele *et al*., 1995). TSNAs are known to be formed when tobacco alkaloids (e.g., nicotine, anabasine and nornicotine) are nitrosated (Tricker and Preussmann, 1988). During the air curing of Burley tobacco, TSNAs form as a result of conversion of nitrate to nitrite by the action of microbes, followed by the reaction of nitrite-derived chemical species with alkaloids present in the tobacco (Peele *et al*.

1995; Chamberlain and Chortyk, 1992; Hecht, 1998; Hamilton *et al*., 1982; Burton *et al*., 1992; Bush *et al*., 1995; Wiernik *et al*., 1995). Other feasible consequences by factors such as temperature of curing, humidity, application of nitrogenous fertilizers, and amount of shade vs. sunlight on the formation of TSNAs have also been extensively studied (Tso, 1990; Davis and Nielsen, 1999). In flue-cured tobacco, the generation of $NO_x$ gases from heating has been shown to be a contributing factor to TSNAs formation (Peele *et al*. 1999). Carcinogenicity of *N*-nitrosamines requires metabolic activation by cytochromes $P_{450}$ leading to several intermediates (Hecht and Tricker, 1999; Atalla and Maser, 1999; Ren *et al*., 1999; Hecht *et al*., 1997). Besides, a genotoxic product of NNK, NNAL may undergo α-hydroxylation resulting in the formation of additional genotoxic metabolites (Hecht and Tricker, 1999; Atalla and Maser, 1999; Ren *et al*., 1999; Hecht *et al*., 1997).

Ames test suggested that both TSNAs and cigarette smoke condensate are mutagenic in the presence of S9 metabolic activation (Padma *et al*., 1989; Lee *et al*., 1996). However, there is no evidence to suggest that the small amount of NNK in cigarette smoke contributes to the mutagenicity observed for cigarette smoke condensate. Approximately 200 μg of pure NNK is required to demonstrate mutagenicity in the Ames assay using strain TA1535, the most sensitive strain for base pair mutagens commonly associated with *N*-nitrosamines (Lee *et al*., 1996). The dose of NNK required to elicit a moderate mutagenic response (200 μg) is equivalent to the amount of NNK yielded by approximately 2985 Kentucky 1R4F reference cigarettes smoked under standard FTC smoking conditions (Borgerding *et al*., 1998). Since the amount of cigarette smoke condensate present in approximately 0.01 1R4F cigarettes (100 μg of CSC) is sufficient to demonstrate a substantial mutagenic response in the Ames test, it follows that the mutagenic response is not being driven by the level of TSNAs in CSC. Furthermore, mainstream smoke from cigarettes generated using low TSNA tobacco failed to demonstrate reduced mutagenic potential within the Ames assay (Doolittle *et al*., 2001). Therefore, several lines of experimental evidence indicated that there are insufficient quantities of TSNAs in tobacco smoke to contribute to the mutagenicity of tobacco smoke observed in the Ames test.

Different approaches have been undertaken for the analysis of TSNAs. Several techniques including Gas Chromatography (GC) (Hoffmann *et al*., 1974; Rhe, 1999; Zhao *et al*., 2000), High-Performance Liquid Chromatography (HPLC) (Hui-jun *et al*., 2000), Gas chromatography/Mass Spectrometry (GC/MS) (Song and Ashley, 1999) and gas chromatography coupled with thermal energy analyzer (TEA) (Stepanov *et al*., 2002), have been employed for analyzing TSNAs. Due to its sensible sensitivity and nitric oxide-specificity, GC coupled with TEA is the most frequently used method. However, this technique has its limitations that include a complex sample cleanup procedure involving liquid-liquid extractions and solid-phase extractions and relative high limit of detection. Analysis of four TSNAs by LC-MS/MS was described by Wagner *et al.,* (2005). LC-MS/MS method provides several advantages over other techniques including the GC-TEA method. It has greater specificity, significantly lower limits of detection, and a larger linear dynamic range and it also proved to be the most consistent method.

Liquid chromatography (LC) is a separation method that is used for isolating the individual constituents of a mixture by mass transfer of a sample through a polar mobile phase and non-polar stationary phase, known as reverse-phase method. The apparatus is made up of a column that consist of the porous medium made of a granular solid material (i.e., stationary phase), such as polymers and silica, where the sample is introduced and the solvent (i.e., mobile phase) passes to transport the sample. On injecting a sample, the stationary phase adsorbed the sample, and the solvent penetrates through the column thereby separating the existing compounds one by one. The compounds get separated on the basis of their relative affinity to the stationary phase materials and the solvent. The component with the highest affinity to the stationary phase is the last to separate as it requires more time to travel to the end of the column.

In Mass spectrometry (MS), the samples (atoms or molecules in gaseous phase or thermally unstable) get ionized to facilitate their separation and detection in accordance with their molecular masses and charges (mass to charge ratio) using a mass analyzer. The species and quantity of each detected ions are determined by a

detector. In case of thermally labile compounds, samples are directly injected to the spectrometer in liquid phase known as direct infusion. In case of direct infusion, syringe pumps are utilized for sample injection and ionization occurs in the condensed phase.

The LC-MS is a hyphenated technology involving the use of MS and HPLC. In LC-MS, the individual components in a mixture are first separated followed by ionization and separation of the ions on the basis of their mass/charge ratio. The separated ions are then directed to a photo or electron multiplier tube detector, which identifies and quantifies each ion. The ion source plays an important role in any MS analysis, as this primarily facilitates efficient generation of ions for analysis. Ion source such as APCI (Atmospheric Pressure Chemical Ionization), ESI (Electron-spray Ionization), etc. are used for ionization of unimpaired molecules without the fragmentation of molecules. The preference of ion source depends on the chemical nature of the analyte of interest i.e. polar or non-polar. The substantial advantages of this technology include sensitivity, specificity and precision as analysis is carried out at molecular level. The structural information of the analyte can also be interpreted.

'Antioxidants' are substances that neutralize free radicals or their actions (Sies, 1996). Reactive oxygen species damage protein, lipids and finally membrane leading to oxidative stress. Antioxidant enzymes such as superoxide dismutase (SOD), catalase, glutathione, glutathione peroxidases and reductase, besides diet derived antioxidants tocopherols, tocotrienols and ascorbate etc., present in the human body fluids which can be easily collected *viz.*, saliva and blood samples. The two main phases of mainstream (smoker inhaled) cigarette smoke, *viz.*, particulate phase (tar) and gas phase (toxic gases, volatile organic compounds, free radicals, etc.), contain abundant free radicals and non-radical oxidants. Cigarette tar comprises exceptionally elevated concentrations of stable free radicals with prolonged lifetimes. The sidestream smoke composed of solid and gas phases, with higher concentrations of toxic and carcinogenic compounds along with other volatile and semivolatile compounds (Pryor and Stone, 1993; Norman, 1977; Brunnemann and Hoffmann, 1974). In the gas phase, stable free radicals and oxidants are continuously generated

and annihilated and their concentration increases with age (Pryor *et al*., 1985; Church and Pryor, 1985). The concentration of the gas phase is around 0.4-0.5 g/cigarette and contains approximately 500 volatile organic and inorganic compounds (Eiserich *et al*., 1996). The particulate phase (tar) consists of very fine particles (0.1-1.0 μm, aerodynamic diameter) that can penetrate deep into the alveoli. Some water-soluble constituents of aqueous cigarette tar (ACT) can produce superoxide anion ($O_2^{\bullet-}$) and then $H_2O_2$ and the reactive hydroxyl radical ($OH^{\bullet}$), resulting in oxidative damage to cellular membrane lipids, proteins, enzymes and DNA (Pryor *et al*., 1990; USEPA 1992). The sidestream smoke is similarly rich in highly reactive and short-lived free radicals with similar chemical components. Passive smoking (or environmental tobacco smoke, ETS) has also been reported to be a major health hazard for non-smokers and major lung diseases (Hackshaw *et al*., 1997; Eisner *et al*., 2005; Heidrich *et al*., 2007).

Electron paramagnetic resonance (EPR) spectral data showed that cigarette tar has high concentrations of stable free radicals such as a semiquinone ($QH^{\bullet}$) and carbon-centered radicals ($C^{\bullet}$) (Pryor, 1992) and were identified as o- and p-benzosemiquinone radicals. The most interesting of which is a quinone/semiquinone/hydroquinone ($Q/QH^{\bullet}/QH_2$) system in the tar polymeric matrix (Pryor *et al*., 1983). The role of these free radicals in the DNA damage, through the mechanism of $HO^{\bullet}$ formation has been established using EPR spin-trapping (Bermudez *et al*., 1994). Cigarette tar can generate significant amounts of $H_2O_2$ in aqueous extracts (Nakayama *et al*., 1989). The engagement of oxidants present in the tar and gas-phase in the discharge of iron from the endogenous enzyme ferritin that may subsequently alter the iron metabolism in the lungs has been studied (Moreno *et al*., 1992; Lapenna *et al*., 1995). The $QH^{\bullet}$ radical reduces $O_2$ into $O_2^{\bullet-}$, which can dismutate to form $H_2O_2$ and generate highly oxidizing hydroxyl radicals ($HO^{\bullet}$) with $Fe^{2+}$ in cigarette tar (present in high concentration) via Fenton reaction. The following mechanisms have been identified:

$$Q + QH_2 \rightleftarrows 2\ QH^{\bullet}$$

$$QH^{\bullet} + O_2 \rightarrow Q + O_2^{\bullet -} + H^+$$

$$O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2$$

$$QH_2 + O_2 \rightarrow H_2O_2 + Q$$

$$QH_2 + O_2 \rightarrow O_2^{\bullet -} + QH^{\bullet} + H^+$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\bullet} + HO^-$$

An imbalance between oxidants and antioxidants with higher concentration of the oxidants, potentially leading to damage, is known as 'oxidative stress' (Sies, 1985, 1986, 1991). Oxidants are reactive molecules that are generated as a normal product of aerobic metabolism but can be produced at intensified rates under pathophysiological conditions. Antioxidants are capable of adapting to changing oxidative status and are responsible for maintaining the balance. Superoxide radical ($O_2^{-\bullet}$), and especially hydroxyl radical ($^{\bullet}OH$), and peroxyl ($ROO^{\bullet}$) have the competence to trigger oxidative damage by lipid peroxidation pathway (Kasap *et al*., 2007). Lipid peroxidation is the oxidative deterioration of lipids where free radicals abstract an electron from the lipids in the cell membrane. The availability of oxidants and free radicals in cigarette smoke have been regarded as a potential mechanism by which smoking exacerbates lipid peroxidation, further instigates atherosclerosis, endothelial dysfunction and acute clinical events, thereby increasing the risk for cardiovascular diseases (Frei *et al*., 1991; Santanam *et al*., 1997; Ambrose and Barua, 2004). The presence of ROS in the cigarette gas-phase plays an additive role in promoting the destruction of endogenous antioxidants (vitamins and enzymatic antioxidants) reducing the vital role of cellular antioxidant defenses (Cross *et al*., 1999). Past studies have revealed that the levels of antioxidant vitamins are lower in smokers that results in systemic oxidative stress (Panta *et al*., 2000; Traber *et al*., 2000).

Oxidative stress is caused by exposure to reactive oxygen intermediates, such as superoxide anion ($O_2^{-\bullet}$), hydrogen peroxide ($H_2O_2$), and hydroxyl radical ($^{\bullet}OH$), that are capable of damaging proteins, nucleic acids, and cell membranes. Various studies have developed the relation between the cumulative damage caused by reactive oxygen species and other several diseases (Aruoma and Halliwell, 1998). Cells intrinsically express enzymes to counteract oxidative stress that detoxify the ROS and repair the damage that goes with it. Oxidative stress is an inevitable by-product of the aerobic metabolism as $O_2^{-\bullet}$ and $H_2O_2$ are produced whenever electron carriers are oxidized. Oxidative stress occurs when ROS submerged the endogenous detoxification channels such as during inflammation, viral and bacterial infections (Ames *et al*., 1993), metabolism of endogenous molecules such as estrogens (Bolton *et al*., 2000), metabolism of drugs such as etoposide (Zheng *et al*., 2004), metabolism of environmental chemicals such as benzo[*a*]pyrene (Park *et al*., 2008), or tobacco smoking (Burke and Fitzgerald, 2003). During oxidative stress, ROS can cause oxidative damage to cellular DNA (Lee and Blair, 2001; Mangal *et al*., 2009) as well as to the trinucleotide precursors of DNA (Cooke *et al*., 2008).

The defence against oxidative stress arising due to the imbalance between the production and the detoxification of reactive oxygen species (ROS) in cigarette is provided by a system of enzymes and antioxidants, capable of preventing excess concentration levels of ROS and neutralizing free radicals (Menshchikova *et al*., 2006) in biological milieu. Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) plays an important defensive role for suppressing oxidative cell damage preventing lipid peroxidation, and oxidation of protein and DNA (Wang *et al*., 2013).

Superoxide dismutase (SOD), which stimulates the dismutation of the superoxide anion ($O_2^{-\bullet}$) into hydrogen peroxide ($H_2O_2$) and molecular oxygen ($O_2$), are one of the essential antioxidative enzymes. In human, three isoforms of SOD are present: the cytosolic copper/zinc (Cu/Zn)-SOD, the mitochondrial manganese (Mn)-SOD, and extracellular (EC)-SOD (Valdivia *et al*., 2009; Miller, 2012; Miao and Clair, 2009; Miriyala *et al*., 2012). Out of which manganese-dependent SOD (Mn-

SOD) plays a major role due to its mitochondrial location, i.e., the main site of superoxide ($O_2^{-\bullet}$) production. (Guilherme *et al*., 2015), and has been considered as a unique tumor suppressor protein delivering a crucial role in regulation of cell death (Miriyala *et al*., 2012). Nevertheless, an increase in the activity of MnSOD under some instances may lead to cell damage by overproduction of $H_2O_2$, particularly in individuals with reduced capacity of removing this highly toxic ROS by catalase (CAT) or glutathione peroxidase (GPx) (Valdivia *et al*., 2009). SOD dismutates the $O_2^{-\bullet}$ radical into $H_2O_2$, which serves as substrate to CAT and GPx. CAT and GPx enzymes further stimulates the reduction of $H_2O_2$ to $H_2O$. GPx exhibits a higher affinity for $H_2O_2$ and requires glutathioine (GSH) for providing a supply of H-atom while GSH is converted into oxidized glutathione (GSSG) (Finaud *et al*., 2006).

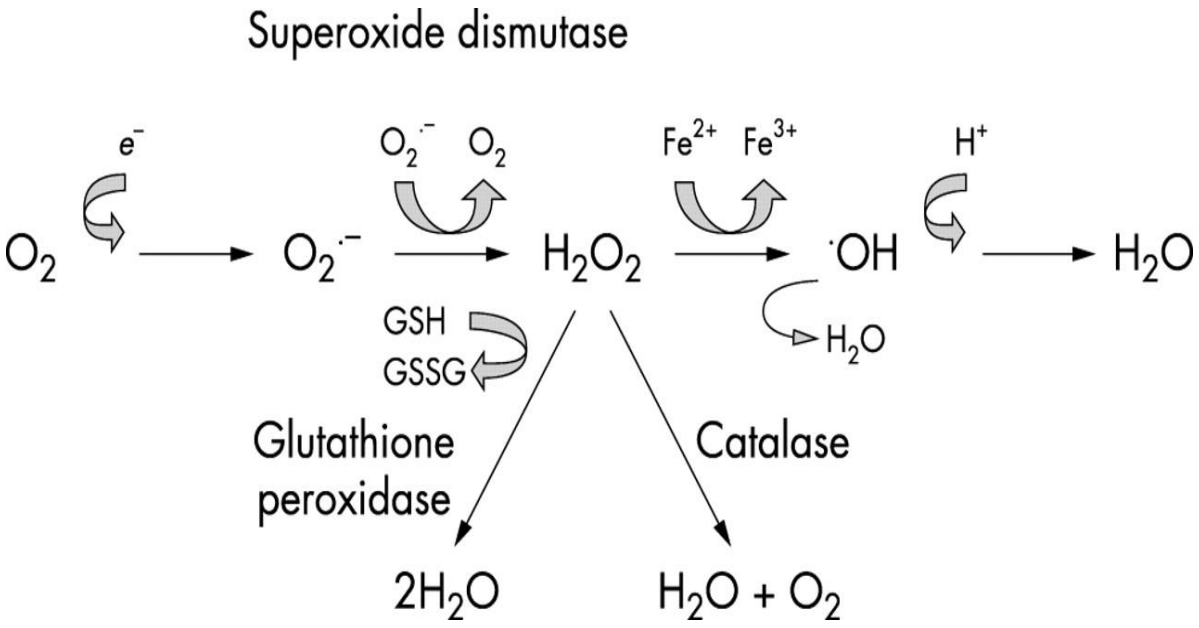



**Figure 1.6**: Generation of reactive oxygen species (Nedeljkovic *et al*., 2003).

Under chronic oxidative stress conditions, apoptosis of the cells can be triggered (Zhao and Wang, 2012). Apoptosis or programmed cell death is an important mechanism in several biological functions, such as cellular damage, teratogenicity, and tumorigenesis (Kang *et al*., 2005) and prevention of cancer is one

of the main functions of apoptosis (Lopez and Tait, 2015). Disruption in normal apoptotic mechanism enables cancer cells to subsist longer providing extended opportunity for the accumulation of mutations thereby increasing its invasiveness during tumor progression, stimulate angiogenesis, deregulate cell proliferation and interfere with differentiation (Hassan *et al*., 2014). Exposure to As(III) and MeHg in leukocytes has been observed to result in apoptosis of cell through generation of ROS (Wang *et al*., 2004; Morcillo *et al*., 2016). Strong up-regulation of Casp-2 and Casp-3 genes was observed in leukocytes on exposure to Cd (Reyes-Becerril *et al*., 2019) where oxidation of cadmium leads to strand breaks in DNA molecules of cells derived from liver or kidney (Forrester *et al*., 2000). Cadmium induced apoptosis occurs through a caspase dependent or independent pathway depending on its concentration (Mao *et al*., 2007; Lee *et al*., 2006). Several animal model studies have indicated that cell apoptosis might be associated with lead-induced cytotoxicity (Pulido and Parrish, 2003; Sharifi *et al*., 2002; Tavakoli-Nezhad *et al*., 2001; Adhikari *et al*., 2001; Iavicoli *et al*., 2001; Shabani and Rabbani, 2000; He *et al*., 2003, He *et al*., 2000). Lead acetate induced apoptosis in pheochromocytoma (PC 12) cells, leading to the activation of caspase-3 (Xu *et al*., 2006). *In vitro* studies on Nickel-induced apoptosis showed the promotion of apoptosis in B cells (Nowak *et al*., 2006), human T hybridoma cells (Guan *et al*., 2007), human hepatoma cells (Kang *et al*., 2004), keratinocytes (Cavani, 2005), human airway epithelial (HEp-2) and human breast cancer (MCF-7) cells (Siddiqui *et al*., 2012), human liver cells ($HepG_2$) (Ahamed *et al*., 2013), and normal rat kidney cells (Chen *et al*., 2010), along with human neutrophils and lymphocytes (Freitas *et al*., 2013;Chen *et al*., 2003). Chromium has also been shown to induce apoptosis in cultured cells (Blankenship *et al*., 1994; Manning *et al*., 1994).

Oxidative stress is considered to be one of the foremost molecular pathways for DNA damage. Reactive oxygen species (ROS) generated during cellular metabolic reactions can preferentially attack DNA nucleotide base guanine resulting in the formation of 8-OHdG lesions, which has been established to have exhibit mutagenic potential, hence used consistently as a putative biomarker for carcinogenesis (Karihtala *et al*., 2009). Few methods for analyzing the extent of 8-

OHdG lesions have been developed such as HPLC (high-performance lipid chromatography), which is often hyphenated with mass spectrometry (HPLC-MS/MS), antibody probes for DNA repair proteins or post treatment with the enzyme formamidopyrimidine DNA N-glycosylase before quantitative analysis with the comet assay to determine DNA strand breaks (Valavanidis *et al*., 2009).

Extensive experimental methodology development strategies have been undertaken demonstrating the occurrence of permanent damage to lipids of cellular membranes, proteins, and DNA due to oxidative stress arising from ROS and/or RNS. Various markers of oxidative damage have been identified (Halliwell and Gutteridge, 1999) most popularly such as malondialdehye (MDA), oxidized LDL, MDA-modified LDL, auto-antibodies against oxidized LDL and MDA-modified LDL, F2-isoprostane, and conjugated diene. In nuclear and mitochondrial DNA, 8-hydroxy-2' -deoxyguanosine (8-OHdG) or 8-oxo-7,8-dihydro-2' -deoxyguanosine (8-oxodG) is among the predominant forms of free radical-induced oxidative damages, and has therefore been widely observed a biomarker for oxidative stress and carcinogenesis (Cooke *et al*., 2001). Oxidation induced damage usually occurs in the nuclear and mitochondrial DNA from tissue and blood lymphocyte (Cooke *et al*., 2002). Among all purine and pyridine bases, guanine is most prone to oxidative processes. Following the oxidation reaction, a hydroxyl group is added to the 8-th position of the guanine molecule (which is most prone to oxidation among all purine and pyridine bases) resulting in the formation of 8-OHdG as one of the major forms of free radical-induced damages of DNA.

Quantification of oxidatively modified DNA in the form of 8-OHdG indicates the extent of DNA damage. Elevated concentration of oxidatively modified DNA has been detected in human tumor tissues (Kasai, 1997). Determination of 8-OHdG have also been performed using samples collected from the workers with high exposure to asbestos in order to determine the oxidative DNA damage caused by asbestos fibers and the concomitant risk of carcinogenesis (Marczynski *et al*., 2000). Study on oxidatively damaged nucleosides stated that concentration of DNA damage also depends on age in both nuclear and mitochondrial DNA (Cooke *et al*., 2001). Since

the oxidized nuclear DNA is typically repaired, the repair products, i.e., oxidized nucleosides and bases, which are relatively water-soluble, will directly be excreted into the urine without further metabolization. Numerous such products have been detected in urine which includes 8-OHdG, 8-OHGua, thymine glycol, and 5-hydroxymethyluracil. These urinary oxidized products include free bases, ribonucleosides from RNA and deoxynucleoside from DNA. Subsequently, urinary 8-OHdG is considered to be a significant biomarker of universal cellular oxidative stress and a DNA repair product in urine (Cooke *et al*., 2000).

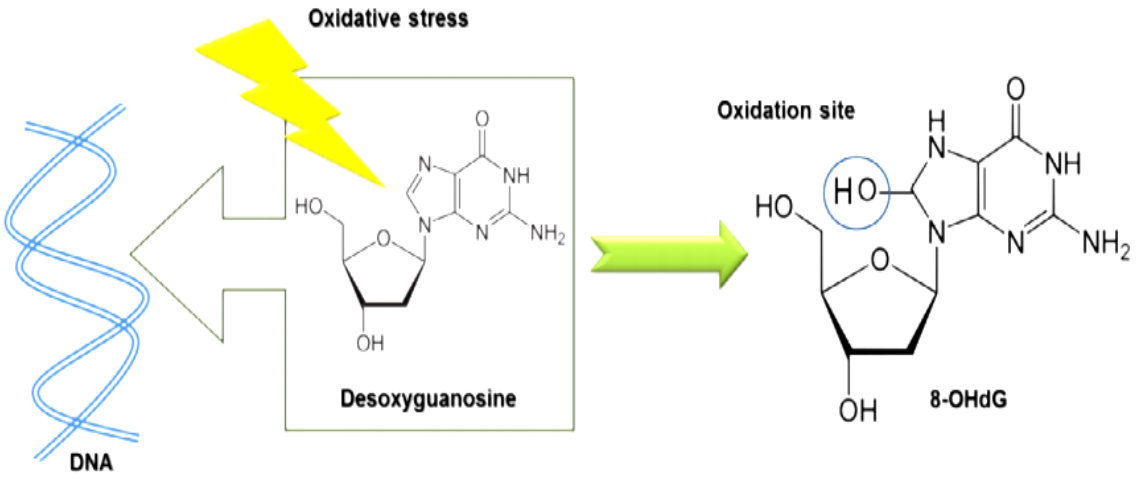



**Figure 1.7**: Formation process of 8-OHdG adduct (Emam *et al*., 2014).

Regardless of the availability of other spectrometry techniques such as HPLC, etc., a much simpler ELISA method has been developed (Tsuboi *et al*., 1998). The enzyme-linked immunosorbent assay (ELISA) is an immunological assay commonly used to measure antibodies, antigens, proteins and glycoproteins in biological samples. It combines the specificity of antibodies with the sensitivity of simple enzyme assays, by using antibodies or antigens coupled to an easily-assayed enzyme. ELISA can provide a useful measurement of antigen or antibody concentration. It combine the specificity of antibodies with the sensitivity of simple enzyme assays, by using antibodies or antigens coupled to an easily-assayed enzyme thereby providing a useful measurement of antigen or antibody concentration. ELISA methods are less cumbersome and more accurate when compared to other

techniques. For urine samples, the study found that ELISA estimates of concentration levels were about twofold higher than that of the HPLC (Shimoi *et al*., 2002).

A micronucleus (MN) test is an assay used in toxicological screening for potential genotoxic compounds by evaluation the presence of micronuclei. The MN assay has been recognized as one of the most successful and reliable assays for genotoxic carcinogens and is an ideal parameter to serve as a biomarker (Motgi *et al*., 2014). MN test is based on the score of number of micronuclei formation in exposed cells (Fenech, 2007). Micronuclei are formed during cell division (mitosis), at the anaphase stage from chromosomal fragments or whole chromosomes that are left behind during division of the nucleus (Heddle, 1973; Schmid, 1975; Fenech, 2000; Fenech and Morley, 1985, 1986). These acentric fragments or lost chromosomes gives rise to small nuclei which appear similar to the main nuclei on staining known as MN (Bolognesi *et al*., 2013; Fenech *et al*., 2011). High frequencies of MN have been demonstrated in exfoliated buccal cells in those individuals occupationally exposed to toxic environmental agents such as solvents, polycyclic aromatic hydrocarbons, emissions of sugarcane straw burning, gasoline, arsenic and anti-neoplastic drugs compared to the control group (Ramos *et al*., 2014).

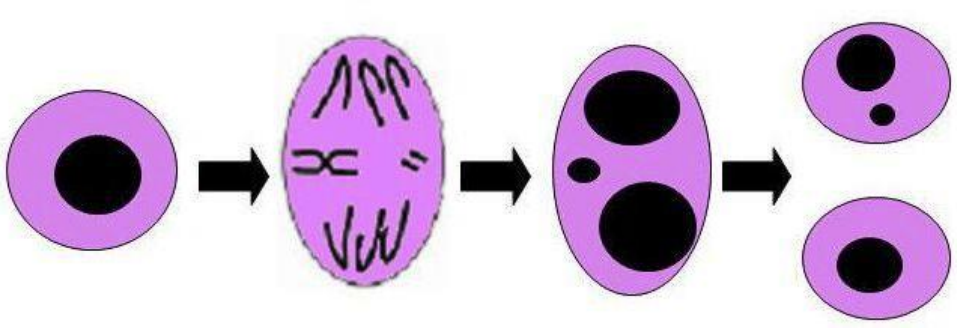


**Figure 1.8**: Formation process of micronuclei (MN) (Doherty *et al*., 2016).

Epithelial cells from the mucosal area of the oral cavity / oral mucosa are acquired from ectodermal embryonic germ layer (Kolltveit *et al*., 2010; Queiroz *et al*., 2010) and are the mechanical and protective barrier for the substantive problems. Cells of buccal mucosa play role as the first barricade against xenobiotic onslaught for inhalation or ingestion route and can metabolize carcinogenic agents to reactive

chemical substances. These epithelial cells execute thermal regulation thereby helping the immune system to release various classes of inflammatory agents (Squier and Kremer, 2001; Yohannes *et al*., 2011; Laheij *et al*., 2013; Bedran *et al*., 2014; Kullaa *et al*., 2014; Shiomi *et al*., 2015). Oral mucosa epithelial cells plays extremely useful source of cell for molecular or DNA research due to its easy access in terms of collection and regeneration capacity. In addition, oral epithelial cells are less exposed to mutation agents as compared to other skin epithelial tissues (Marchetto *et al*., 2004; Souto *et al*., 2006). Epithelial oral mucosal DNA has been used in various diagnostic studies (Aidar and Line, 2007), such as observed to resolve mutations in the Prader-Willi syndrome (PWS) (Munce *et al*., 2008), verifying the elevated risk for gonadoblastoma in Turner syndrome patients (Bianco *et al*., 2006), analyzing segregation in DNA 3243 A>G mutation in mitochondrial disorders (Uusimaa *et al*., 2007). Epithelial oral mucosal smears has been used in diagnosis of HNF1B mutation, a cancer related gene, to evaluate cancer risk for patients using Fluorescent In Situ Hybridization (FISH) technique (Laffargue *et al*., 2013). They are often considered as an appropriate site for assessment of early cytogenetic damage as about 90% of human cancers are epithelial in origin (Holland *et al*., 2008).

Application of liquid-based cytology (LBC) was introduced first as gynecological cytology (Bibbo and Wilber, 2008). LBC is a method of preparing samples for cytological examination which, unlike the conventional 'smear' preparation, involves making a suspension of cells from the sample which is then used to develop a thin layer of cells on a slide (Karnon *et al*., 2004). Several laboratories have subsequently established the application of LBC to non-gynecological sample and exfoliated buccal epithelial cells (Zeppa, 2014). Consistently prepared slides for the analysis of MN consists of bacteria, staining deposits, nuclear fragments and other cytoplasmic cells and necrotic cores that are easily confused with MN, which may further lead to generation of false positive results. However, slides prepared under LBC method can eliminate or reduce these confounding factors thereby serving as a reliable technique for MN analysis (Holland *et al*., 2008; Beerman *et al*., 2009; Norimatsu *et al*., 2013; Samanta and Dey, 2012).

Tobacco of Northeast India has never been characterized. The indigenously processed zozial without the so-called 'protective' filter is more preferred by the local population, residing mainly at rural areas, due to its abundance and cheaper in terms of cost. Generally, heavy metal species may directly psychologically influence behavior by impairing mental and neurological functions, influencing neurotransmitter production and utilization, and altering numerous metabolic processes (Dauwe *et al.,* 2004). Therefore, it is indeed imperative to monitor tobacco use as a lifestyle product for the protection of the environment and human health (Zhang *et al.,* 2005). Populace of Mizoram is generally the least knowledgeable with regard to health risks associated with zozial consumption. It is pertinent to mention here that zozial, manufactured on a cottage industrial scale, is sold without any regulation or restriction in Mizoram besides it does not have any health warnings on the pack. Zozial smoking is considered to be putatively posing a major health risks due to the potential synergistic effects of various xenobiotic compounds that are potentially hazardous to ingest and a detailed scientific study on the oxidative/carcinogenic effects of heavy metal species in the presence of various tobacco-specific carcinogens is essential. In order to understand the cellular and molecular mechanisms of induced pathophysiological processes due to the consumption of zozial, it is, therefore, the appropriate time to carry out the physico-chemical characterization of zozial and the evaluation of altered biochemical profile of the biological fluids of zozial consumers.

The present study focuses on the scientific study on the physico-chemical properties of the zozial tobacco and evaluation of its potential induced adverse health effects on the consumers with the following broad objectives:

i) Identification of various heavy metal species present in zozial, using Inductively Coupled Plasma - Optical Emission Spectroscopy (ICP-OES) methods.
ii) Determination of various elemental concentration using Instrumental Neutron Activation Analysis (INAA) methods.
iii) Determination of tobacco-specific nitrosamines (TSNAs) by Liquid chromatography–mass spectrometry (LC-MS/MS).

iv) Evaluation of the induced oxidative stress levels and the anti-oxidant capacity of zozial using the biological fluid samples (blood, urine and saliva) of zozial consumers in comparison with commercial cigarette users and non-smokers (control) among the rural Mizo population (free from urban pollution).

# CHAPTER 2

## MATERIALS AND METHODS

# CHAPTER 2

# MATERIALS AND METHODS

Zozial has been one of the few indigenous tobacco consumption habits by the Mizo population and it is imperative to carry out a detailed scientific study on locally sourced tobacco. No scientific research on the various chemical constituents of zozial has been so far performed due to which scarce scientific data is available on the adverse health effects of the chronic consumption of zozial. It is pertinent to carry out a detailed characterization of zozial, identifying important chemical constituents present in the tobacco matter and their potential detrimental effects on human health. This chapter provides details about the scientific methods for the characterization of tobacco leaves grown in Mizoram and zozial filler matter and assays employed for the assessment of the adverse effects on the biological matrices of zozial consumers.

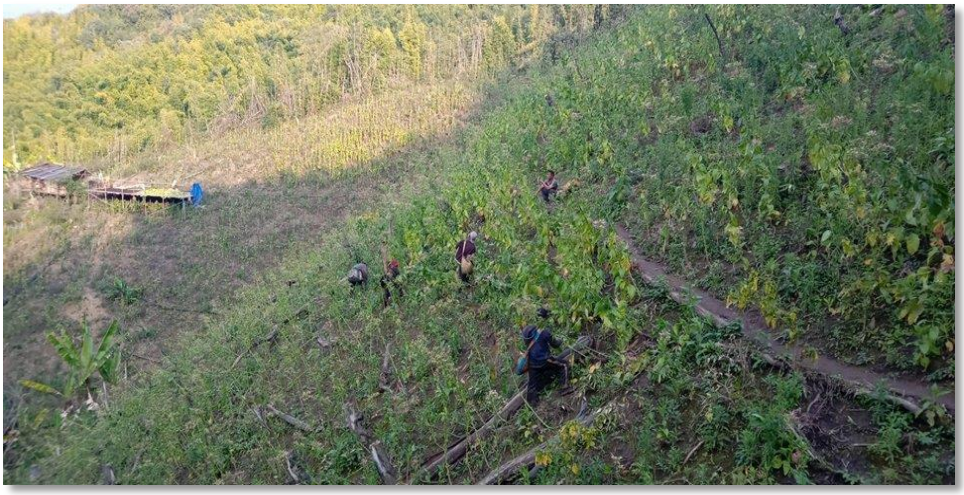
## 2.1. Zozial manufacturing

Zozial is idiosyncratic in its own way from other smoked tobacco products available in the Northeast Indian market as it is possibly the only, or, perhaps among the few entirely handmade tobacco products that is still made on a cottage industrial scale and consumed in the world. Though its consumption is rather limited over a small territory in Northeast India, viz., concentrated mostly within the state of Mizoram, most of the regular smoking consumers tend to favor and choose zozial despite the availability of a plethora of commercial cigarettes produced in India, Myanmar as well as imported brands. Like every other tobacco products, the raw tobacco leaves produced in Mizoram need to be ‘cured’ and subsequently processed for the production of zozial too; but, unlike most tobacco products, processing is carried out without the involvement of processes such as reconstitution of tobacco waste, conditioning, blending, fumigating, etc. The production of zozial is also undertaken entirely in the absence of modern ‘automated’ machineries albeit on a cottage level industry.

The tobacco (*Nicotiana tabacum*) used for *zozial* is exclusively grown within the state of Mizoram. During harvesting season (winter season), matured leaves of yellowish-green color, which were organically grown (without the amendment of chemical fertilizers or applications of herbicides/insecticides) are harvested by priming method (Removing 4 or 5 leaves at each priming in four or five installments). 'Oriental curing' is employed after the leaves are thoroughly squashed by using bare foot on a bamboo mat called "*pher"*. During curing, due to the loss of chlorophyll, progressively, the green color changes to yellow and concomitantly as a result of microbial 'fermentation' changes to golden brown color. After curing for ~ 10-15 days, the stems, petioles and large veins are stripped from the completely dried leaves are then made into coarse flakes using wooden mortar and pestle. Subsequently the coarse tobacco flakes are packed inside rolling paper, mostly by women folk, using bare hands. For long term usage, dried leaves are packed and stored in dry places away from moisture. Majority of Mizo rural population being farmers, most family would often grow their own tobacco for rolling their zozial; besides, as a practice where few families would collectively cultivate tobacco in a particular field is also traditionally followed; both of which resulted in availability of freshly harvested tobacco each year for consumption. It is also important to note that various anthropological activities which may lead to the deposition of heavy metals on the soil, in general, may also be absent in the rudimentary agricultural practices of Mizoram.

Domestically grown and harvested tobacco plants (Nicotiana tabacum) are used exclusively as the raw materials for zozial manufacturing. During the early days of Mizo settlements, smoking tobacco was practiced using pipes where the tobacco fillers were stuffed on one end of the bamboo pipe and lighted, and were sucked from the other end of hollow bamboo pipe. Since the arrival of papers which were brought in by the British on the later part of the 1880s, papers were then employed as rolling papers. Subsequently, specific cigarette papers were then introduced in Lushai hills area and used in later years for rolling zozial. After the introduction of cigarette paper, the production and marketing of zozial has been undertaken as cottage scale industry. The crushed tobacco fillers would be collected from

cultivation sites/villages and transported to the larger towns/sub-urban area where they would be sold in the local market. Persons engaged in rolling zozial (mostly women) would then roll the zozial with their bare hands. Zozial sticks (mostly 10 and 20 sticks per packet) are then packed in small polyethylene bags and sold [Figure 2.2 (F)].



**(A)**

**(B)**

**Figure 2.1: Tobacco harvesting in Mizoram.**
**(A) Harvesting tobacco by priming method.**
**(B) Sun curing of freshly harvested tobacco.**

**(A)**

**(B)**

**(C)**

**(D)**

**(E)**

**(F)**

**Figure 2.2**: **Tobacco processing and marketing.**

**(A) Squashing tobacco leaves using bare foot on a bamboo mat.**

**(B) Oriental Curing of tobacco leaves.**

**(C) A Mizo woman hand rolling zozial.**

**(D) Sun-cured tobacco leaves flaked using wooden mortar and pestle.**

**(E) Contents of one stick of zozial – tobacco flakes and rolling paper.**

**(F) Pack of commercially available Zozial.**

## 2.2. Collection of tobacco samples.

For the present study, tobacco leaf samples were collected for spectrometric analysis as the leaves were used for *zozial* as such without any chemical additives/flavors. Though majority of tobacco cultivation for cottage scale industry is mainly carried out on the vicinity of the eastern region of Mizoram, collection of samples was carried out as much diverse as possible covering the maximum possible regions of Mizoram where tobacco cultivation is undertaken. All samples were collected from selected cultivation sites that were well isolated from exposure to possible petrol and diesel pollutants with the nearest sampling site located 10 km(s) (approx.) away from the main road (rural roads).

### 2.2.1. Samples for ICP-OES -

Tobacco leaf samples from 23 different plantation sites from different districts of Mizoram were collected on 2015 and 2016 during harvesting season (December to February). Collected samples were grouped according to the area they were collected from as shown in Table 2.1. From each sampling site, approximately 10 fresh tobacco leaves each were collected. The collected samples were separately packed using fresh polyethylene bags. The collected leaf samples were then left under direct sunlight for curing. Sun-curing is carefully monitored in such a manner that tobacco leaves are under direct sunlight exposure at maximum possible duration. After sun curing for about a week, the completely dried leaf samples were packed in clean, dry and air-tight plastic containers and stored in room temperature in a dry area away from sunlight for further spectrometric analysis.

### 2.2.2. Samples for INAA –

Tobacco leaf samples were collected during harvesting season (December 2019 – February 2020) from 8 different cultivation sites from Northern and Eastern area of Mizoram, covering 4 districts. 2-3 sets each of 5-10 matured tobacco leaves from each tobacco plant were collected from each sampling sites. Each leaf samples were packed separately using clean polyethylene bags. The samples were orientally

cured. After curing, all samples were carefully labelled [Table 2.1 (b)] and stored in a clean and dry area preceding to analysis.

**Table 2.1: Sample information (ICP-OES).**

| Sl No | Location | Sample ID | | Sl No | Location | Sample ID |
|---|---|---|---|---|---|---|
| **1** | Mamit Site 1* | **T-1** | | **13** | South Lungpher Site 1* | **T-7** |
| **2** | Mamit Site 2* | | | **14** | South Lungpher Site 2* | |
| **3** | Hmunpui Site 1* | **T-2** | | **15** | Lungpher Site 1* | **T-8** |
| **4** | Hmunpui Site 2* | | | **16** | Lungpher Site 2* | |
| **5** | Lawngtlai Site 1* | **T-3** | | **17** | East Lungdar Site 1* | **T-9** |
| **6** | Lawngtlai Site 2* | | | **18** | East Lungdar Site 2* | |
| **7** | Saitual Site 1* | **T-4** | | **19** | Lunglei Site 1* | **T-10** |
| **8** | Saitual Site 2* | | | **20** | Lunglei Site 2* | |
| **9** | Tualbung Site 1* | **T-5** | | **21** | Dilkhan Site 1* | **T-11** |
| **10** | Tualbung Site 2* | | | **22** | Dilkhan Site 2* | |
| **11** | Champhai Site 1* | **T-6** | | **23** | Kolasib Site | **T-12** |
| **12** | Champhai Site 2* | | | | | |

* *Site 1 and Site 2 are from same village area but different locations.*

**Table 2.2: Sample information (INAA).**

| Sl No | Location | Sample ID | | Sl No | Location | Sample ID |
|---|---|---|---|---|---|---|
| **1** | Puilo | **T-13** | | **5** | Bungtlang | **T-17** |
| **2** | Chhawrtui | **T-14** | | **6** | Hmuntha | **T-18** |
| **3** | Tualte | **T-15** | | **7** | Lungdai | **T-19** |
| **4** | Khuangleng | **T-16** | | **8** | Model Veng | **T-20** |

## 2.3 Physical comparison of zozial and commercial cigarettes.

Commercial zozial packets available in the market were collected and comparison regarding length, amount of loaded tobacco and other physical comparisons were undertaken against available commercial cigarettes from Indian and Myanmar manufacturers.

## 2.4. Analysis of heavy metals by ICP-OES.

Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) is a destructive analytical technique that analyzes using plasma and depends on optical emission. In ICP-OES, the sample is introduced to the plasma in the form of aerosol using either nebulizers, laser ablation (LA-ICP-OES) or electrothermic vaporization (ETV). The ICP torch using thermal energy, volatilizes, atomizes, ionizes the aerosol sample and promotes the atoms/ions in their gaseous state to their excited orbital states. Concomitantly, 'excited' atomic species emit electromagnetic radiation to return back to the ground orbital state. The resultant 'poly'chromatic electromagnetic radiation (emr) in the UV-Visible region is dispersed to their constituent wavelengths, using high resolution optics, corresponds to the specific atomic constituents of the sample. The intensity of the emitted emr is correlated to the concentration of a given element.

In ICP-OES, the sample is introduced into the instrument as a stream of liquid sample which is converted into aerosol (a very fine mist of sample droplets) through a process called nebulization. The sample aerosol is then transported to the plasma by the nebulizer. Firstly, the high temperature plasma desolvates the aerosol resulting in microscopic salt particles. The microscopic particles are then vaporized as gaseous molecules and concomitantly atomized in the preheating zone (PHZ). After desolvation, vaporization and atomization, the gaseous atoms are then excited and ionized. The excitation and ionization process occur predominantly in the initial radiation zone (IRZ) and the normal analytical zone (NAZ). The analyte emission is usually measured in NAZ region. Though the precise mechanism of excitation and ionization in the ICP are not completely elaborated, it is considered that it takes place

as a result of collisions between analyte atoms and high energy electrons (Hasegawa *et al*., 1992). The main analytical advantage of the ICP over other emission sources is the ability of ICP to vaporize, atomize, excite and ionize efficiently a wide variety of elements present in several sample types in a reproducible manner. The higher temperature of ICP rather than flames and furnaces improves the excitation and ionization efficiencies besides reduces several chemical interferences experienced in the flames/furnaces.

The light emitted by the excited atoms and ions in the plasma region is measured to obtain qualitative as well as quantitative information about the atomic constituents of a sample. As the excited species in the plasma emit light at several different wavelengths, the emission from the plasma is polychromatic. This polychromatic radiation must be dispersed into the constituent wavelengths so that the emission from each excited species can be identified and its intensity can be measured without interference from emission at other wavelengths. The separation of light according to wavelength is generally done using a monochromator or a grating. The actual detection of the light, once it has been separated from other wavelengths, is done using a photosensitive detector such as a photo-multiplier tube (PMT) or advanced detector techniques such as charge-injection device (CID) or a charge-coupled device (CCD).Obtaining qualitative information involves identifying the presence of emission at the wavelengths characteristics of the elements of interest. Quantitative information, on the other hand, can be accomplished using plots of emission intensity versus concentration called calibration curves.

The elemental analysis of the collected samples was performed using ICP-OES (PERKIN ELMER OPTIMA 5300 DV ICP-OES) at IIT Madras. All the collected samples were labeled properly and were thoroughly ground using mortar and pestle. To meet the pre-requisite of the elemental analysis, all samples were acid digested using 4 ml Nitric Acid (ICP Grade) and 2 ml Hydrochloric Acid (ICP Grade). Digested samples were heated to 90 -110°C using hot plate for finer decomposition of the matrix. After cooling to room temperature, the samples were sonicated for 2-3 minutes. Appropriate dilutions were performed on the supernatant liquid using de-ionized water in 100 ml volumetric flasks. Concentrations of selected

metals: *viz.*, Arsenic, Cadmium, Lead, Mercury, Nickel and Chromium were analyzed at specific wavelengths as shown in Table 2.2.

**Table 2.3: Specific wavelengths (nm) used in ICP-OES readings.**

| Elements | Wavelength (nm) |
|---|---|
| As | 188.979 |
| Cd | 228.802 |
| Cr | 267.716 |
| Ni | 231.604 |
| Pb | 220.353 |
| Hg | 253.652 |

The analyzed concentrations of heavy metals were compared against the permissible limits of heavy metals (Indian standard) and the heavy metal concentrations of Indian commercial cigarettes.

## 2.5. Elemental analysis by INAA.

For INAA, sample irradiation was carried out at the installation REGATA of the pulsed fast reactor IBR-2 of the Frank Laboratory of Neutron Physics (JINR, Dubna, Russia). For neutron activation analysis (NAA), all the samples were dried at 105 °C to obtain constant weight and then homogenized. Plant material was pelletized. Then, samples (about 300 mg of plant material) were packed in polyethylene bags for short term irradiation and in aluminum bags for long-term irradiation. Two procedures of samples irradiation were applied. To determine elements with short-lived radionuclides (Mg, Al, Si, Cl, Ca, Ti, V, Mn, and Dy), samples were irradiated at a thermal neutron flux of 1.6 × 1012 $ncm^{-2}$ $s^{-1}$ for 3 min and measured for 15 min. To determine elements with long lived radionuclides (Na,

K, Sc, Cr, Fe, Co, Ni, Zn, As, Br, Rb, Sr, Zr, Sb, Cs, Ba, La, Ce, Nd, Sm, Eu, Tb, Yb, Hf, Ta, W, Th, and U), samples were irradiated for 4 days at a neutron flux 3.31 × 1011 $ncm^{-2}$ $s^{-1}$, re-packed, and measured twice using HP germanium detectors after 4 and 20 days of decay, respectively. The NAA data processing and determination of element concentrations were performed using Genie2000 and software developed in JINR.

The quality control of NAA results was ensured by simultaneous analysis of the examined samples and standard reference materials: NIST 1633b (Constituent Elements in Coal Fly Ash), NIST 2709 (San Joaquin Soil), NIST 1547 (Peach Leaves), NIST 1575a (Trace Elements in Pine Needles), and NIST 1632c (Trace Elements in Coal). The measured concentrations were in good agreement with the recommended values.

**2.6. Statistical analysis.**

Means ± standard deviations (SD) of each experimental analysis were calculated. Pearsons' correlation was used to determine the correlations between the pH of the soil and the analyzed concentration of various metals. SPSS statistical package (V 16.0) was used to perform statistical analysis.

**2.7. Estimated Daily Intake of Heavy Metals through *zozial.***

In order to determine the putative daily exposure of chemical residues arising from zozial smoke, the Estimated Daily Intake (EDI) of toxic heavy metals (As, Cd, Pb, Hg, Ni, and Cr) were calculated as follows:

$$\mathbf{EDI} = \frac{\mathbf{MC} \times \mathbf{Cons}}{\mathbf{BW}}$$

where MC is the mean concentration of heavy metal in zozial (µg/L); Cons is the daily average consumption of zozial in the study region (g/person/d); BW is the

average body weight of the consumers. The calculated EDI of heavy metals were compared with the tolerable daily intakes (TDI) of metals recommended by the JECFA (JECFA, 2004), USEPA (USEPA, 2010), ISIRI (ISIRI, 2010) and EFSA (EFSA, 2015).

**2.8. Target Hazard Quotient.**

Target Hazard Quotient (THQ) expressed the potential health risks due to zozial consumption. THQ is a ratio between the exposure to toxic element and the reference dose (RfD). RfD values of As, Cd, Pb, Hg, Ni, and Cr are 0.0003, 0.001, 0.004, 0.0005, 0.02, and 0.003 (µg/g bw/day), respectively (USEPA, 2010).

The THQ was calculated according to the method presented in the USEPA Region III risk-based concentration table (USEPA, 2007) and in Wang *et al*. (2005), according to the following equation:

$$THQ = \frac{EFr \times ED \times FIR \times MC}{RfD \times BW \times AT} \times 10^{-3}$$

where EFr is frequency of exposure (365 days/year); ED is the duration of exposure (68 years for our study population); FIR is food ingestion rate (g/person/d); MC is the mean level of metal in food (µg/g, on fresh weight basis); RfD is the oral reference dose (mg/kg/ d); BW is the average body weight, 66.9 Kg for age group ranging between 20-35 years, 66.79 Kg for age group ranging between 36-50 years, 68.41 Kg for age group ranging between 51-60 years; AT is the mean exposure time (365 days/year × number of exposure years, assuming 68 years in this study). ***THQ less than 1 indicates that the user will not be exposed to health risk***.

**2.9. Hazard Index.**

Zozial induced potential risk was assessed using hazard index (HI). The calculated hazard quotients were aggregated to evaluate the HI (USEPA, 2010) according to the following equation:

$$HI = \sum HQ = HQAs + HQCd + HQPb + HQHg + HQNi + HQCr$$

where Σ HQ is the summation of hazard quotients of metals and HQ As, HQ Cd, HQ Pb, HQ Hg, HQ Ni and HQ Cr and are the hazard quotients for Arsenic, Cadmium, Lead, Mercury, Nickel and Chromium respectively. ***Calculated HI value greater than 1 indicates a potential adverse health effect*** (Huang *et al*., 2008).

**2.10. Carcinogenic Risk.**

Carcinogenic risk (CR) is a probability of a person to develop cancer over the period of life due to prolonged exposure to a potential carcinogen. Cancer risk due to exposure to As, Cd, Pb, Ni and Cr were determined using cancer slope factor (CSF), provided by USEPA (2010) with the following equation:

$$CR = CSF \times EDI$$

where CSF is the carcinogenic slope factor of 1.5 $(mg/kg/day)^{-1}$ for As, 15 $(mg/kg/day)^{-1}$ for Cd 0.0085 $(mg/kg/day)^{-1}$ for Pb, 0.91 $(mg/kg/day)^{-1}$ for Ni and 0.5 $(mg/kg/day)^{-1}$ for Cr set by USEPA (2010). EDI is the estimated daily intake of heavy metals. $E^{-4}$ (1 in 10,000) to $E^{-6}$ (1 in 1,000,000) is the acceptable range of CR and a CR value away from the acceptable range indicates the probability of developing cancer by the exposed population.

**2.11. Determination of tobacco-specific nitrosamines (TSNAs) by Liquid chromatography–mass spectrometry (LC-MS/MS).**

Liquid chromatography–mass spectrometry (LC-MS/MS) is an effective analytical technique that combines the separating power of liquid chromatography with the highly sensitive and selective mass analysis potential of triple quadrupole mass spectrometry. In LC-MS/MS, a sample solution is pumped at high pressure through a stationary phase (LC column) by a mobile phase. The interactions between the constituents of the sample, the stationary phase and the mobile phase influence the flow rates through the LC column entail the separation mechanism. Availability of broad range of stationary phase and mobile phase combinations helps in facilitating effective and efficient separation thereby widening its application to adapt for several complex systems.

Following the elution of the sample, the effluent is directed to the mass spectrometer where it is nebulized, desolvated and ionized generating charged particles. These resulting charged particles generated under high vacuum eventually pass through a series of mass analyzers (quadrupole) by under the exposure of applied electromagnetic fields. A specified mass/charge precursor ion (or parent ion) is focused to pass through the first quadrupole, collided with an inert gas and gets fragmented into product ions (or daughter ions) in the collision cell. The third quadrupole targets a specific product ion fragments. The consequent isolated ions are then quantified using an electron multiplier. The equipment set up of High performance liquid chromatography coupled to tandem mass spectrometer (LC-MS/MS) with electrospray ionization (ESI) source consists of a binary pump, autosampler, column oven, tandem mass spectrometer and data collection system.

### 2.11.1. Sample preparation for TSNA analysis.

Solid tobacco samples were allowed to settle at room temperature for at least 12 hours prior to sample preparation. 0.1 g of the sample was taken and 1.5 mL of 100 mm ammonium acetate buffer (pH=6.8) was added and were thoroughly mixed using a stirrer for 60 minutes at 200 rpm. The solution was then centrifuged at 8000 rpm for 5 minutes and then filtered using 13 mm, 0.22 µm Nylon syringe filter. 50 µl of the extract was collected and then mixed with 50 µl internal standard solution [$d_5$-

NNAL (8 ppb), $^{13}C_6$-NNN (2 ppb) and $d_3$-NNK (8 ppb)]. Quantitative Analysis was done using LC-ESI-triple quad MS/MS system.

### 2.11.2. Sample preparation for $NO_2^-$/$NO_3^-$ analysis.

To each of 0.1 g of cut and ground tobacco samples, 5 mL each of deionised water was added and thoroughly mixed using a shaker at 200 rpm for 60 minutes. The solutions were then centrifuged at 3800 rpm for 10 minutes to separate the undissolved particles. After centrifugation, the solutions were then filtered using Nylon syringe filter of 13 mm and 0.22 µm to remove the particulate matters and diluted to 10× using deionised water. For $NO_2^-$ analysis, 250 µl of $^{15}$N-$NO_2^-$ and 50 µl of derivatization agent, 2,3-diaminonaphthalene (6.25 mM DAN) were added to the prepared solution and heated in an oven at 37°C for 30 minutes. The solution was cooled to room temperature and 50µl of NaOH (0.58 M) was added and diluted to 10× using 10% MeOH/1 mM

LC-MS/MS was performed using CORESTA recommended Method No. 72 "Determination of Tobacco-Specific Nitrosamines in tobacco products by LC-MS/MS".

### 2.12. Evaluation of the oxidative potential of zozial.

In addition to physico-chemical characterization, this study aimed to evaluate the oxidative potential of zozial among zozial consumers in the Mizo population. Biological samples were collected from a pool of willing donors within the Mizo population from the state of Mizoram. The samples were classified depending on the demographic conduct of the person as habituated *viz.,* tobacco consumption, dietary habits, etc. Different approach were undertaken to evaluate the effect of zozial on the consumers.

Oxidative stress is the imbalance between the production of free radicals and antioxidants in the biological system caused mostly by the lifestyle habits. Free radicals are oxygen-containing molecules with number of unpaired electrons which renders them in unstable configuration, and therefore, they are highly reactive

allowing them to react rapidly with other ‘electron-rich’ molecules resulting in a large cascade of chemical reactions in the body. These reactions leading to the degradation of ‘electron-rich’ molecules are called oxidation. They can either be advantageous or damaging. The damaging action of free radicals can be neutralized by antioxidants which donate electron(s) to free radicals without destabilizing themselves.

Oxidative stress can results in various pathophysiological conditions such as gene mutations and cancers, Alzheimer’s disease, Parkinson's disease, chronic fatigue syndrome, heart and blood vessel disorders, atherosclerosis, heart failure, heart attack and inflammatory diseases.

### 2.12.1. 8-hydroxy 2 deoxyguanosine (8-OHdG) ELISA (enzyme-linked immunosorbent assay).

The concentration of 8-hydroxy 2 deoxyguanosine (8OHdG) within a cell essentially reflects the extent of oxidative stress levels as it is one of the major by-products of DNA damage and therefore considered as a potential biomarker for oxidative stress. In biological specimen such as blood plasma, cell lysates, and tissue samples, 8-OHdG is detectable either as the free nucleoside or assimilated along with the DNA fragments. However, the kidney can filter free 8-OHdG into the urine leaving the larger DNA fragments in the bloodstream due to which urine is considered as a more suitable matrix for the quantification of free 8-OHdG content than plasma. In this study, the concentration of urinary 8-OHdG was measured and assessed for *zozial* consumers in comparison with non-smokers.

#### 2.12.1 (a) Sample collection.

For 8-OHdG Assay, urine sample were collected to assess the difference in the concentration of 8-OHdG in urine specimen between the aforementioned study groups. Autoclaved sample vials (5 ml glass vials) were distributed to 48 consented participants (24 *zozial* consumers and 24 controls) after a brief explanation to collect their first urine in the morning after sleep. The collected urine samples were then immediately retrieved and filtered using filter paper (0.2 µm) and then stored in

–20°C. The participants for this study comprised the donors living in rural area with biological age group ranging from 25-55 years (Mean age = 40.42 ± 9.95) for controls and 29-60 years (Mean age = 41.79 ± 8.84) for *zozial* consumers from selected villages within Mizoram. Questionnaires containing information regarding their demographic details along with their lifestyle habits, *viz.*, tobacco habits, alcohol consumption, daily lifestyle, dietary habits, etc. were also collected from the volunteers. The undertaken protocol were reviewed and approved by the Institutional Review Board of all institutes involved in the study.

**2.12.1 (b) 8-OHdG Assay.**

Two sets of 8-hydroxy 2 deoxyguanosine (8-OHdG) *in vitro* ELISA Kit (ab201734) purchased from Abcam (Abcam Inc., UK) were used to perform the quantitative measurement of urinary 8-OHdG following the protocols given in the Kit. All reagents were equilibrated to room temperature (18-25°C) prior to use. 1× Wash Buffer was prepared by diluting the supplied 10× wash buffer using deionized (UltraPure) water. Antibody (1× 8-hydroxy-2-deoxyguanosine: HRP Conjugate Monoclonal Antibody) was prepared by dilution of 8-hydroxy-2-deoxyguanosine:HRP Conjugate Antibody Concentrate 1:100 with 8-hydroxy-2-deoxyguanosine Antibody Diluent. TMB (3,3',5,5'-Tetramethylbenzidine) and stop solutions were provided at working strength and used as such.

Standard solution is a solution with known concentrations prepared for the quantitative measurement of solutions with unknown concentrations. For standard solution preparation in the present study, 8-hydroxy-2-deoxyguanosine Standard (Stock) vial was first centrifuged for the maximum availability of standard solution. 8 autoclaved centrifuge tubes were labeled Tube 1 – Tube 8. 500 µl each of Sample and Standard Diluent solution were first added to centrifuge tubes labeled as Tube 1 and 8; 250 µl each of the Sample and Diluent solution were added to tubes 2-7. 10 µl of 3.06 µl/mL 8-hydroxy-2-deoxyguanosine Standard was added to Tube 1 to make a concentration of 60 ng/mL and was thoroughly mixed. 250 µl of the solution from Tube 1 was pipetted out and then added and mixed with Tube 2. 250 µl of Tube 2 solution was then again pipetted out and added to Tube 3 and the trend was

continued till Tube 7 which results in the final concentration of standards 1-8 (Tubes 1-8) in the order 60 µl/mL, 30 µl/mL, 15 µl/mL, 7.5 µl/mL, 3.75 µl/mL, 1.875 µl/mL, 0.94 µl/mL and 0 µl/mL respectively as shown in Table 2.3. These standard solutions with known concentrations were then used in the measurement of concentrations of the samples.

**Table 2.4: Standard preparation and concentrations (8-OHdG)**

| Standard # | Sample to Dilute | Volume to Dilute (µL) | Volume of Diluent (µL) | Starting Conc. (ng/mL) | Final Conc. (ng/mL) |
|---|---|---|---|---|---|
| 1 | Stock | 10 | 500 | 3060 | 60 |
| 2 | Standard #1 | 250 | 250 | 60 | 30 |
| 3 | Standard #2 | 250 | 250 | 30 | 15 |
| 4 | Standard #3 | 250 | 250 | 15 | 7.5 |
| 5 | Standard #4 | 250 | 250 | 7.5 | 3.75 |
| 6 | Standard #5 | 250 | 250 | 3.75 | 1.875 |
| 7 | Standard #6 | 250 | 250 | 1.875 | 0.94 |
| 8 | Diluent | 0 | 500 | 0 | 0 |

All urine samples were diluted with the supplied Sample and Standard Diluent in the ratio 1:20 (V:V) prior to testing. 50 µl of the prepared Standard solutions (Standard 1-8) were pipetted in triplicates to wells labeled S1-S8 on the supplied 8-OHdG : Bovine Serum Albumin (BSA) Coated Plate. The diluted samples were also loaded in triplicates to the wells labeled 1-24 (1-12 for zozial users and 13-24 for controls). 50 µl each of Antibody solution was added to each well except the Zero standard (S8) 50 µl of Standard and Sample Diluent was added to the Zero standard well. Antibody solution detects and amplifies the target molecule using the catalytic property of the enzyme. The plate was covered using the

supplied adhesive plate cover and then incubated at room temperature for 1 hour. All the contents of the plate was then emptied and washed with 1 × wash buffer (300 µl each) for four times using multichannel pipette while drying it by tapping gently against paper towel in between the washes. After washing was completed, 100 µl of TMB Substrate was added to each well and then covered with a fresh adhesive plate cover. The plate was then incubated again in the dark at room temperature for 30 minutes. After incubation, the reaction was terminated by adding the Stop Solution (100 µl) to each well. The absorbance was measured using a micro plate reader (SpectraMax M2e) at 450 nm.

**2.12.1.(c) Statistical Analysis.**

Independent Sample T Test, Bivariate correlation test and linear regression analysis were performed to find the relationship between the various quantitative and qualitative variables and the level of the resultant concentrations. All statistical analyses were performed using SPSS statistical package (V 16.0) and ORIGIN Pro (V 8.0).

**2.12.2. Superoxide Dismutase (SOD) ELISA (enzyme-linked immunosorbent assay).**

Superoxide dismutase (SOD), which catalyzes the dismutation of the superoxide anion ($O_2^{-\bullet}$) into hydrogen peroxide and molecular oxygen, is one of the most important antioxidative enzymes. The current SOD Assay utilizes the highly water soluble tetrazolium salt, WST-1 [2-(4-Iodophenyl)- 3-(4-nitrophenyl)-5-(2,4-disulfophenyl)- 2H-tetrazolium, monosodium salt] that produces a water-soluble formazan dye upon reduction with a superoxide anion. The rate of the reduction with $O_2$ is proportional to the xanthine oxidase (XO) activity, and is inhibited by SOD. Therefore, the $IC_{50}$ (50% inhibition activity of SOD) can be determined by a colorimetric method.

**2.12.2 (a) Sample collection.**

For this study, 2 ml of blood samples were intravenously collected from each of 145 zozial consumers and 56 controls (non-smokers) with the age distribution between 20 to 62 years (Mean age = 44.63 ± 10.18) for zozial consumers and aged between 21 and 59 years (Mean age = 40.61 ± 11.4) for controls from selected rural areas of Mizoram who were willingly participating in this study. Serum was extracted from each blood samples following the method of Tuck *et al*. (2009). Serum contains proteins and other molecules that could portray the entire physiological system eliminating cells and clotting factors. The cells and clotting factors were removed from the blood sample by keeping the samples undisturbed for 30 mins at room temperature allowing a clot to form. The timing should be carefully monitored as serum samples that are allowed to sit less than 30 min are likely to retain cellular elements and samples that sit longer than 60 min are likely to experience cell lysis, releasing cellular components impacting future analysis (Timms *et al*., 2007). The separated serum samples were then stored in –20°C for further analysis. All volunteers were personally briefed about the study and were engaged with their full agreement. Consent of the participants was taken and the participants were interviewed with standardized structured questionnaires which contain demographic information besides their lifestyle habits, *viz*., their tobacco habits, alcohol consumption, daily lifestyle, dietary habits, etc. The undertaken protocol were reviewed and approved by the Institutional Review Board of all institutes involved in the study.

**2.12.2 (b) SOD Assay.**

SOD Assay was performed using SOD Determination Kit purchased from Sigma-Aldrich (Cat. 19160, Sigma, St. Louis MO, USA) on the collected serum samples using the prescribed standard protocol available at www.sigmaaldrich.com. Working solutions were prepared using the components supplied along with the kit as follows; water-soluble tetrazolium salt (WST - 1[2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)- 2H-tetrazolium, monosodium salt] ) working solution was prepared by diluting 1 ml of WST Solution with 19 ml of Buffer

solution. Enzyme working solution was prepared by mixing 15 µl centrifuged Enzyme Solution with 2.5 ml of Dilution Buffer.

Plain 96-well microplate was taken and four wells were labeled as 'Sample', 'Blank 1', 'Blank 2' and 'Blank 3'. Detailed summary of loading solutions to microplate is displayed in Table 2.4.

**Table 2.5: Solution loading pattern for Sample and Blanks 1, 2 and 3 wells.**

| | Sample | Blank 1 | Blank 2 | Blank 3 |
|---|---|---|---|---|
| Sample solution | 20 µl | - | 20 µl | - |
| Ultrapure H2O | - | 20 µl | - | 20 µl |
| WST Working solution | 200 µl | 200 µl | 200 µl | 200 µl |
| Enzyme working solution | 20 µl | 20 µl | - | - |
| Dilution buffer | - | - | 20 µl | 20 µl |

After equilibrating the serum samples at room temperature, 20 µl of each of the samples were pipetted to 'Sample' and 'Blank 2' wells. 20 µl each of ultrapure $H_2O$ was added to 'Blank1' and 'Blank 3' wells. 200 µl of WST Working Solution was added to each well that produces a water-soluble formazan dye upon reduction with a superoxide anion. The contents of each well were diluted with 200 µl of Dilution Buffer. 20 µl of Enzyme Working Solution each was added to 'Sample' and 'Blank 1' wells to catalyze the reaction. The plate was then incubated at 37°C for 20 minutes and absorbance reading was taken at 450 nm using a microplate reader (SpectraMax M2e).

SOD activity (inhibition rate %) was calculated using the equation:

$$\textbf{SOD activity (inhibition rate \%)} = \frac{(\mathbf{A_{Blank\ 1} - A_{Blank\ 3}}) - (\mathbf{A_{Sample} - A_{Blank\ 2}})}{(\mathbf{A_{Blank\ 1} - A_{Blank\ 3}})} \times \mathbf{100}\%$$

### 2.12.3. Micronucleus (MN) Assay.

The genotoxic interruption leading to disorder in the typical cell repair mechanism, DNA damage and chromosomal anomaly may stimulate the formation of MN bringing about cell death, genomic instability or development of cancer. *In vitro* MN assay was performed to analyze the difference in the extent of DNA damage between *zozial* consumers, commercial cigarette users and controls.

### 2.12.3 (a) Sample collection.

For this study, 100 each of epithelial cell samples from oral mucosa (EOM) of zozial users, commercial cigarette consumers and controls (with no tobacco consumption history) were collected from voluntary participants from selected rural areas of Mizoram. EOM were obtained by gently scraping the mucosal surfaces (palate and gum) using an interdental brush, right after the participants are requested to thoroughly rinse their mouth with water. The brush containing the cell samples were dipped and the cells were suspended in 1× PBS solution and stored under appropriate condition for further analysis.

The age distribution for each groups are in the range of 21 and 58 years (Mean age = 39.94 ± 10.86) for controls, 21 to 57 years (Mean age = 40 ± 10.4) for *zozial* consumers and 22 to 54 years (Mean age = 36.88 ± 9.53) for commercial cigarette users. Volunteers were briefly informed about the study and participated with their full consent. Information regarding their demographic factors, tobacco habit, alcohol habit, life style, dietary habits, etc was also collected from the study subjects using a standardized questionnaire along with their consent. The undertaken protocol were reviewed and approved by the Institutional Review Board of all institutes involved in the study.

### 2.12.3 (b) MN Assay.

50 µl of the cell suspensions were uniformly smeared over a clean glass slides. The samples were fixed using 3:1 Methanol:Acetic Acid solution and were dried at room temperature. Staining was performed using Giemsa's staining solution

(Stanbio ISO 9001 : 2000) with staining time of 20 min. The stained glass slide was washed with Acetonitrile to remove the excess staining. The slides were evaluated using light microscope (MLX-B Plus) with 100-fold magnification using oil immersion. 1000 cells were counted for each staining procedure and the frequency of MN formation was recorded for each slides. The images of the cells with micronucleus (MN) and normal cells were captured for comparative observations using mobile phone camera attached to the lens of the microscope.

### 2.12.4 Statistical Analysis.

Independent Sample T Test, Bivariate correlation test and linear regression analysis were performed to find the relationship between the various quantitative and qualitative variables and the level of the resultant concentrations. All statistical analyses were performed using SPSS statistical package (V 16.0) and ORIGIN Pro (V 8.0).

# CHAPTER 3

## RESULTS AND DISCUSSIONS

# CHAPTER 3

# RESULTS AND DISCUSSIONS

## 3A. RESULTS:

### 3A.1. Chemical characterization of *Zozial*

The elemental contents (Macro-, micro-, and trace element distributions) of zozial filler (tobacco) samples were qualitatively determined using instrumental neutron activation analysis (INAA) and inductively coupled plasma-optical emission spectrometry (ICP-OES) method. Tobacco samples under investigation were collected from different conditions and cultivation sites of Mizoram, India. Differences in metal concentration of tobacco samples may be due to the agro climatic and environmental conditions on which the plant is grown. Concentration of various heavy metal species in tobacco products may vary widely depending on country specific and product specific (Verma *et al*., 2010) various parameters such as the agro climatic factors including the production practices and soil characteristics and the environmental conditions such as humidity, temperature, sunlight, rainfall etc., have been reported to influence the accumulation of metals in the leaf (IARC, 2004).

#### 3A.1.1 Elemental analysis (INAA).

Using INAA, alkaline earth metals such as Na, Mg, K, Ca, Rb, Cs, Sr, Ba along with Transition elements such as V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Zr, and Rare earth elements such as La, Ce, Nd, Sm, Eu, Tb, Dy, Yb, Hf, Ta, W, Th, and U were detected in the leaf samples at ppm (µg/g) levels. The concentrations (Average ± S.D) of various elements for the collected samples are presented in Table 3.1.

**Table 3.1: Elemental concentrations (µg/g) in tobacco leaf samples.**

| Elements | Concentrations (µg/g) | | | Elements | Concentrations (µg/g) | | |
|---|---|---|---|---|---|---|---|
| | Min. | Max. | Average ± S.D | | Min. | Max. | Average ± S.D |
| **Na** | 309 | 753 | 410.75 ± 267.42 | **Rb** | 14.6 | 29.4 | 20.7 ± 9.26 |
| **Mg** | 7800 | 9300 | 8562.5 ± 2116.56 | **Sr** | 165 | 165 | 173.25 ± 33.85 |
| **Al** | 1240 | 3140 | 1445.75 ± 829.3 | **Zr** | 7.5 | 13 | 7.27 ± 3.03 |
| **Si** | 15900 | 64600 | 37500 ± 15303.2 | **Sb** | 0.071 | 0.192 | 0.1 ± 0.06 |
| **Cl** | 643 | 42100 | 16926.6 ± 15475.5 | **Cs** | 0.135 | 0.304 | 0.16 ± 0.08 |
| **K** | 57000 | 64000 | 49050 ± 11309.5 | **Ba** | 107 | 200 | 159.25 ± 44.79 |
| **Ca** | 22600 | 30300 | 26050 ± 7335.82 | **La** | 1.7 | 4.8 | 3.09 ± 1.09 |
| **Sc** | 0.287 | 0.82 | 0.34 ± 0.22 | **Ce** | 2.3 | 7.3 | 3.44 ± 1.62 |
| **Ti** | 134 | 172 | 114.25 ± 34.78 | **Nd** | 1 | 3.9 | 1.78 ± 0.97 |
| **V** | 1.53 | 4.8 | 2.05 ± 1.27 | **Sm** | 0.255 | 0.79 | 0.38 ± 0.18 |
| **Cr** | 2.4 | 8.4 | 4.33 ± 2.14 | **Eu** | 0.07 | 0.162 | 0.07 ± 0.04 |
| **Mn** | 123 | 681 | 214.25 ± 190.57 | **Tb** | 0.027 | 0.093 | 0.04 ± 0.02 |
| **Fe** | 809 | 3390 | 1183.63 ± 951.06 | **Dy** | 0.31 | 0.75 | 0.46 ± 0.15 |
| **Co** | 0.83 | 3.5 | 1.32 ± 0.93 | **Yb** | 0.063 | 0.276 | 0.1 ± 0.07 |
| **Ni** | 5.6 | 10 | 5.76 ± 2.44 | **Hf** | 0.26 | 0.52 | 0.29 ± 0.12 |
| **Cu** | 19 | 49 | 38.95 ± 15.28 | **Ta** | 0.029 | 0.072 | 0.03 ± 0.02 |
| **Zn** | 101 | 203 | 149.75 ± 70.86 | **W** | 0.2 | 0.34 | 0.29 ± 0.08 |
| **As** | 0.277 | 0.91 | 0.36 ± 0.23 | **Th** | 0.325 | 1.03 | 0.43 ± 0.26 |
| **Br** | 25.3 | 76 | 61.54 ± 34.85 | **U** | 0.086 | 0.23 | 0.1 ± 0.06 |

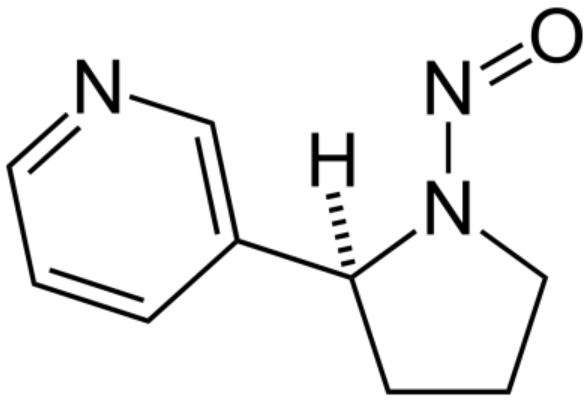
### 3A.1.2. Trace metal analysis (ICP-OES).

Using ICP-OES, the concentrations of trace metals viz., As, Cd, Pb, Hg, Ni and Cr was analyzed from the tobacco samples. The concentration range of various trace elements for the collected 23 samples (grouped accordingly) is presented in Table 3.2. The ICP-OES data showed the presence of arsenic at a concentration ranging between 1.78 – 9.69 µg/g, cadmium 0.64 – 6.52 µg/g, lead 1.78 – 5.29 µg/g, mercury 0.12 – 0.67 µg/g, nickel 0.63 – 1.96 µg/g and chromium 0.51 – 3.49 µg/g on the tobacco samples. The mean concentrations of the analyzed heavy metals are 3.60 µg/g for As, 1.92 µg/g for Cd, 2.55 µg/g for Pb, 0.36 µg/g for Hg, 1.12 µg/g for Ni and 1.21 µg/g for Cr. As is the most abundant and Hg is the least abundant among the elements analyzed. Among the study samples, Arsenic from T-12 site showed the highest concentration and Hg from T-1 sampling site has the lowest concentration. The list of concentrations of each element from each samples are presented in Table 3.2.

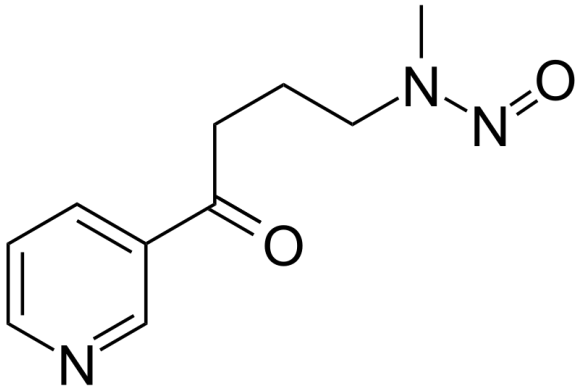
| Table 3.2: ICP-OES analysis of concentrations* of heavy metals (µg/g) in tobacco samples. | | | | | | |
|---|---|---|---|---|---|---|
| **SAMPLE ID** | **As** | **Cd** | **Pb** | **Hg** | **Ni** | **Cr** |
| **T-1** | 1.78 ± 1.19 | 1.44 ± 0.98 | 2.76 ± 2.17 | 0.12 ± 0.09 | 1.01 ± 0.75 | 0.80 ± 0.61 |
| **T-2** | 1.97 ± 1.16 | 1.14 ± 0.69 | 2.41 ± 1.53 | 0.47 ± 0.26 | 1.12 ± 0.8 | 0.54 ± 0.37 |
| **T-3** | 2.19 ± 0.99 | 1.17 ± 0.62 | 1.78 ± 1.46 | 0.58 ± 0.24 | 0.63 ± 0.52 | 1.04 ± 0.63 |
| **T-4** | 2.91 ± 1.44 | 1.64 ± 1.01 | 2.33 ± 1.38 | 0.17 ± 0.15 | 0.74 ± 0.48 | 0.51 ± 0.31 |
| **T-5** | 2.02 ± 1.27 | 1.09 ± 0.94 | 2.29 ± 1.24 | 0.52 ± 0.24 | 1.37 ± 0.71 | 1.17 ± 0.57 |
| **T-6** | 2.28 ± 1.22 | 0.64 ± 0.41 | 1.82 ± 1 | 0.30 ± 0.15 | 0.79 ± 0.36 | 0.75 ± 0.57 |
| **T-7** | 3.72 ± 1.62 | 1.89 ± 0.92 | 2.75 ± 2.03 | 0.23 ± 0.12 | 0.71 ± 0.47 | 1.30 ± 0.85 |
| **T-8** | 2.80 ± 1.35 | 1.66 ± 0.80 | 2.78 ± 1.46 | 0.67 ± 0.35 | 1.10 ± 0.61 | 1.21 ± 0.74 |
| **T-9** | 3.05 ± 1.41 | 1.28 ± 0.61 | 1.98 ± 0.96 | 0.25 ± 0.15 | 0.66 ± 0.51 | 1.27 ± 0.94 |
| **T-10** | 2.93 ± 1.4 | 0.98 ± 0.75 | 2.43 ± 1.31 | 0.17 ± 0.12 | 1.85 ± 1.25 | 1.65 ± 1 |
| **T-11** | 7.90 ± 5.16 | 6.52 ± 4.18 | 5.29 ± 3.04 | 0.60 ± 0.67 | 1.96 ± 1.63 | 3.49 ± 1.98 |
| **T-12** | 9.69 ± 7.64 | 3.64 ± 3.09 | 1.99 ± 1.73 | 0.19 ± 0.11 | 1.45 ± 1.22 | 0.79 ± 0.58 |
| *Mean ± Standard deviation | | | | | | |

### 3A.1.3. Determination of tobacco-specific nitrosamines (TSNAs) by Liquid chromatography–mass spectrometry (LC-MS/MS).

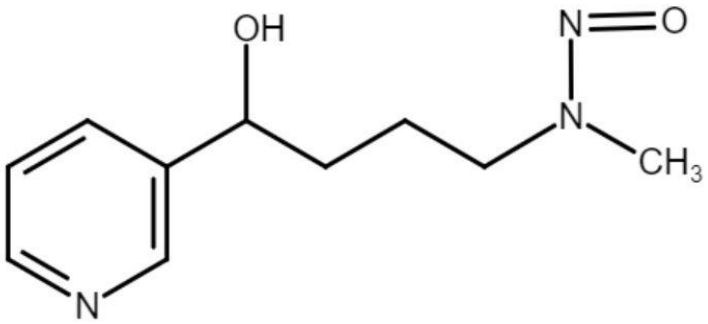
Using LC-MS/MS methods, the determination of TSNAs present in zozial samples, Indian and Myanmar commercial cigarette samples was performed. Zozial samples and Tobacco leaf fillers (freshly collected pre-rolled tobacco fillers that will further be used for rolling zozial) along with Indian and Myanmar commercial cigarette samples were analyzed. N-nitrosonornicotine (NNN), Nicotine-derived nitrosamine ketone (NNK) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) were detected in the analyzed samples. Concentrations (ng/g) of the detected TSNAs are given in Table 3.3 (a) (b) (c).




**(A)**




**(B)**




**(C)**

**Figure 3.1: Structures of TSNAs analyzed**

**(A) N-nitrosonornicotine (NNN)**

**(B) Nicotine-derived nitrosamine ketone (NNK)**

**(C) Nicotine-derived nitrosamine aldehyde (NNAL)**

**Table 3.3: Concentrations (in ng/g) of (a) NNN, (b) NNK and (c) NNAL in tobacco samples.**

**(a) Concentration of NNN (ng/g)**

| Sl No. | NNN (ng/g) | | | |
|---|---|---|---|---|
| | **Z** | **TLF** | **CC-M** | **CC-I** |
| 1 | 3.7 | 2 | 887.5 | 46.4 |
| 2 | 11.69 | 1.8 | 594.61 | 40.48 |
| 3 | 20.04 | 2.6 | 65.56 | 46.75 |
| 4 | 2.47 | 2.9 | 595 | 40 |
| 5 | 12 | 1.97 | 66 | 47 |
| 6 | 20 | 1.79 | 1190 | 80 |
| 7 | 23 | 2.59 | 130 | 90 |

Limit of detection (LOD) for NNN: 0.05 ng/g

Z – Zozial

TLF – Tobacco leaf filler

CC-M – Commercial cigarette (Myanmar)

CC-I – Commercial cigarette (Indian)

**(b) Concentration of NNK (ng/g)**

| Sl. No. | NNK (ng/g) | | | |
|---|---|---|---|---|
| | **Z** | **TLF** | **CC-M** | **CC-I** |
| 1 | N.D | ND | 58.8 | 24.1 |
| 2 | 13.96 | ND | 90.59 | 35.52 |
| 3 | 18.12 | 3.4 | 56.12 | 31.42 |
| 4 | N.D | 2.5 | 91 | 36 |
| 5 | 14 | ND | 56 | 31 |
| 6 | 18 | ND | 180 | 70 |
| 7 | 28 | 3.45 | 110 | 60 |

**ND** – Below detection limits (**LOD**)

Limit of detection (LOD) for NNK: 0.59 ng/g

Z – Zozial

TLF – Tobacco leaf filler

CC-M – Commercial cigarette (Myanmar)

CC-I – Commercial cigarette (Indian)

**(c) Concentration of NNAL (ng/g)**

| Sl. No. | NNAL (ng/g) | | | |
|---|---|---|---|---|
| | **Z** | **TLF** | **CC-M** | **CC-I** |
| 1 | ND | ND | 6.2 | 4.5 |
| 2 | 1.95 | ND | 9.24 | 5.66 |
| 3 | 5.75 | ND | 32.06 | 5.18 |
| 4 | ND | ND | 9.2 | 5.7 |
| 5 | 1.9 | ND | 32 | 5.2 |
| 6 | 5.7 | ND | 18 | 11 |
| 7 | 3.9 | ND | 64 | 10 |

**ND** – Below detection limits (**LOD**)

Limit of detection (LOD) for NNAL: 0.25 ng/g

Z – Zozial

TLF – Tobacco leaf filler

CC-M – Commercial cigarette (Myanmar)

CC-I – Commercial cigarette (Indian)

### 3A.2. DNA Damage Study.

Oxidative stress is considered to be one of the major molecular pathways of DNA damage. Reactive oxygen species (ROS) generated during cell metabolic reactions are able to attack DNA base guanine resulting in the formation of 8-OHdG lesions, which has been established to have mutagenic potential. These defective DNA bases would be removed by DNA repair proteins and concomitantly excreted. Hence excreted 8-OHdG levels have been used consistently as a biomarker for carcinogenesis (Karihtala *et al.*, 2009).

#### 3A.2.1. 8-OHdG Assay.

In our study, we have used urine specimen to determine 8-OHdG levels as the biomarker of oxidative DNA damage using ELISA assay as this technique is non-invasive and 8-OHdG is persistently stable in the urine (Poulsen et al., 1998). Results of the present study using ELISA assay has demonstrated that zozial consumption can cause considerably elevated urinary 8-OHdG levels in comparison with non-smokers (controls) samples of the study population.

Samples from 48 consented participants (24 *zozial* consumers and 24 controls) were analysed using 8-hydroxy 2 deoxyguanosine (8-OHdG) *in vitro* ELISA Kit (ab201734) purchased from Abcam (Abcam Inc., UK) and the test were performed in triplicate sets. The urinary 8-OHdG concentration of controls ranges from 1.33-7.55 ng/mg creatinine (Table 3.4) and that of zozial users ranges from 1.93-11.64 ng/mg creatinine (Table 3.5). The mean concentration of urinary 8-OHdG for zozial consumers was 5.43 (SD=2.98) ng/mg creatinine and for controls was 3.87 (SD=1.77) ng/mg creatinine (Figure 3.2).

**Table 3.4: Urinary-8OHdG concentrations (ng/mg) of controls.**

| CONTROLS | | | | |
|---|---|---|---|---|
| **ID** | **8-OHdG** | | **ID** | **8-OHdG** |
| H-1 | 2.19 | | H-13 | 3.36 |
| H-2 | 2.13 | | H-14 | 3.68 |
| H-3 | 1.33 | | H-15 | 4.90 |
| H-4 | 2.79 | | H-16 | 3.63 |
| H-5 | 3.43 | | H-17 | 4.52 |
| H-6 | 2.69 | | H-18 | 3.69 |
| H-7 | 2.65 | | H-19 | 5.47 |
| H-8 | 3.58 | | H-20 | 7.22 |
| H-9 | 2.23 | | H-21 | 7.55 |
| H-10 | 2.07 | | H-22 | 7.01 |
| H-11 | 2.47 | | H-23 | 5.61 |
| H-12 | 2.78 | | H-24 | 6.00 |

**Table 3.5: Urinary-8OHdG concentrations (ng/mg) of zozial users.**

| ZOZIAL USERS | | | | |
|---|---|---|---|---|
| **ID** | **8-OHdG** | | **ID** | **8-OHdG** |
| Z-1 | 2.58 | | Z-13 | 7.45 |
| Z-2 | 4.04 | | Z-14 | 6.96 |
| Z-3 | 2.41 | | Z-15 | 6.41 |
| Z-4 | 3.54 | | Z-16 | 9.51 |
| Z-5 | 2.13 | | Z-17 | 7.55 |
| Z-6 | 2.10 | | Z-18 | 10.66 |
| Z-7 | 3.23 | | Z-19 | 3.97 |
| Z-8 | 1.93 | | Z-20 | 6.24 |
| Z-9 | 3.98 | | Z-21 | 6.53 |
| Z-10 | 3.19 | | Z-22 | 9.13 |
| Z-11 | 3.55 | | Z-23 | 11.64 |
| Z-12 | 2.63 | | Z-24 | 8.87 |

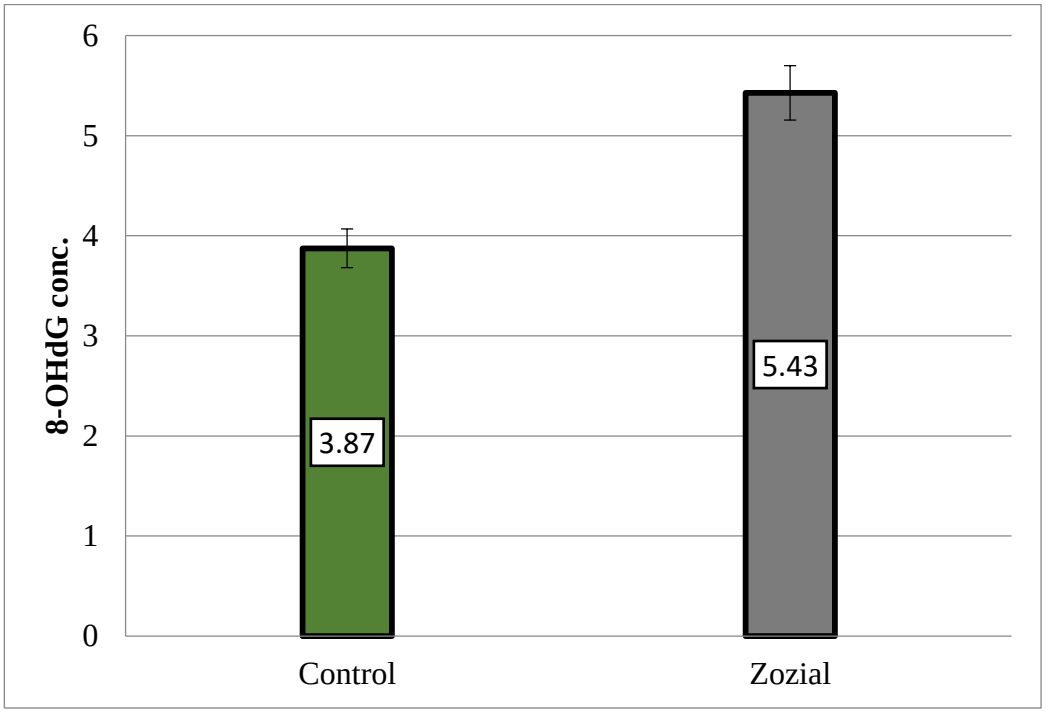



**Figure 3.2: Mean Urinary-8OHdG concentrations (ng/mg creatinine) of Zozial users and controls.**

### 3A.2.2. Superoxide dismutase (SOD) Assay.

Antioxidant enzymes, such as SOD are critical 'safety valves' against overproduction of reactive oxygen species (ROS) (Flora, 2011; Jomova *et al*., 2015). SOD converts superoxide anion into hydrogen peroxide ($H_2O_2$) and molecular oxygen ($O_2$). Samples from 201 consented participants (145 *zozial* consumers and 56 controls) were analysed using SOD Determination Kit purchased from Sigma-Aldrich (Cat. 19160, Sigma, St. Louis MO, USA) and the test were performed in triplicate sets and the inhibition rate (%) was calculated using the provided equation:

$$\textbf{SOD activity (inhibition rate \%)} = \frac{(\mathbf{A_{Blank\ 1}} - \mathbf{A_{Blank\ 3}}) - (\mathbf{A_{Sample}} - \mathbf{A_{Blank\ 2}})}{(\mathbf{A_{Blank\ 1}} - \mathbf{A_{Blank\ 3}})} \times \mathbf{100}\%$$

The activity of SOD in serum samples of zozial users and controls are summarized in Table 3.6. The SOD activity significantly increased in serum of zozial smokers by heavy metal exposure. The percentage of inhibition between zozial consumers and controls were compared against each other to evaluate the existing differences (Figure 3.3). Right from the initial stage, elevated percentage of inhibition was observed for zozial users when compared to control. This elevation was time-dependent after one hour. Our results demonstrated that reactive oxygen species (ROS) generation is elevated in zozial consumers when compared to the control population. Thus, high levels in $H_2O_2$ could exacerbate high ROS production.

**Table 3.6: SOD Activity (percentage of inhibition) of Zozial users and controls.**

| TIME (mins) | PERCENTAGE OF INHIBITION | |
|---|---|---|
| | **Zozial users (%)** | **Controls (%)** |
| 0 | 23.51 ± 1.78 | 8.21 ± 1.2 |
| 10 | 31.76 ± 7.78 | 13.27 ± 0.87 |
| 20 | 54.56 ± 3.91 | 19.02 ± 0.85 |
| 30 | 76.74 ± 2.58 | 24.16 ± 0.78 |
| 40 | 82.93 ± 1.7 | 32.01 ± 4.79 |
| 50 | 92.55 ± 3.2 | 58.96 ± 12.43 |

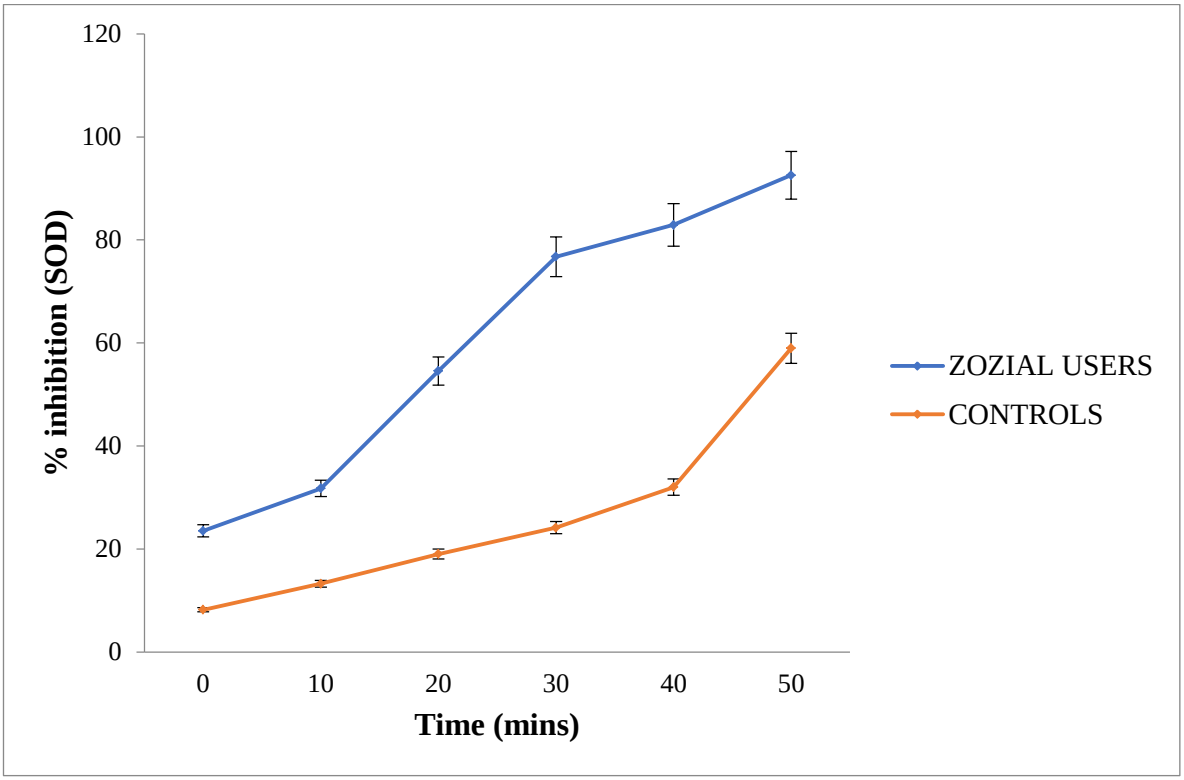



**Figure 3.3: SOD Activity (percentage of inhibition) of Zozial users vs. controls.**

### 3A.2.3. Micronucleus (MN) Assay.

As cells of buccal mucosa play role as the first barricade against xenobiotics, MN Assay was also performed along with oxidative stress-related assays to evaluate DNA damage [Figure 3.4(a) and Figure 3.4(b)]. The frequency of micronuclei was studied in 100 each of controls, commercial cigarette users and zozial users. The mean frequency of MN count per 1000 cells was highest for zozial consumers with mean MN count of 40, followed commercial cigarette users with mean MN frequency of 16.23. The mean frequency of MN for controlled was found to be 2.3 MN per 1000 cells (Fig 3.5).

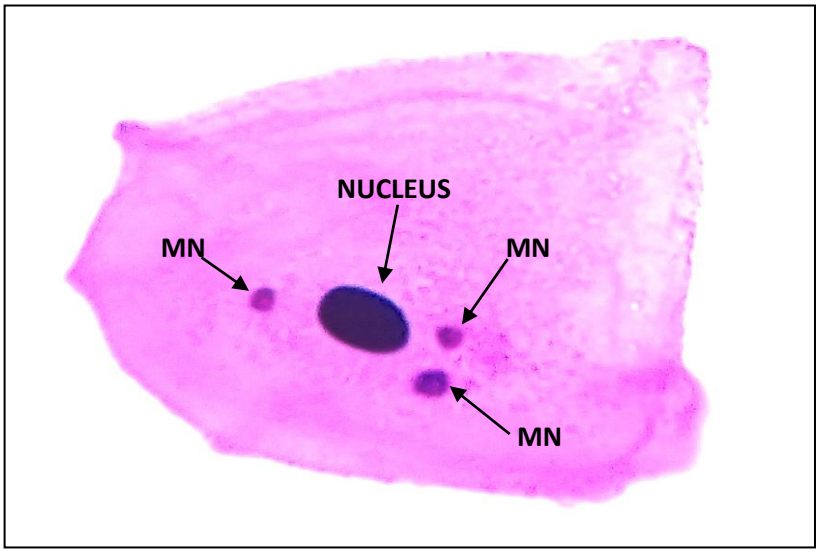



**Figure 3.4(a): Exfoliated human buccal mucosa cell showing nucleus and micronuclei using Giemsa dye observed under light microscope.**

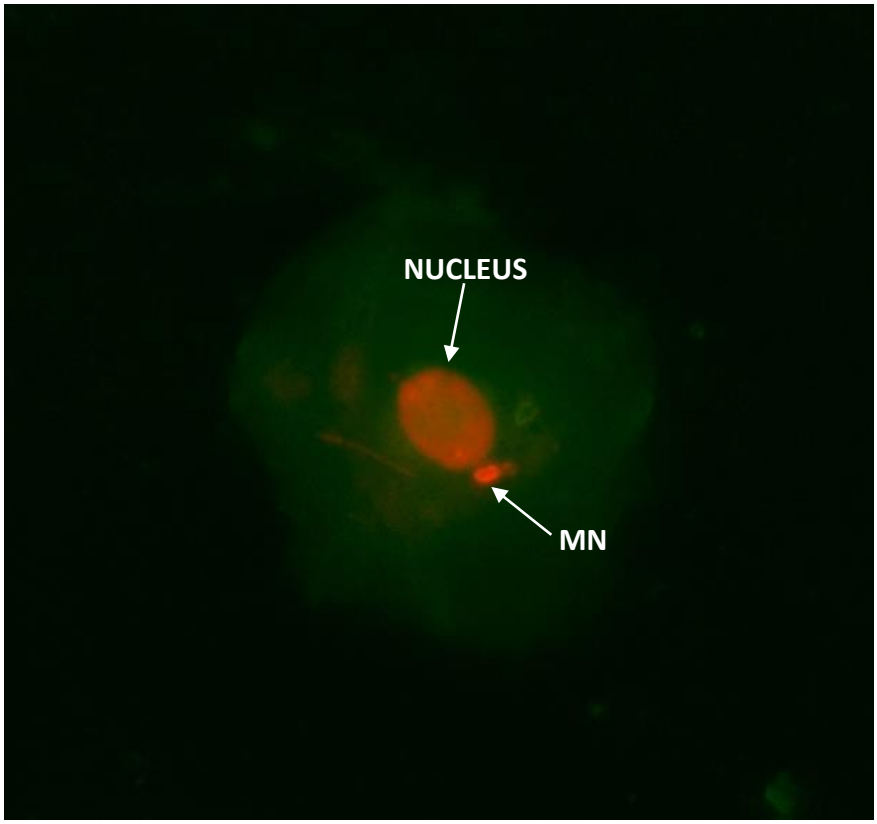



**Figure 3.4(b): Exfoliated human buccal mucosa cell showing nucleus and micronuclei using Acridine orange dye observed under fluorescence microscope.**

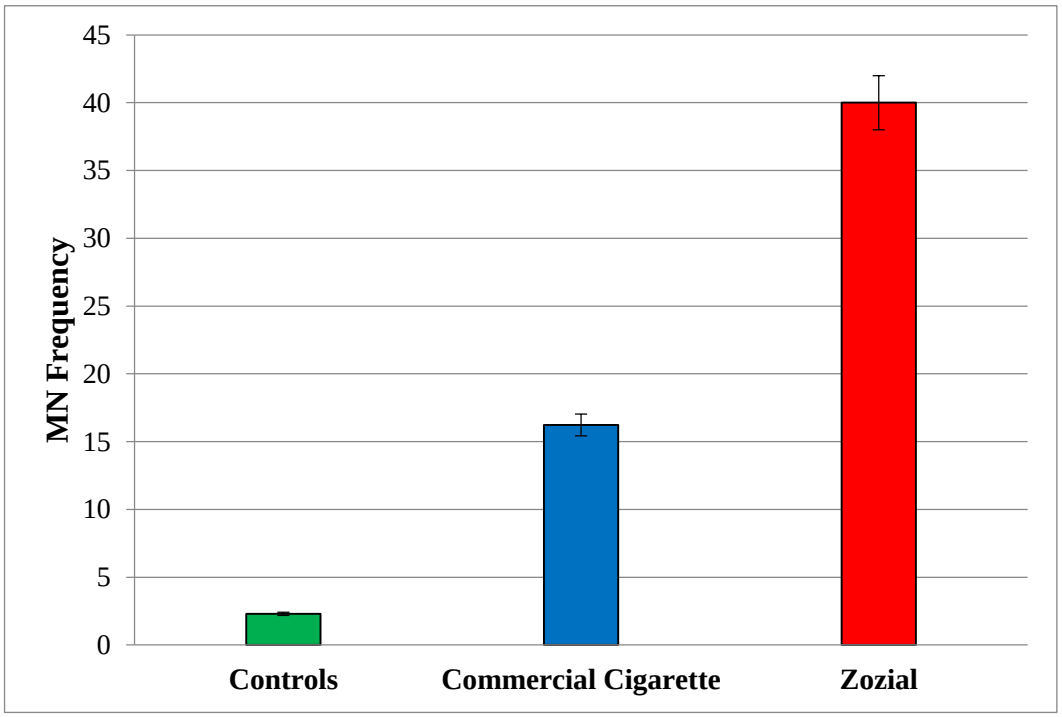



**Figure 3.5: Frequency of MN count.**

## 3B. DISCUSSIONS:

### 3B.1. Physical comparison of zozial and commercial cigarettes.

Comparative study on the physical characteristics between zozial and commercial cigarettes from Indian and Myanmar manufacturers which are commercially available in the study area was conducted. Both the variety of commercial cigarettes selected for this comparative study, viz. 'V', a Myanmar cigarette, and 'Silk cut', an Indian cigarette, are among the most customarily consumed commercial cigarettes in the study area. Comparisons on the basis of their physical appearance, length, amount of tobacco matter loaded per sticks, and other distinctions were noted to report the peculiarity of zozial among other smoked tobacco products.

Packaging of commercial Myanmar and Indian cigarettes are properly carried out with a commercial grade sealed-box pack consisting of 20 and 10 cigarette sticks per pack respectively. On the other hand, zozial is hand-packed using custom-made polyethylene bags and heat-sealed and mainly packed in a group of 15 to 20 sticks depending on the price of the available tobacco fillers (per Kg). Packaging of zozial is done in such a manner that any information such as manufacturing dates, place and health warning signs of any kind is totally absent which is against the Prevention of Food Adulteration Act & Rules (PFA, 2004). Zozial has a pronounced difference among the three brands due to the absence of cellulose acetate type filter. Length wise, Myanmar cigarette is the longest (86 mm) while Indian cigarette (66 mm) and zozial (67 mm) were nearly of the same length. Though shorter in length than Myanmar cigarette, the average amount of tobacco loaded (in grams) on zozial was the most (830 mg). This is due to the absence of cigarette filter in zozial that the whole length of the stick is fully loaded with tobacco fillers. The tobacco for zozial is darker in color compared to both the commercial cigarettes indicating that TSNA formation proceeds predominantly through leaf-borne microbial facilitated reaction of tobacco alkaloids with nitrite (Bush *et al*., 2001). A detailed comparison between the three is shown in Figure 3.6.


(a)


(b)

(i) Approx length of : Zozial = 67 mm
Silk cut = 66 mm
V = 86 mm

(ii) Approx weight of tobacco loaded on :
Zozial = 830 mg
Silk cut = 543 mg
V = 766 mg

(c)

(d)

(e)

**Figure 3.6: Comparison between Zozial and commercial cigarettes.**

**a) (i) 'V' (Commercial Myanmar cigarette)**
**(ii) 'Silkcut' (Commercial Indian cigarette)**
**(iii) 'Zozial' (Indigenous Mizo Cigarette)**

**b) (i) Comparison based on length of sticks.**
**(ii) Comparison based on weight of loaded tobacco filler.**

**c) Tobacco filler from Indian Commercial Cigarette.**

**d) Tobacco filler from Zozial.**

**e) Tobacco filler from Myanmar Commercial Cigarette.**

**3B.2. Elemental analysis.**

As already pointed out on 3A.1.1, alkaline earth metals such as Na, Mg, K, Ca, Rb, Cs, Sr, Ba along with Transition elements such as V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Zr, and Rare earth elements such as La, Ce, Nd, Sm, Eu, Tb, Dy, Yb, Hf, Ta, W, Th, and U were detected in the tobacco leaf samples at ppm (µg/g) levels. *Nicotiana tabacum* necessitate various essential elements for growth and survival. These elements enter the plant system from the soil, flows through the roots, through the stem, and ultimately get absorbed in the leaves which are the main destination for all elements.

The most abundant elements analyzed from our tobacco samples were Mg, Al, Si, Cl, K, Ca and Fe. Potassium is the most abundant cation in plants playing essential roles in enzyme activation, protein synthesis, photosynthesis, osmoregulation, stomatal movement, energy transfer, phloem transport, cation–anion balance and stress resistance (Wang *et al*., 2013).

Calcium is an essential element and is required for various structural roles in the cell wall and membranes; it is a counter - cation for inorganic and organic anions in the vacuole (White and Broadley, 2003). Chlorine is another essential element thar maintains the normal plant growth and development by means of osmotic and stomatal regulation, evolution of oxygen in photosynthesis, disease resistance and tolerance (Chen *et al*., 2010). Magnesium is important for chlorophyll biosynthesis and carbon fixation existing as a cofactor of a series of enzymes involved in carbon metabolism (Ducu *et al*., 2010; Konieczyski and Wesoowski, 2012; Guo *et al*., 2015). Iron is an essential element for majority of the living organisms as it contributes in an extensive array of metabolic processes, including oxygen transport, deoxyribonucleic acid (DNA) synthesis, and electron transport (Abbaspour *et al*., 2014).

Elements such as Na, Ti, Mn, Cu, Zn, Br, Rb, Sr and Ba were found to be present at minor levels, and Sc, V, Cr, Co, Ni, As, Zr, Sb, Cs, La, Ce, Nd, Sm, Eu, Tb, Dy, Yb, Hf, Ta, W, Th and U at trace levels. Fe, Mn, Zn, and Br are considered

as essential nutrients for human body. Manganese triggers enzymes that hepls in the metabolism of carbohydrates, amino acids, and cholesterol and is also engaged in photosynthesis (Demoz *et al*., 2016). Zinc is essential for the normal growth and the reproduction of all higher plants and animals, and of humans (Frassinetti *et al*., 2006). Bromine is essential element required for several enzymes activity (Scott *et al*., 2014). For significant periods, rare earth elements (REEs) were assumed to have no biological function; despite that, study conducted by Liu et al. (2012) suggested that REEs can regulate the chlorophyll activities and photosynthetic rate in plants. Recent studies have shown that at low concentrations, REEs have a positive effect on the growth of medicinal plants and negative effects at comparatively high concentrations (Zhang *et al*., 2013). In the current study, REE elements, namely La, Ce, Nd, Sm, Eu, Tb, Dy, Yb, Hf, Ta, W, Th and U were determined at ppm concentration. Al, Cs, Rb, Sb, Sr, and Th have no known biological functions and their major source can be considered soil. WHO has established maximum amounts in medicinal plants for lead, cadmium and arsenic (WHO, 1998).

**3B.3. Trace metal analysis.**

The ICP-OES data showed the presence of trace metals such as arsenic at a concentration ranging between 1.78 – 9.69 µg/g, cadmium 0.64 – 6.52 µg/g, lead 1.78 – 5.29 µg/g, mercury 0.12 – 0.67 µg/g, nickel 0.63 – 1.96 µg/g and chromium 0.51 – 3.49 µg/g on the tobacco samples (3A.1.2). The variation of heavy metal concentrations in different samples suggests the difference in the source and growing conditions of the tobacco samples. Though the potential source for heavy metals such as industrial pollution, amendments of chemical fertilizers, application of insecticides/pesticides and several other anthropological activities are not practiced within the sampling areas, the concentration levels of the analyzed heavy metals are considerably high. Majority of heavy metals are recognized to be carcinogenic (Tchounwou *et al*., 2012; IARC, 2004). The effects of heavy metal exposure have been reported on the biological system such as cell membrane, mitochondrial, lysosome, endoplasmic reticulum, nuclei, and certain enzymes entailed in metabolism, detoxification, and damage repair (Wang and Shi, 2001).

Metal ions can cause DNA damage and conformational changes which may further induce carcinogenesis or apoptosis (Chang *et al*., 1996; Wang and Shi, 2001; Beyersmann and Hartwig, 2008). Generation of reactive oxygen species (ROS) and induced oxidative stress are the dominant characteristics of the toxicity and carcinogenicity of metals such as arsenic (Yedjou and Tchounwou, 2006, 2007; Tchounwou *et al*., 2004), cadmium (Tchounwou *et al*., 2001), chromium (Patlolla *et al*., 2009), lead (Yedjou and Tchounwou, 2008; Tchounwou *et al*., 2004), and mercury (Sutton and Tchounwou, 2007; Sutton *et al*., 2002). As a result of their significant extent of toxicity, these elements are proven toxicants even on exposure to lower concentrations, and are known to induce multiple organ dysfunctions. These metals have been classified as human carcinogens by the United States Environmental Protection Agency (USEPA) and the International Agency for Research on Cancer (IARC).

Arsenic, which is known to be highly toxic even at very low concentration, was found in the range of 1.78 – 9.69 µg/g and is the most abundant element in zozial samples. Tobacco smoke has been reported to contain arsenic, particularly when the tobacco has been treated with lead arsenate insecticide (WHO, 2002). Cigarette smoking lowers methylation capacity of arsenic thereby retards the elimination of arsenic from the body and thus smoking can act synergistically with arsenic exposure to cause DNA damage in lungs (Hays *et al.*, 2006). Arsenic is known to induce intracellular signal transduction, activates transcription factors, and alters the expression of genes that are involved in promoting cell growth, proliferation, and malignant transformation (Schoen *et al.*, 2004). It is also important to note that for zozial samples collected at T-12 (Kolasib site), the observed concentration of mercury is exceptionally high.

The second most abundant element in zozial samples, lead, was detected in the range of 1.78 – 5.29 µg/g. Lead has been classified as Group 2A human carcinogen by the international agency for research on cancer (IARC) (IARC, 2004). Lead plays a substantial role in the toxicity of tobacco as it contributes to lung cancer through the release of radiation from Pb-210. The nervous system is the most

susceptible target of lead poisoning. Lead is capable of causing serious effects on the brain and bone mineral density (Amodio-Cocchieri *et al*., 1996; USEPA, 2002). Recent studies found that antioxidant enzyme levels of workers exposed to lead are remarkably higher than that found in non-exposed workers (Bechara *et al*., 1993). In lead-induced oxidative stress, the reactive oxygen species (ROS) impose an oxidative imbalance causing damage to membranes, DNA and proteins (Patra *et al*., 2011). Lead exposure is known to entail certain biological processes such as induced cell death & oxidative stress (Yedjou and Tchounwou, 2008; Yedjou *et al*., 2006), transcriptional activation of stress genes (Tchounwou *et al*., 2004), and DNA damage (Yedjou and Tchounwou, 2008).

Cadmium is among the several chemicals in cigarette smoke generating ROS thereby placing smokers at a greater risk of free radicals (Kim *et al*., 2010). Cadmium has been conjectured to provoke cell damage mainly through the generation of ROS (Stohs and Bagchi, 1995), causing DNA damage thereby impeding the synthesis of nucleic acids and proteins (Mitra, 1984). Tobacco plants are capable of absorbing cadmium from the soil and accumulate it in high concentrations. Table 3.1 depicts the concentration of cadmium of different zozial samples. Smoking is among the preventable source of cadmium exposure (WHO, 2010); where substantial portion of it passes into smoke thus posing a risk to passive smokers as well (Chiba and Masironi, 1992). Cadmium has a remarkably long half-life in humans (Tribowo *et al*., 2014). Contribution of long-term occupational exposure to cadmium to the development of various types of cancer is evident enough that the international agency for research on cancer (IARC) has classified cadmium as Group 1 carcinogens to humans (IARC, 2004).

Mercury is identified in the concentration range of 0.12 – 0.67 µg/g. Mercury induced oxidative stress demonstrated mechanisms of sulfhydryl reactivity by which reaction with cystine residues of proteins occurs depleting the cellular antioxidants which serves as a line of cellular defense (Valko *et al*., 2006). Mercury exposure also triggers the generation of oxidative damage by the accumulation of reactive oxygen species (ROS). These ROS are eliminated by cellular antioxidants in normal

conditions (Tchounwou *et al*., 2012). Mercury is classified as Group 3 carcinogen by international agency for research on cancer (IARC) (IARC, 2004).

Concentration levels of chromium was in the range of 0.51 – 3.49 µg/g. Chromium in its trivalent compound is an essential elemental species (Mertz and Reginsky, 1987) as it in modulates the carbohydrate levels of the body. In recent study, cellular damage due to exposure to high concentrations of $Cr^{3+}$ has been reported (Eastmond *et al*., 2008). $Cr^{6+}$ produces hyper-reactive hydroxyl radicals and the exposure to high concentration of $Cr^{6+}$ results in red blood cell damage due to oxidation and the concomitant functional degradation of the liver and kidney (Shi and Dalal, 1993; Hamilton and Wetterhahn, 1986; Dartsch *et al*., 1998). Recent study showed that chromium mainly induce apoptosis, oxidative stress and DNA damage (Kim *et al*., 2015). As $Cr^{6+}$ species is gravely toxic in nature with potent carcinogenic effects, it is classified by International Agency for Research on Cancer (IARC) as a group I carcinogen (IARC, 2004).

Nickel (Ni) is an essential element in the biological system at lower levels, but exposure to high concentration is toxic. It was found in the range of 0.63 – 1.96 µg/g in zozial samples. High level exposure to Ni has been linked with neurotoxicity, hepatotoxicity, nephrotoxicity, gene toxicity, reproductive toxicity, and elevated risk of cancer (Xu *et al*., 2015; Gathwan *et al*., 2012; Scutariu and Ciupilan, 2007; Goodman *et al*., 2009; Forgacs *et al*., 2012; Duman and Ozturk, 2010). Nickel has been reported to be mutagenic (Werfel *et al*., 1998) and is accountable for several types of cancer cases such as cancer of the respiratory tract including dermal, lung, and nasal sinus cancers (Bernhard *et al*., 2005; Awofolu *et al*., 2005). Nickel is stable and consistent environmental contaminant and it is impossible to biologically or chemically eliminate unlike many other organic toxic pollutants. Although nickel is an essential trace element, exposure or ingestion of large amount is toxic. Due to the high concentration of carbon monoxide in tobacco smoke, nickel can produce nickel carbonyl which is known to be the most toxic form of nickel (Ellenhorn, 1997) and is considered to be a potential carcinogen (Sunderman, 1961; Chiba and Masironi, 1992). Epidemiological and experimental studies have demonstrated the carcinogenic

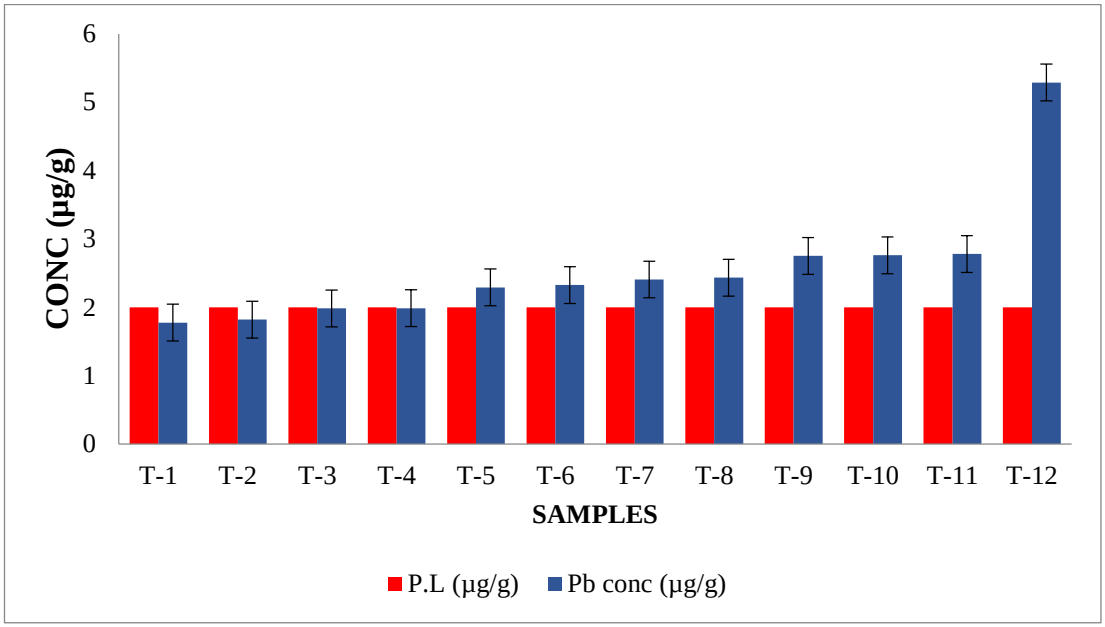
potential of nickel compounds (Shakya, 2007). The international agency for research on cancer (IARC) has also classified nickel as a Group I carcinogenic to human (IARC, 2004).

The heavy metal analysis of zozial has yielded very useful information about the exposure and indirect intake of heavy metals by the consumers. Permissible limit is a legal or safe limit for exposure of a person to a chemical substance or physical agent. Exposure to certain chemicals with concentration beyond their permissible limits may results in toxic effects. Several consumable products have recommended limits for concentration of heavy metal content. The permissible limit concentrations (in µg/g) in plants for Cd, Pb, Hg, Ni and Cr according to WHO, (1996) and Patra and Sharma, (2000) are shown in Table 3.3. Such guideline values for plant systems are not available for Arsenic (Sanyal, 2018). Comparative analysis between the mean concentrations of heavy metals detected from zozial samples and their corresponding permissible limits was performed.

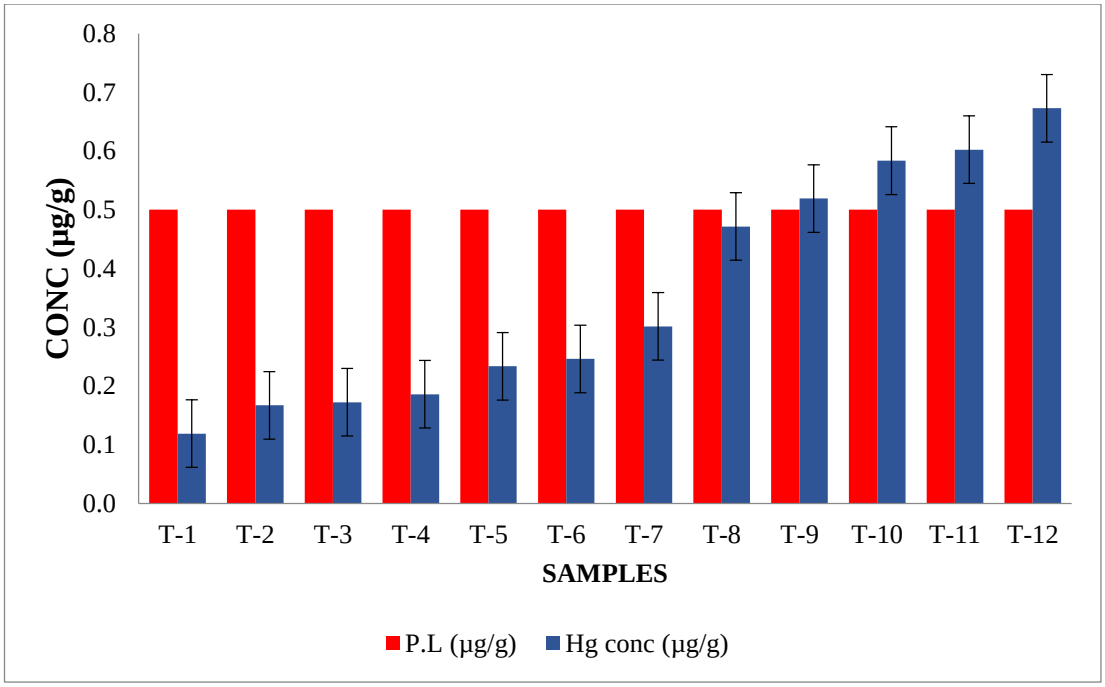
**Table 3.7: Permissible limit for some heavy metals in plants**

**(* WHO, 1996; ** Patra and Sharma, 2000).**

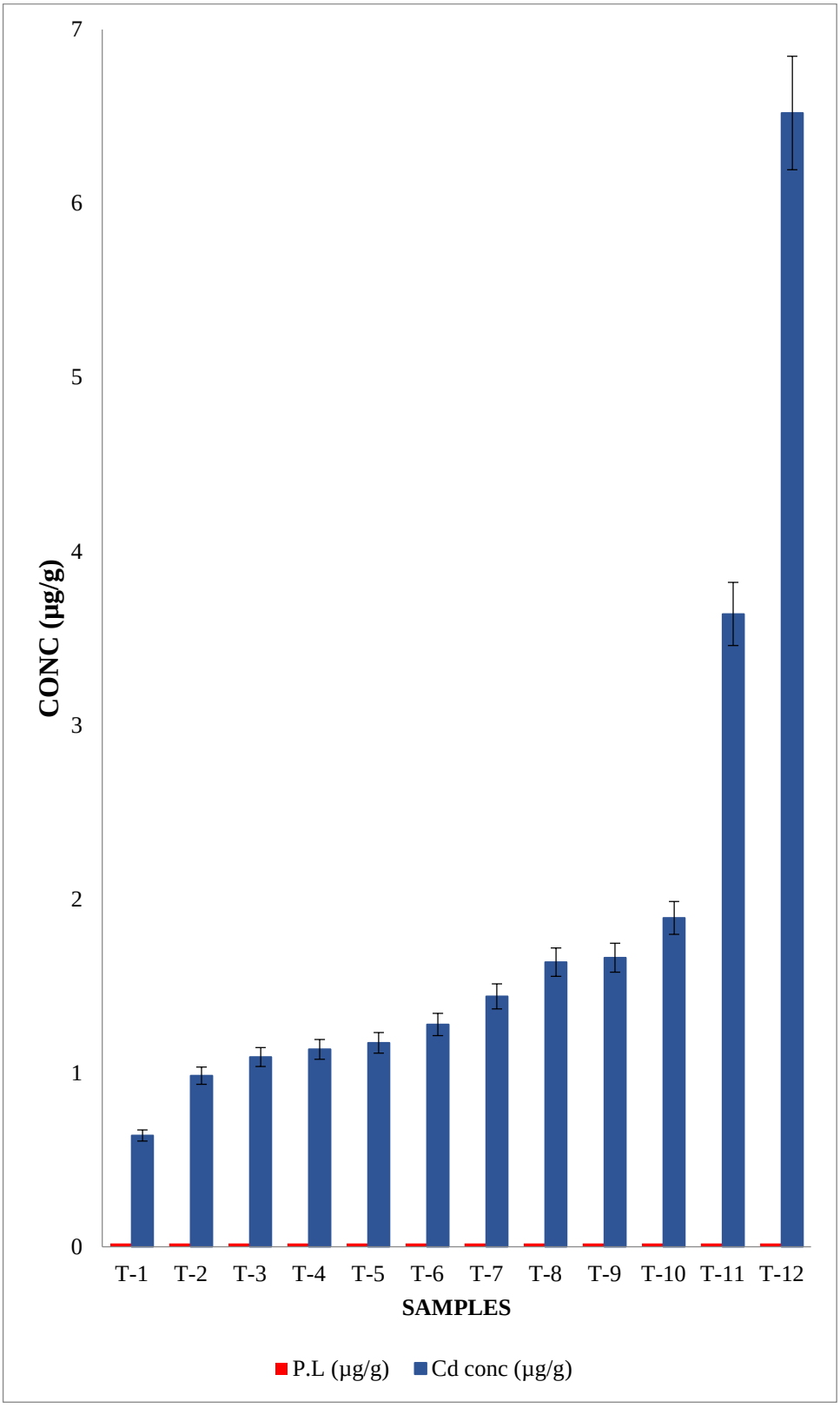
| Elements | Permissible limits (µg/g) |
|---|---|
| **As** | N.A |
| **Cd** | 0.02 * |
| **Pb** | 2 * |
| **Hg** | 0.5 ** |
| **Ni** | 10 * |
| **Cr** | 1.3 * |



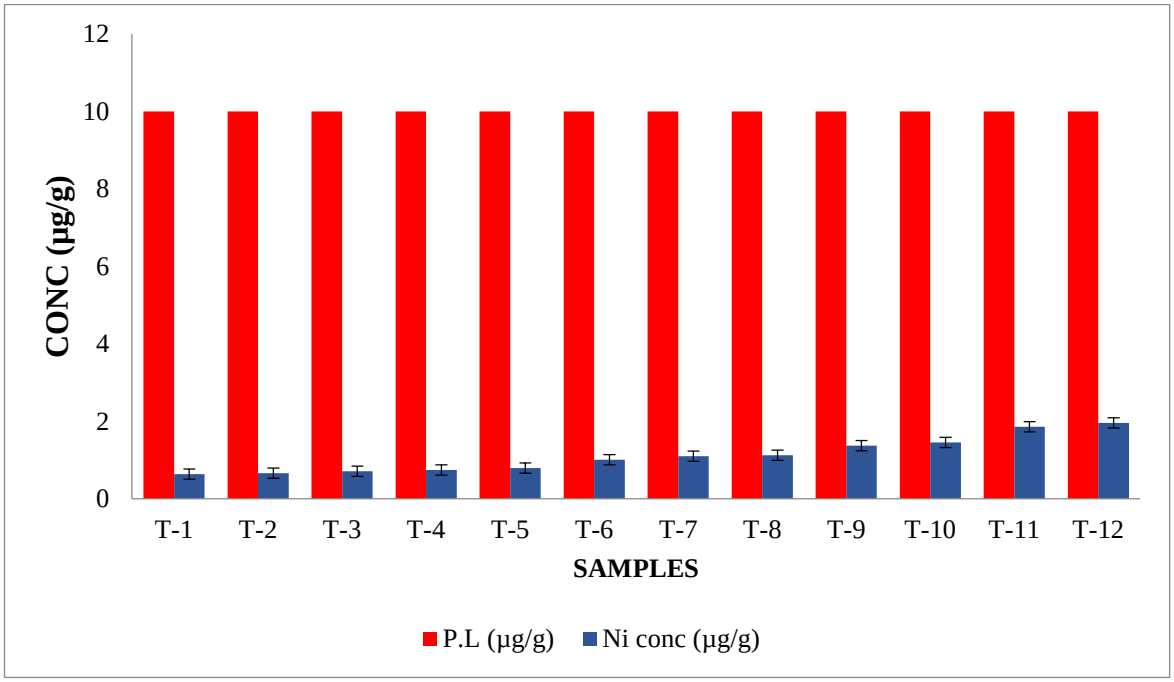

**(a) Lead (Pb); Permissible limit 2 µg/g**



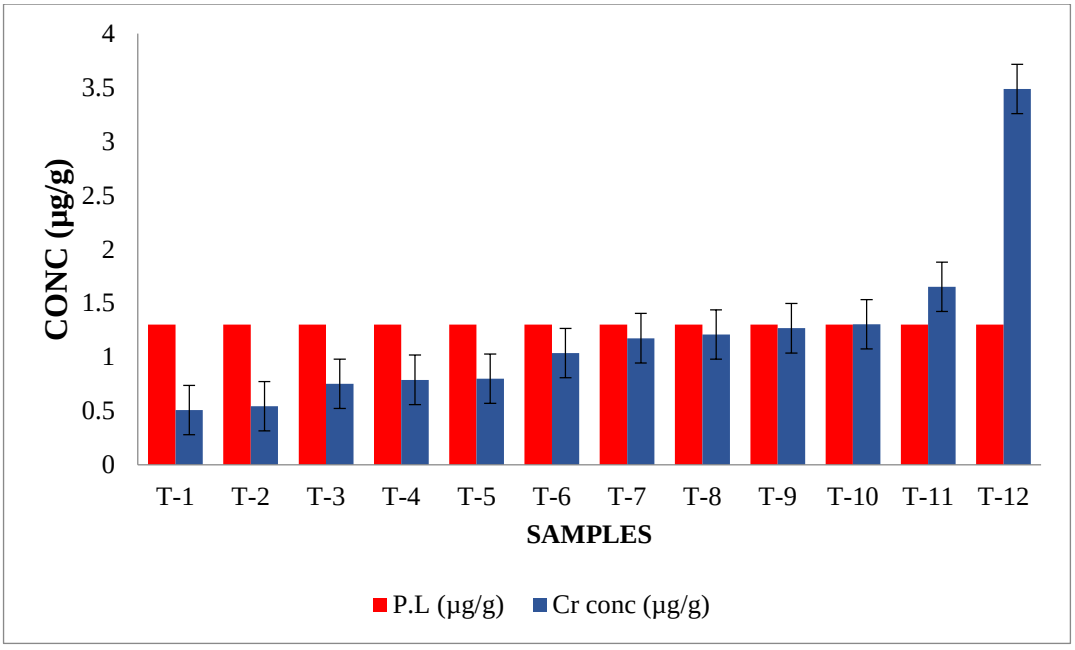

**(b) Mercury (Hg); Permissible limit 0.5 µg/g**




**(c) Cadmium (Cd); Permissible limit 0.02 µg/g**




**(d) Nickel (Ni); Permissible limit 10 µg/g**




**(e) Chromium (Cr); Permissible limit 1.3 µg/g**

**Figure 3.7: Permissible limits vs concentrations of heavy metals**

**[(a) Lead (Pb), (b) Mercury (Hg), (c) Cadmium (Cd), (d) Nickel (Ni), and (e) Chromium (Cr)]**

Figure 3.7 shows the comparison between the analyzed heavy metal concentrations of zozial and their corresponding permissible limits according to available literatures. The comparison between of the heavy metal concentrations with their corresponding permissible limits indicated that the mean concentrations of Cadmium (1.92 µg/g) and Lead (2.55 µg/g) are above their permissible limits while those of Mercury (0.36 µg/g), Nickel (1.12 µg/g) and Chromium (1.21 µg/g) were within the permissible limits. All samples display significantly high concentrations of Cd, which is around 31 times more even on the lowest detected concentration when compared to their permissible limits. Samples from T-1 to T-4 for Lead, T-1 to T-8 for Mercury, all samples for Nickel, and T-1 to T-9 for Chromium were below their corresponding permissible limits whereas all the other samples shown concentrations higher than their corresponding permissible limits [Figure 3.7(a),(b),(c),(d), & (e)]. The comparison of concentration levels of heavy metals available in zozial with their corresponding permissible limits showed that zozial contains concentrations of heavy metals such as of Cd, Pb, Hg and Cr are in toxic level.

**3B.3(a). Statistical analysis.**

All type of cash crop cultivation including tobacco cultivation in Mizoram is practiced without the applications of chemical fertilizers and pesticides. Various anthropological activities that could exacerbate the deposition of heavy elements were virtually non-existent at the tobacco cultivation sites. Samples were collected from locations that are well isolated from diesel pollutants with the nearest sampling site located 10 km(s) (approx) away from the main road (rural roads). Soil pH, soluble organic matter, soil type and chemical contents of soil are known to play an important role in the translocation of heavy metals in plants (Noler *et al*., 2006). So, it is pertinent that soil character of Mizoram plays a significant role in the concentration of heavy metal species on collected tobacco plants. Mizoram has primarily medium to deep loamy red and yellow soils which are highly to moderately acidic in nature with average pH value varying from 4.1 to 5.9 (Misra and Saithantluanga, 2003; Mallik and Rai, 2013).

Bivariate correlation analysis was applied to assess the correlation between the concentration of heavy metals and pH data of the soil collected from the data base of Department of Agriculture, Govt. of Mizoram (Table 3.8); correlations between different heavy metals within the elemental concentrations were also assessed.

**Table 3.8: pH of soil samples.**

| SAMPLE ID | pH |
|---|---|
| **T-1** | 4.82 |
| **T-2** | 5.32 |
| **T-3** | 5.24 |
| **T-4** | 5.06 |
| **T-5** | 5.66 |
| **T-6** | 4.78 |
| **T-7** | 5.08 |
| **T-8** | 5.01 |
| **T-9** | 4.84 |
| **T-10** | 5.34 |
| **T-11** | 5.07 |
| **T-12** | 5.2 |

Typically, the soil pH is the principal factor in determining the plant uptake of trace metals, whose mobility usually increase under acidic conditions (Gondola and Kadar, 1994; Golia *et al*., 2007). Therefore, bioaccumulation of heavy-metal species is more reasonable to be a bigger concern in acidic soils. Depending on the soil properties, the chemical forms of metals in soil can also vary and influence accumulation property of plants (Lehoczky and Kiss, 2002).

Bivariate correlation analysis (Pearsons' correlation) showed positive correlations between the pH of the soil and As, Hg, Ni and Cr while negative correlation exist between soil pH and Cd and Pb but were not statistically significant. Between the elemental concentrations, a highly significant positive correlation was observed between the concentrations of Cd and As ($p < 0.01$), Pb and Cd ($p < 0.01$), Cr and Cd ($p < 0.01$), Cr and Pb ($p < 0.01$), Ni and Cd ($p < 0.05$), Ni and Pb ($p < 0.05$), Ni and Cr ($p < 0.05$). Non-significant negative correlation was observed between Hg and As. Non-significant positive correlations were also observed between As and Pb, As and Ni, As and Cr, Cd and Hg, Pb and Hg, Hg and Ni, Hg and Cr (Table 3.9).

The positive correlation existing between the pH of the soil and the concentrations of the heavy metals exhibited that the increase of pH value influence the increase in concentrations of all the elements and vice versa; expressing the role of soil parameters in the translocation of heavy metals in tobacco plants.

**Table 3.9: Pearson's correlation between pH and concentration of elements (ICP-OES)**.

| | pH | As | Cd | Pb | Hg | Ni | Cr |
|---|---|---|---|---|---|---|---|
| **pH** | 1 | | | | | | |
| **As** | 0.007 | 1 | | | | | |
| **Cd** | -0.041 | .838** | 1 | | | | |
| **Pb** | -0.056 | 0.429 | .828** | 1 | | | |
| **Hg** | 0.314 | -0.032 | 0.238 | 0.341 | 1 | | |
| **Ni** | 0.422 | 0.518 | .585* | .602* | 0.156 | 1 | |
| **Cr** | 0.034 | 0.462 | .770** | .867** | 0.381 | .644* | 1 |

**. Correlation is significant at 0.01 level (2-tailed).

*. Correlation is significant at 0.05 level (2-tailed).

### 3B.3(b) Daily Intake of Heavy Metals.

The estimated daily intakes (EDI) of heavy elements for zozial users are given in Table 3.6. The EDIs of As, Cd, Pb, Ni and Cr were calculated using the formula; $\mathbf{EDI} = \frac{\mathbf{MC} \times \mathbf{Cons}}{\mathbf{BW}}$ (Chapter 2.7), and compared with the available tolerable daily intake (TDI) values from JECFA, USEPA, ISIRI and EFSA (Table 3.10). All the EDIs of As, Cd, Pb, Ni and Cr due to zozial consumption were numerically smaller than TDI. Therefore, intake of toxic metals through zozial by zozial smokers is low in the study region.

**Table 3.10: Estimated daily intake of heavy metals (µg/kg bw/day).**

| Sample ID | As | Cd | Pb | Ni | Cr |
|---|---|---|---|---|---|
| **T-1** | 0.004 | 0.003 | 0.006 | 0.002 | 0.002 |
| **T-2** | 0.005 | 0.003 | 0.006 | 0.003 | 0.001 |
| **T-3** | 0.006 | 0.003 | 0.005 | 0.002 | 0.002 |
| **T-4** | 0.008 | 0.005 | 0.006 | 0.002 | 0.001 |
| **T-5** | 0.006 | 0.004 | 0.006 | 0.003 | 0.003 |
| **T-6** | 0.006 | 0.002 | 0.005 | 0.002 | 0.002 |
| **T-7** | 0.010 | 0.004 | 0.007 | 0.002 | 0.004 |
| **T-8** | 0.007 | 0.004 | 0.006 | 0.003 | 0.003 |
| **T-9** | 0.009 | 0.003 | 0.006 | 0.002 | 0.004 |
| **T-10** | 0.007 | 0.004 | 0.006 | 0.005 | 0.005 |
| **T-11** | 0.009 | 0.007 | 0.005 | 0.002 | 0.003 |
| **T-12** | 0.032 | 0.012 | 0.006 | 0.005 | 0.003 |
| **TDI (µg/kg/day)** | **2.1[a]** | **1[b]** | **3.6[c]** | **2.8[d]** | **1500[b]** |

a – JECFA ; b – USEPA; c – ISIRI; d – EFSA.

### 3B.3(c). Target Hazard Quotient.

Target Hazard Quotient (THQ) was calculated using the formula; $\boldsymbol{THQ = \frac{EFr \times ED \times FIR \times MC}{RfD \times BW \times AT} \times 10^{-3}}$ (Chapter 2.8) where THQ value > 1 signifies the probability of occurrence of adverse effects due to the exposure to certain toxic elements (USEPA, 2000). Meanwhile, in our current study, all of the calculated THQ values were below 1 (Figure 3.8). Among the samples, noticeably high THQ value of As in sample T-12 compared with other samples was observed.

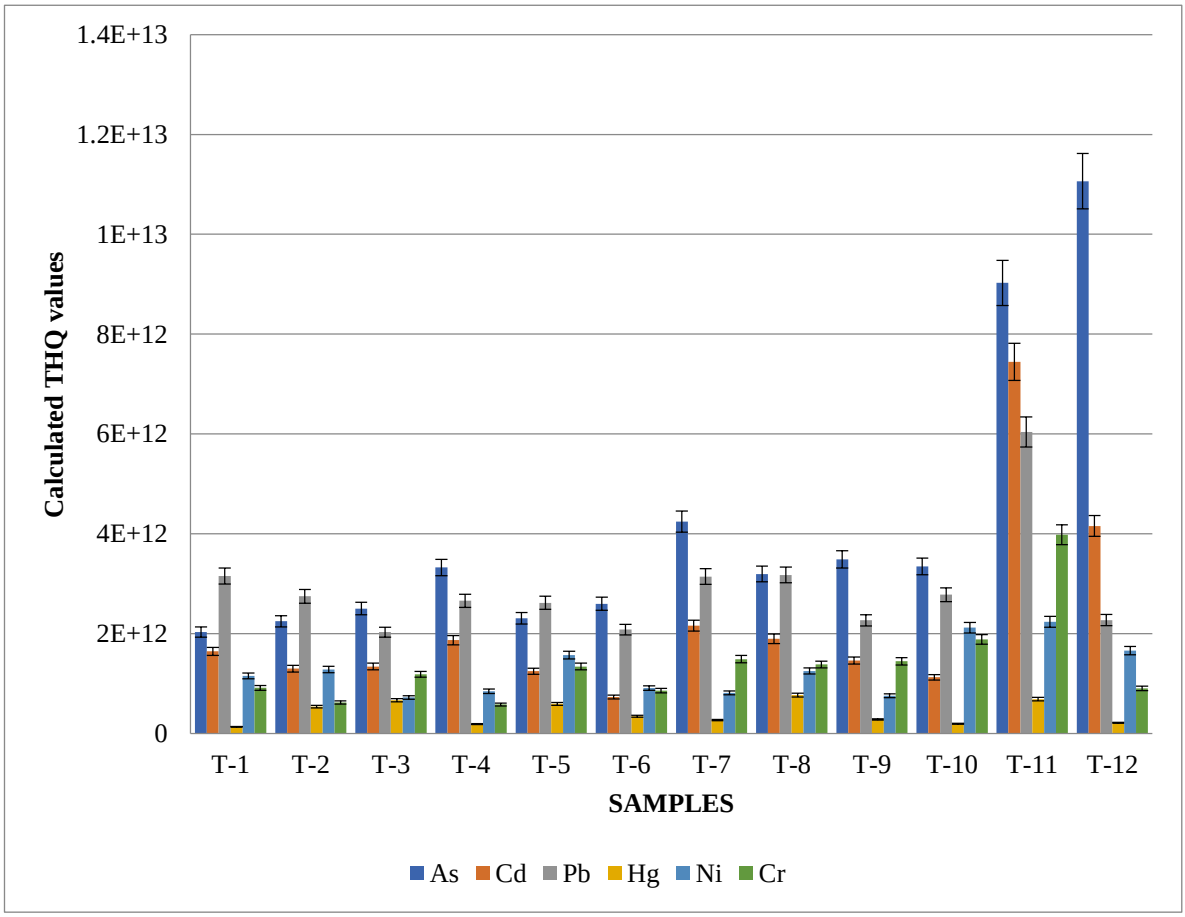



**Figure 3.8: Target hazard quotients (THQs) of As, Cd, Pb, Hg, Ni and Cr.**

**3B.3(d). Hazard Index.**

The Hazard Index (HI) was calculated for all the heavy metals using the formula; $\boldsymbol{HI = \sum HQ = HQAs + HQCd + HQPb + HQHg + HQNi + HQCr}$ (Chapter 2.9), on each of the samples (Figure 3.9). All heavy elements showed tremendously high values of HI with numerical values ranging from 8617.24 – 37138.26. The HI value > 1 indicates a potential adverse effect on the exposed population (Huang *et al*., 2008).

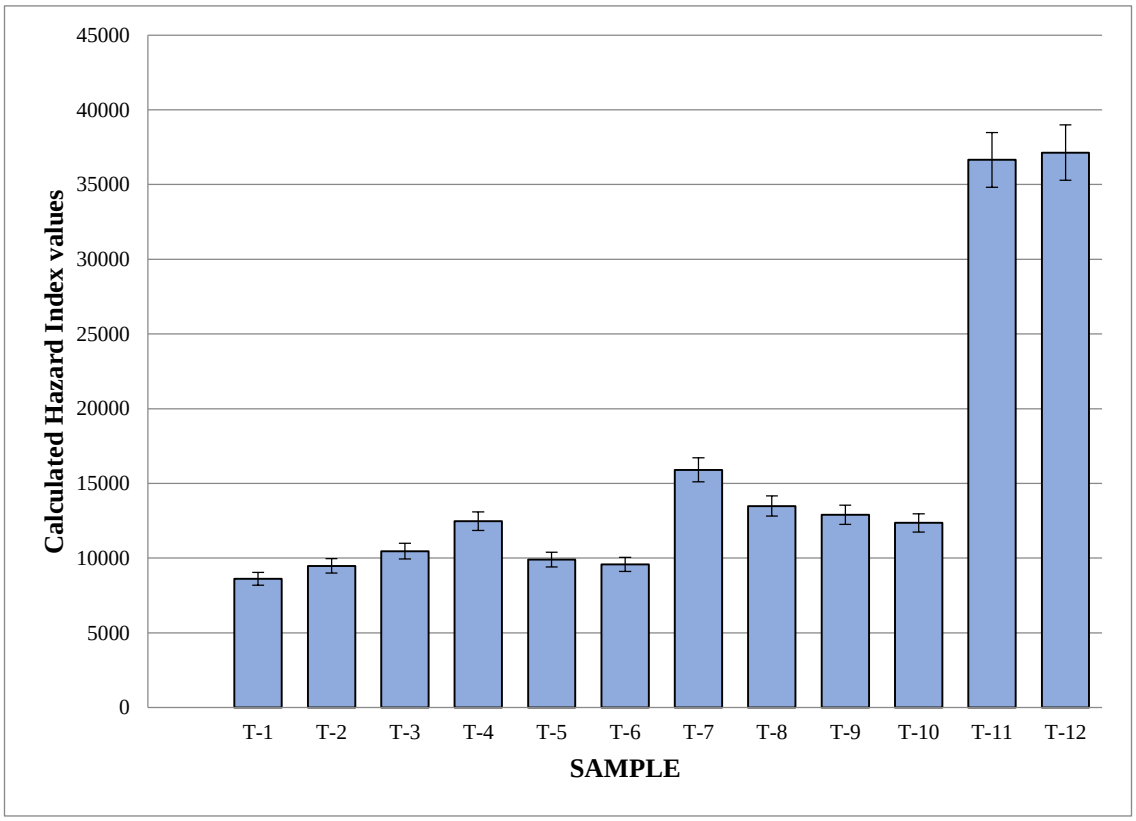



**Figure 3.9: Hazard index of heavy metals.**

**3B.3(e). Carcinogenic Risk.**

The Carcinogenic risk (CR) was calculated using the equation; $\boldsymbol{CR = CSF \times EDI}$ (Chapter 2.10) and the CR values of As, Cd, Pb, Ni and Cr for zozial consumers due to the consumption of analyzed tobacco samples as zozial are presented in Table 3.11. Generally, the CR values below E-6 are insignificant, values higher than E-4 is unacceptable, and so the acceptable range is between E-6 to E-4 (USEPA, 2010). In the current study, calculated CR values for As, Cd, Ni and Cr were numerically higher than the unacceptable range where as that of Pb falls in the acceptable range.

**Table 3.11: Carcinogenic risk* of As, Cd, Pb, Ni and Cr.**

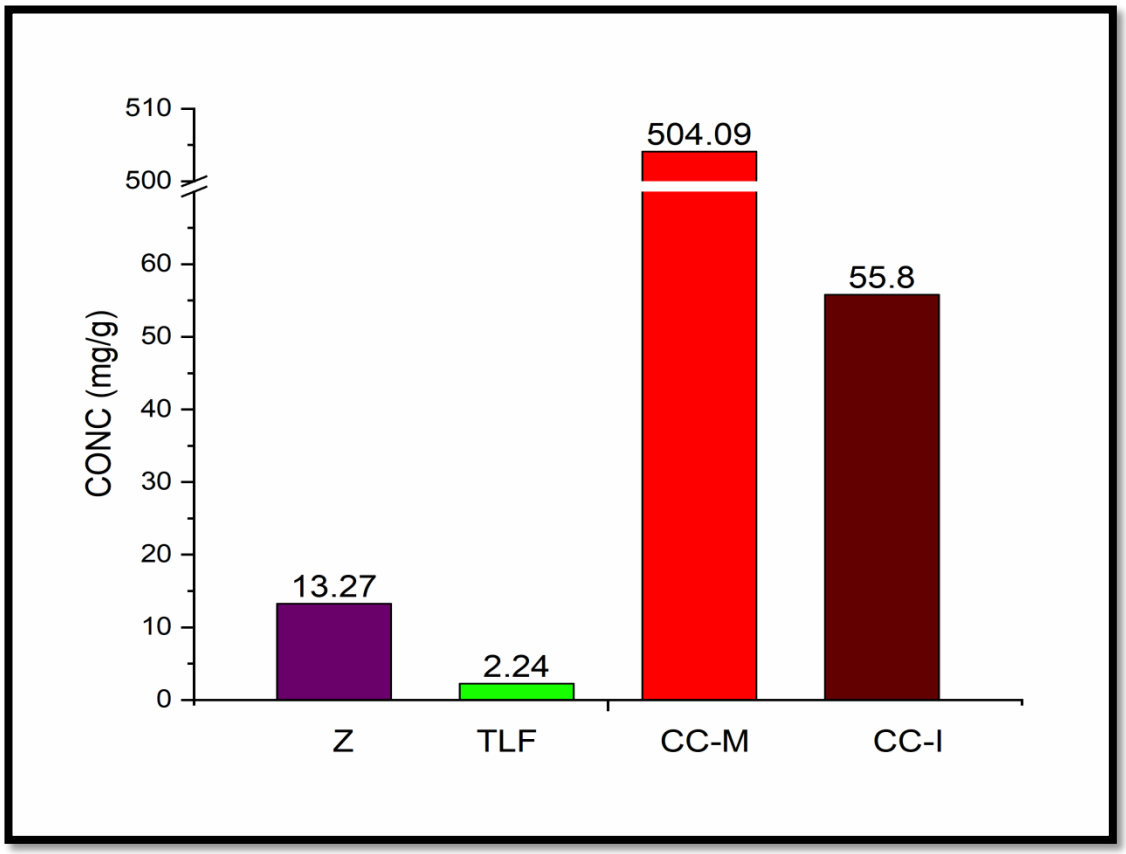
| SAMPLE | As | Cd | Pb | Ni | Cr |
|---|---|---|---|---|---|
| **T-1** | **0.006** | **0.001** | 4.80863E-05 | **0.002** | **0.001** |
| **T-2** | **0.008** | **0.001** | 5.51062E-05 | **0.003** | **0.001** |
| **T-3** | **0.009** | **0.001** | 4.49274E-05 | **0.002** | **0.001** |
| **T-4** | **0.013** | **0.002** | 5.39128E-05 | **0.002** | **0.001** |
| **T-5** | **0.009** | **0.001** | 4.86479E-05 | **0.003** | **0.001** |
| **T-6** | **0.009** | **0.001** | 4.46466E-05 | **0.002** | **0.001** |
| **T-7** | **0.015** | **0.002** | 6.12837E-05 | **0.002** | **0.002** |
| **T-8** | **0.011** | **0.002** | 5.45446E-05 | **0.002** | **0.002** |
| **T-9** | **0.013** | **0.001** | 4.71737E-05 | **0.002** | **0.002** |
| **T-10** | **0.011** | **0.001** | 5.01221E-05 | **0.005** | **0.002** |
| **T-11** | **0.013** | **0.003** | 4.48572E-05 | **0.002** | **0.002** |
| **T-12** | **0.048** | **0.004** | 5.37022E-05 | **0.004** | **0.001** |

*CR > E-4 is unacceptable; E-6 < CR < E-4 is acceptable; CR < E-6 is negligible.

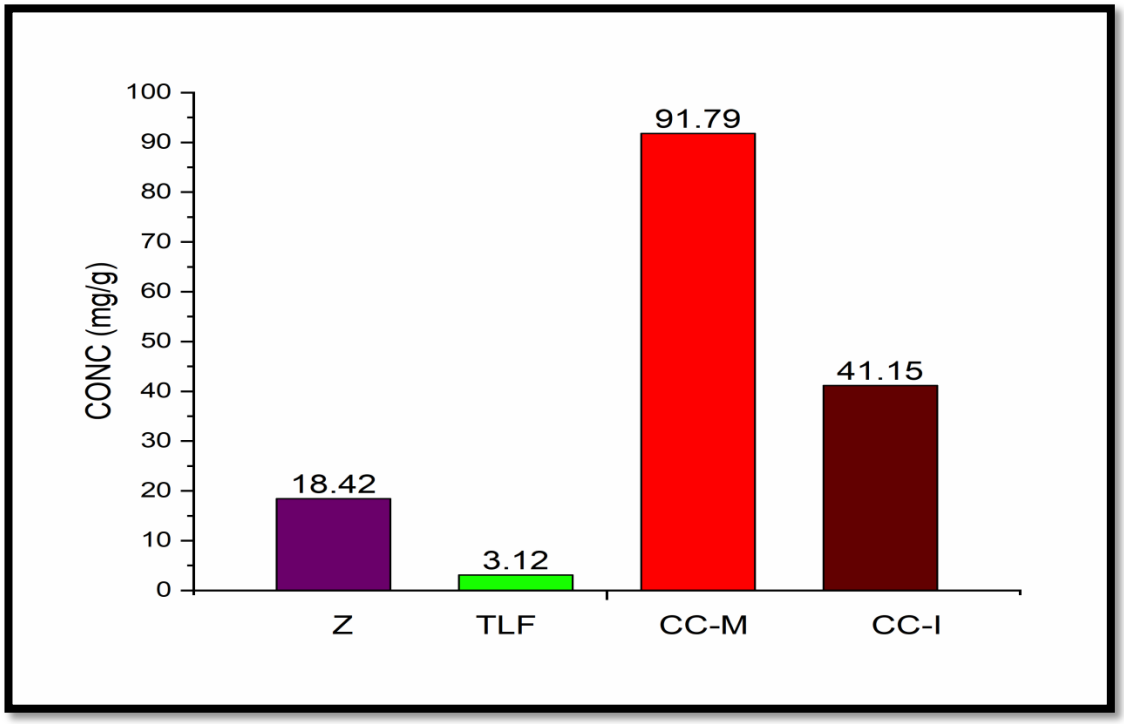
### 3B.4. Tobacco-specific nitrosamines (TSNAs).

TSNAs detected from commercial cigarettes are comparatively higher than that of zozial and Tobacco leaf fillers. Among the commercial cigarette samples, the concentrations of all TSNAs detected in Myanmar commercial cigarette are significantly higher than those of commercial Indian cigarettes. Zozial and tobacco leaf fillers have lower levels of NNN and NNK compared to both the commercial cigarettes. Concentrations of NNK and NNAL from some zozial samples and fillers were below the limit of detection of the spectrometer.

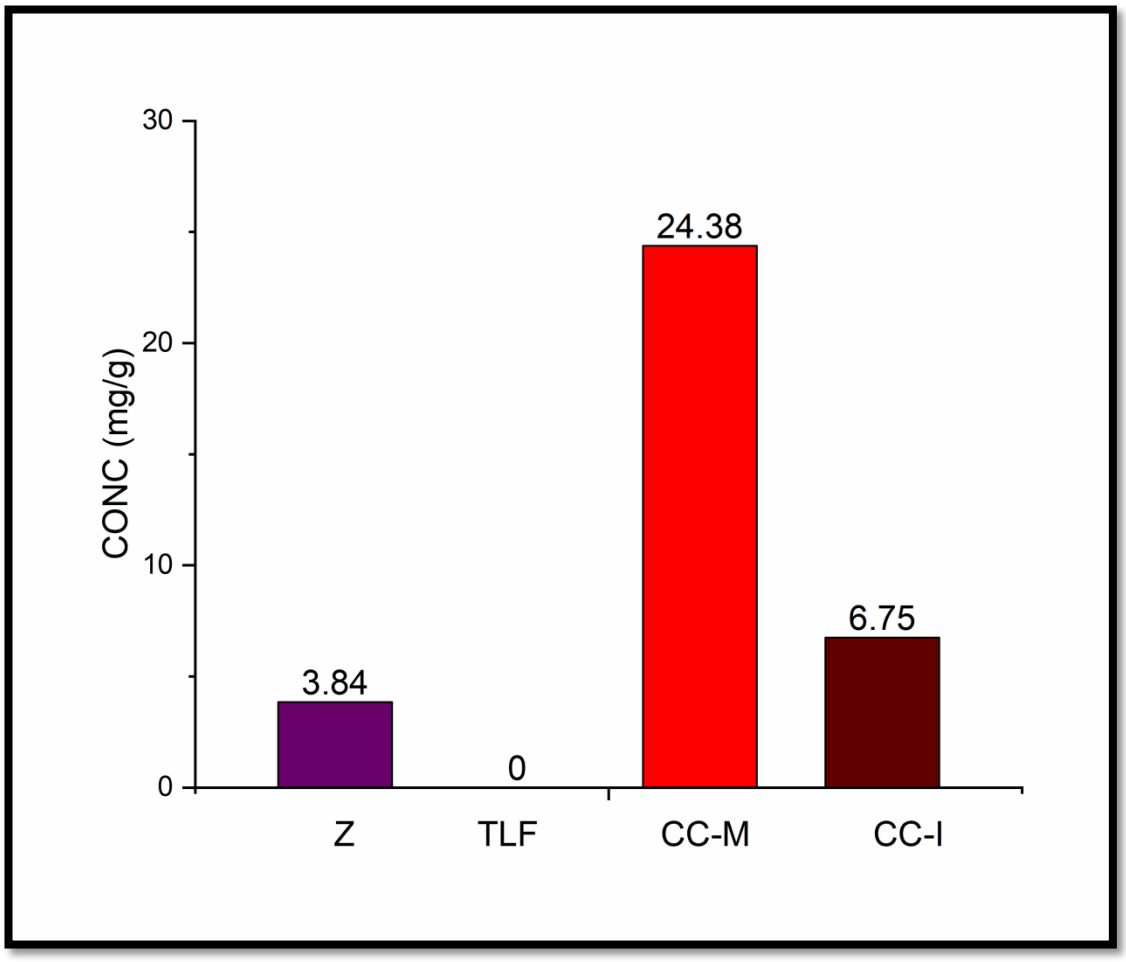
In all tested samples, the average TSNA contents were comparatively high in the case of commercial cigarettes from Myanmar with an average concentration of 504.1 ng/g, 91.79 ng/g and 24.38 ng/g for NNN, NNK and NNAL, respectively. The average concentrations of TSNAs in Indian commercial cigarettes are 55.8 ng/g, 41.15ng/g and 6.75 ng/g for NNN, NNK and NNAL respectively. The concentrations of TSNAs in zozial and Tobacco leaf fillers are significantly lower than those of the commercial cigarettes. The average concentration of TSNA for zozial is 15.32 ng/g for NNN, 21.35 ng/g for NNK and 5.12 ng/g for NNAL and that of Tobacco filler is 3ng/g for NNN, 4 ng/g for NNK and NNAL is below the detection limit in all the collected samples as shown in Figure 3.10 (a) (b) and (c).


CONC (mg/g)
510
500
60
50
40
30
20
10
0
504.09
55.8
13.27
2.24
Z
TLF
CC-M
CC-I

(a) NNN.


CONC (mg/g)
100
90
80
70
60
50
40
30
20
10
0
91.79
41.15
18.42
3.12
Z
TLF
CC-M
CC-I

(b) NNK.




(c) NNAL.

**Figure 3.10: Comparison between average concentrations of (a) NNN, (b) NNK and (c) NNAL in Zozial (Z), Tobacco leaf filler (TLF), Myanmar Commercial Cigarettes (CC-M) and Indian Commercial Cigarettes (CC-I).**

TSNAs are a group of carcinogenic components of tobacco and tobacco smoke among which NNN and NNK are strong carcinogens (Gupta *et al*., 1996; Hecht, 1998). The significant difference in the concentrations of TSNAs between zozial, tobacco leaf fillers and commercial cigarettes may be indicative of the difference in curing and processing. Zozial, along with Tobacco leaf fillers are sun-cured for an average period of 15 days while commercial cigarettes are cured for

longer duration (generally between 6 months to 1 year depending on the kind of curing process employed and desired type of tobacco products) that reflects in the difference in concentration of TSNAs.

TSNAs are produced by the process of nitrosation of alkaloids during the curing and storage period of tobacco (Bush *et al*., 2001; Djordjevic *et al*., 1989; Shi and Zhang, 2004; Stepanov *et al*., 2011). Cui (1998) reported that during air-curing of tobacco, TSNAs levels in the leaf lamina and midrib significantly increased during the $4^{th}$ to $7^{th}$ weeks which is totally absent in the case of zozial and tobacco leaf fillers thereby potentially resulting in the low concentrations of TSNAs when compared with commercial cigarettes. During this period, the microbial activities reduced the nitrate ion to nitrite ion and concomitantly it is engaged in the nitrosation reactions with the naturally existing alkaloids thereby forming TSNAs (Burton *et al*., 1994). Earlier studies have shown that TSNAs may be formed as a result of nitrosation with the elevated levels of $NO_x$ in the leaves during curing (Ellington and Boyette, 2013) and the temperature at which tobacco matter is stored also plays a significant factor (Wang *et al*., 2017).

The difference in curing and processing between zozial along with tobacco leaf filler when compared to commercial cigarettes may further be explained by comparing the concnentrations of Tobacco alkaloids, TSNAs and Nitrogen oxides as shown in Table 3.12.

**Table 3.12: Comparison between Zozial (Z), Myanmar Commercial cigarette (CC-M) and Tobacco leaf filler (TLF).**

| ID | Measured concentrations | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | pH | Tobacco Alkaloids (TAs) (mg/g) | | | | Tobacco Specific N-nitrosamines (TSNAs) (mg/g) | | | Nitrite (mg/g) | Nitrate (mg/g) |
| | | Nicotine | Cotinine | Nornicotine | | NNK | NNAL | NNN | | |
| **Z** | 6.5 | 16.5 | 0.11 | 0.46 | | 0.036 | 0.0115 | 0.04 | 1.3 | 854 |
| **CC-M** | 6.5 | 32 | 0.17 | 4.3 | | 0.18 | 0.018 | 1.19 | 2 | 8436 |
| **TLF** | 7 | 23.3 | 0.03 | 0.54 | | N.D | N.D | 0.004 | 0.8 | 430 |

Alkaloids measured from the samples were compared against each other (Table 3.9). LC-MS/MS data showed commercial cigarette with highest nicotine concentration followed by TLF then zozial which was also the same in the case of nornicotine concentration. Nornicotine is understood to be almost entirely a demethylated derivative of nicotine (Lewis *et al.*, 2010). The lower concentration of nornicotine in zozial compared to TLF (which are more or less the same tobacco) could be the difference in duration of storage. Zozial samples were collected 6 months earlier than TLF samples providing more time to form NNN from nornicotine. This further reflects in the lower concentration of NNN of TLF as nornicotine reacts with nitrosating agents to form NNN. The concentration of alkaloids and TSNAs in commercial cigarettes are relatively higher than those of tobacco samples collected from Mizoram (zozial and TLF). The nitrogen oxide species detected from commercial cigarette were also of significantly higher concentrations as compared to zozial and TLF. The concentration of nitrate in commercial cigarette is tenfold higher of that measured in zozial. The significant difference in concentrations of alkaloids, TSNAs and nitrogen oxide species observed between the samples is an indicative of the difference in the process and duration of curing.

NNN is classified as a group I carcinogen by the International Agency for Research on Cancer (IARC, 2007). An animal model study on the carcinogenic activity suggested that NNN plays an important role in inducing esophageal cancer in smokers (Hecht and Hoffmann, 1989; Hecht, 2003) and oral cancer in smokeless tobacco users (IARC, 2007). Moreover, NNN is also responsible for inducing primarily papilloma and carcinoma of the nasal cavity (Hoffmann and Hecht, 1985). Recent studies have mentioned NNK induced gene variation in cells playing a crucial role in gene silencing, modification and functional disruption resulting in an early development of carcinogenesis (Akopyan and Bonavida, 2006). Although NNN and NNK are known to be mutually present in all commercial tobacco products, there is a considerable variation in the extent of these compounds in smoking and smokeless tobacco products. The variation is mainly due to factors such as differences in tobacco types used for various tobacco products, agro climatic conditions and

agricultural practices, anthropogenic activities, curing and processing methods and in manufacturing processes (IARC, 2007). NNN and NNK, being classified strong carcinogens, functions as a bridge between nicotine and tobacco-related cancers (Hoffmann and Hecht, 1985). Animal model studies showed that NNN and NNK induce benign and malignant tumors by causing DNA adducts and mutations as well as promoting tumor growth through receptor mediated effects (Hecht, 2003; Takahashi *et al*., 2010).

**3B.5. 8-OHdG Assay.**

Analysis of urinary 8-OHdG has been reputable as a significant biomarker to assess oxidative stress and to evaluate carcinogenic risks after exposure to various carcinogenic substances, environmental pollutants, and lifestyle factors (Kasai *et al*., 2001; Cooke *et al*., 2002). Hydroxyl radical ($HO^{\bullet}$), which is the most significant oxygen-free radical, when reacting with the nucleobases of the DNA strand (such as guanine) forms C8-hydroxyguanine (8-OHGua) or its nucleoside form deoxyguanosine (8-hydroxy-2′-deoxyguanosine). While reaction between the other nucleobases of DNA with $HO^{\bullet}$ takes place analogously, the 8-OHdG lesion is the most predominant DNA lesion as it is relatively easily formed and is promutagenic, and is therefore being established as an important biomarker of carcinogenesis and oxidative stress (Kasai, 1997; Shigenaga *et al*., 1989).

Oxidative properties and its carcinogenic potential of tobacco smoking are well known (Pryor, 1997). Recent studies have shown elevated levels of 8-OHdG in human tissues of smokers (Kiyosawa *et* al., 1990; Asami *et al*., 1996) and in passive smokers in exposed to environmental tobacco smoke at workplace (Howard *et al*.,1998). The urinary excretion of products of damaged nucleotides has been reported to be an important biomarker of risk for lung cancer (Loft *et al*., 2006).

Recent studies have shown exposure to Cd resulting in elevated 8-OHdG concentrations, with a strong association between urinary Cd and 8-OHdG (Engström *et al*., 2010). The inhibitory property of Pb on antioxidant enzymes imparts cells more liable to oxidative stress (Ercal *et al*., 2001). In the current study,

the urinary 8-OHdG concentration of controls ranges from 1.33-7.55 ng/mg creatinine and that of zozial users ranges from 1.93-11.64 ng/mg creatinine. The mean concentration of urinary 8-OHdG for zozial consumers was 5.43 (SD=2.98) ng/mg creatinine and for controls was 3.87 (SD=1.77) ng/mg creatinine (3A.2.1). The significant difference in the concentration of 8-OHdG between zozial users and controls where high 8-OHdG concentration in zozial may be due to prolonged exposure to high concentration of heavy metal species from zozial by zozial users.

**3B.5(a). Statistical Analysis – 8OHdG Epidemiological Data.**

The study populace for 8OHdG Assay consists of 48 male participants who were further classified as zozial users (n=24) and controls (n=24) with no history of tobacco consumption. The demographic characteristics were compared between the groups (Table 3.13).

**Table 3.13: Characteristics of study population (8OHdG Assay).**

| CHARACTERISTICS | | ZOZIAL USERS (N=24) | CONTROL (N=24) |
|---|---|---|---|
| **AGE** | (year) (mean ± SD) | 41.79 ± 8.84 | 40.42 ± 9.95 |
| **DOSE** | Sticks per day | 21.86 ± 7.91 | 0.00 ± 0.00 |
| **TOBACCO** | (g/day) | 18.14 | 0.00 |
| **DURATION OF SMOKING** | (year) (mean ± SD) | 24.375 ± 10.87 | 0.00 ± 0.00 |
| **ANNUAL INCOME** | Below 3 Lakh (%) | 79.16 | 41.67 |
| | Above 3 Lakh (%) | 20.84 | 58.33 |

Control (healthy) groups have higher annual income with 58.33% earning more than 3 lakh annually. Zozial users have lower annual income with 79.16% earning less than 3 lakh. Commercial zozial sticks are of the average 6.7 cm in length, and an average 0.830 grams of tobacco is loaded per stick. The daily dose of zozial smokers is on average, 21.86 sticks per day, *viz.*, 18.14 g of tobacco consumed daily.

The mean age of zozial consumers is 41.79 (SD=8.84) and that of controls is 40.42 (SD=9.95). The sampling carried out in such a way to nullify the consequence owing to age as it has been reported to affect 8-OHdG level (Witherell *et al*., 1998). Pearson's correlation established a significant ($p<0.05$) positive correlation between the ages of zozial consumers (Z) and control (H) and the concentration of 8-OHdG in urine while the correlation between 8-OHdG concentration and tobacco dose (amount of zozial consumed per day) was not found to be significant. The number of smoking years (history of smoking) revealed a highly significant ($p<0.01$) correlation with 8-OHdG levels in urine (Table 3.14).

**Table 3.14: Pearson's Correlation coefficient of 8OHdG with Age and Dose.**

| | AGE (Z) | AGE (H) | DOSE | DURATION |
|---|---|---|---|---|
| 8OHdG conc. | .448* | .509* | .189 | .680** |

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Smoking is known to exacerbate the risk of cancer (Carbone, 1992), and in this study we were able to establish a relationship between smoking and 8-OHdG concentration. Current findings may indicate dietary habits such as daily fat intake (saturated fats and trans-fats, which should not exceed 10% and 1% of the total daily energy intake respectively; WHO, 2018), saum (indigenously fermented pig fat), salt intake (WHO recommended rate is < 5 g per day; WHO, 2012), smoked meat and smoked vegetables are positively associated with 8-OHdG concentration which

commensurates well with previous studies (Balakrishnan *et al*., 2002; Raimondi *et al*., 2007; Taioli *et al*., 2007; Dani *et al*., 2014). DNA adducts due to PAHs as a result of consumption of smoked food has been observed (Raimondi *et al*., 2007; Taioli *et al*., 2007) which was also observed in our assay. The relation between the concentration of 8-OHdG and consumption of oily foods and saum (fermented pig fat) was also established. A significant influence of salt intake on the concentration of urinary 8-OHdG was observed as high consumption of NaCl induce structural chromosomal damage (Balakrishnan *et al*., 2002).

Linear regression analysis was performed using SPSS statistical package (V 16.0) for testing the statistical significance to check whether demographic, food habit, tobacco habit and other daily habits are significantly related with the concentration of 8-OHdG. The p-value $<0.05$ indicates that there is no significance difference among the variables within samples with different parameters. Linear regression analysis illustrates the significant relation between the qualitative variables and 8-OHdG levels in urine as shown in Table 3.15. Linear regression analysis reflects that daily fat intake ($p<0.01$), consumption of saum ($p<0.01$), salt ($p<0.05$), smoked meat ($p<0.01$) and smoked vegetables ($p<0.05$), and physical exercises ($p<0.05$) shows significant relation with the concentration of 8-OHdG.

The genotoxic effect of alcohol may be considered as a promoter of tobacco induced buccal carcinogenesis (Wynder *et al*., 1977) which was not the case for 8-OHdG concentration in our study. Interestingly, other lifestyle habits which were considered such as frequency of fruit intake, daily water intake, and other tobacco products such as tuibur, kuhva, zarda and sahdah were statistically not significant for 8-OHdG concentration in our study samples. The rate of physical exercise shows significant association with concentration of 8-OHdG ($p<0.01$) but DNA effects identified as a result of intense physical activity were not due to apoptosis or necrosis (Hartmann *et al*., 1998).

**Table 3.15: General linear regression of 8OHdG frequency with potential confounders.**

| | 8-OHdG | | |
|---|---|---|---|
| | **B** | **Std. Error** | **Sig.** |
| Alcohol | 1.019 | 0.846 | 0.235 |
| Fat intake | -1.22 | 0.429 | 0.007 |
| Saum | -1.089 | 0.313 | 0.001 |
| Salt intake | -0.724 | 0.359 | 0.05 |
| Fruits | -0.993 | 0.63 | 0.122 |
| Water intake | 0.181 | 0.646 | 0.781 |
| Exercise | 1.758 | 0.533 | 0.002 |
| Smoked meat | -1.49 | 0.376 | 0.001 |
| Smoked veg. | -1.166 | 0.498 | 0.024 |
| Tuibur | 0.207 | 1.052 | 0.845 |
| Kuhva | 0.536 | 0.797 | 0.505 |
| Zarda | -0.152 | 1.212 | 0.901 |
| Sahdah | -0.817 | 0.929 | 0.384 |

### 3B.6. Superoxide dismutase (SOD) Assay.

Chapter 3A.2.2 showed the increase in the SOD activity among zozial smokers potentially due to heavy metal exposure. The current study showed an elevated generation of reactive oxygen species (ROS) in zozial consumers when compared to the control population. Thus, high levels in $H_2O_2$ could exacerbate high ROS production. $H_2O_2$ is highly unstable with a half-life ~1 ms and thus induced antioxidant mechanisms use enzymatic and non-enzymatic $H_2O_2$ scavengers (Hossain *et al*., 2015).

Several antioxidant enzymes such as catalase, peroxidases, transferases, reductases, and peroxyredoxin are involved on ROS conversion and degradation, and all of them cooperatively function to attenuate and maintain the homeostatic levels of $H_2O_2$ (Miller *et al*., 2010; Kapoor *et al*., 2015). Predominantly, heavy metals can inhibit their enzyme activity whose inhibition mechanisms remain elusive (Jackim, 1974; Singh and Sivaling, 1982). If oxidative stress is sustained, apoptosis of the cells might be evoked. Apoptosis plays a key role in several biological functions, such as cellular damage, teratogenicity, and tumorigenesis (Kang *et al*., 2005). Wang *et al*. (2004) and Morcillo *et al*. (2016) evaluated the exposure of As(III) and MeHg in leukocytes and observed apoptosis cell death through ROS generation. Cadmium oxidizes and forms DNA strand breaks in cells derived from liver or kidney (Forrester *et al*., 2000). These and other biochemical changes have been associated to apoptosis induced by cadmium exposure (Robertson and Orrenius, 2000).

### 3B.6(a). Statistical Analysis – SOD Epidemiological Data.

The study population for SOD Assay consists of 201 male participants who were further classified as zozial users (n=145) and controls (n=56) with no history of tobacco consumption. The characteristics were compared between the groups (Table 3.16).

Comparison between our study populations suggested that control (healthy) group individuals have higher annual income with 82.14% earning more than 3 lakh annually. Zozial users have lower income with 95.17% earning less than 3 lakh a

year. On a daily basis, 20.258 g (an average of 24.41 zozial sticks per day) of tobacco is being consumed by zozial users. The mean age of zozial consumers is 44.63 (SD = 10.18) and that of controls is 40.61 (SD = 11.4). The sampling carried out in such a way to nullify the consequence owing to age as biological aging has been reported to affect the level of DNA damage (Witherell *et al*., 1998). Duration of smoking years indicates how long (in years) a person has been smoking. The current study population consumes zozial for a mean duration of 26.903 years (SD=10.84).

**Table 3.16: Characteristics of study population (SOD Assay).**

| CHARACTERISTICS | | ZOZIAL USERS (N=145) | CONTROL (N=56) |
|---|---|---|---|
| **AGE** | (year) (mean ± SD) | 44.63 ± 10.18 | 40.61 ± 11.4 |
| **DOSE** | Sticks per day | 24.41 ± 10.16 | 0.00 ± 0.00 |
| **TOBACCO** | (g/day) | 20.258 | 0 |
| **DURATION OF SMOKING** | (year) (mean ± SD) | 26.903 ± 10.84 | 0.00 ± 0.00 |
| **ANNUAL INCOME** | Below 3 Lakh (%) | 95.17 | 17.86 |
| | Above 3 Lakh (%) | 4.83 | 82.14 |

**3B.7. Micronucleus (MN) Assay.**

The frequency of micronuclei formation, studied using buccal swab samples of 300 volunteers (100 each of controls, commercial cigarette users and zozial users) provided a significant result. The mean frequency of MN count per 1000 cells was highest for zozial consumers, followed commercial cigarette users and then that of controlled. While using Giemsa stain for the identification of micronuclei (DNA) it was observed that Geimsa dye was yielding false positive data. Therefore, acridine orange was employed for the identification of micronuclei which commensurate with

the precious studies (Nersesyan *et al*., 2006). Optical microscope was used for detection of MN in buccal swab samples with the application of Geimsa dye, whereas Fluoresence microscopy was employed when AO was used for the detection of MN on oral cells.

In spite of the genotoxicity of chemical constituents of tobacco smoke, the frequency of MN formation as a putative biomarker has shown variation in the data. The inconsistent genotoxicity data could be due to the differences in the type and the concentration levels of these chemical constituents that distinguishes cigarette smoke as well as zozial smoke besides the extent of the corresponding smoke exposure. Early identification of smoking hazard is crucial to reduce exposure and carcinogenic risk.

**3B.7 (a). Statistical Analysis – Micronucleus (MN).**

Interestingly, as an important cytogenetic markers, the micronuclei frequency of the smokers (commercial cigarette and zozial) in comparison with tobacco non-user groups indicated significantly higher frequency of total micronuclei in smokers than in non-users ($P < 0.001$) (Table 3.17). Among the group of exposed subjects who were subdivided into commercial cigarette smokers and zozial smokers, a significant differences in the frequencies of micronuclei/buccal cell ($p < 0.001$) was also observed between the experimental groups (Table 3.17).

**Table 3.17: Inter-group comparison of mean MN frequency**

| Group | MN frequency | | |
|---|---|---|---|
| | *n* | Mean ± SD | Range |
| Controls | 100 | 2.3±0.99♠ | 1-4 |
| Commercial cigarette users | 100 | 16.23 ± 4.56♠ | 7-34 |
| Zozial users | 100 | 40 ± 10.40♠ | 21-57 |

♠ $P < 0.001$.

The study populace for MN Assay consists of 300 male participants who were classified as zozial users (n=100), commercial cigarette users (n=100) and controls (n=100) with no history of tobacco use. The demographic characteristics were compared between the groups (Table 3.18).

**Table 3.18: Characteristics of study population (MN Assay).**

| **CHARACTERISTICS** | | **ZOZIAL USERS (N=100)** | **COMMERCIAL CIGARETTE USERS (N=100)** | **CONTROLS (N=100)** |
|---|---|---|---|---|
| **AGE** | (year) (mean ± SD) | 40 ± 10.4 | 36.88 ± 9.53 | 39.94 ± 10.86 |
| **DOSE** | Sticks per day | 7.85 ± 9.33 | 4.24 ± 6.2 | 0.00 ± 0.00 |
| **TOBACCO** | (mean ± SD) (g/day) | 6.515 | 2.302 | 0.00 |
| **ANNUAL INCOME** | Below 3 Lakh (%) | 94 | 40 | 17 |
| | Above 3 Lakh (%) | 6 | 60 | 83 |

Control (healthy) groups have highest annual income with 83% earning more than 3 lakh annually followed by the annual income of commercial cigarette users with 60% earning above 3 lakh.. Zozial users have lowest annual income with 94% of the population earning less than 3 lakh. Between the two cases of smokers, *viz.*, zozial (Z) users and commercial cigarette (CG) users, Z users tends to smoke more with an average of 7.85 (SD=9.33) sticks per day while CG users smoke an average of 4.24 (SD=6.2) sticks a day. Z users smoke an average of 6.515 g of tobacco in a day while CG users consume an average of 2.302 g of tobacco a day.

The mean age of Z consumers is 40 (SD=10.4), CG users is 36.88 (SD=9.53) and that of controls is 39.94 (SD=10.86). The sampling carried out in such a way to nullify the consequence owing to age as it has been reported to affect the level of DNA damage (Witherell *et al*., 1998). Pearson's correlation established a significant ($p<0.01$) positive correlation between the ages of Z consumers and CG users with the MN frequency while the correlation between MN frequency and controls was not significant. A positive correlation was observed between the dose and MN frequency ($p<0.01$) for both Z and CG users (Table 3.19).

**Table 3.19: Pearson's correlation of MN with age and dose.**

| | Age(Z) | Age(C) | Age(H) | Dose(Z) | Dose(C) |
|---|---|---|---|---|---|
| MN | .478** | .806** | 0.048 | .587** | .674** |

**Correlation is significant at 0.01 level (2-tailed).

Linear regression analysis was performed using SPSS statistical package (V 16.0) for testing the statistical significance to check whether demographic, food habit, tobacco habit and other daily habits are significantly related with the frequency of Micronucleus. The p-value <0.05 indicates that there is no significance difference among the variables within samples with different parameters. Linear regression analysis illustrates the significant relation between the qualitative variables and MN frequency as shown in Table 3.20. Daily habits and dietary habits such as alcohol consumption ($p<0.01$), salt intake ($p<0.01$), consumption of smoked meat ($p<0.01$) and smoked vegetables ($p<0.01$) shows significant relation with the score of number of Micronuclei formation in the exposed cell.

In this study, we have evaluated the correlation between tobacco consumption and the micronuclei frequency. Current findings has indicated dietary habits such as high salt intake, smoked meat and smoked vegetables were positively associated with MN frequency which corresponds with previous studies (Balakrishnan *et al*., 2002;

Raimondi *et al*., 2007; Taioli *et al*., 2007; Dani *et al*., 2014). A significant influence of salt intake on both the frequency of MN was observed as high consumption of NaCl may not only in a subtle manner alter the pH conditions but it may also induce structural chromosomal damage which may have been exhibited as Micronuclei (Balakrishnan *et al*., 2002). DNA adducts due to PAHs as a result of consumption of smoked food has been observed (Raimondi *et al*., 2007; Taioli *et al*., 2007) which also commensurated with the results of our assay studies.

The genotoxic effect of alcohol may be regarded as a promoter of tobacco induced buccal carcinogenesis (Wynder *et al*., 1977) which, in our study shows a significant relation with MN frequency. Kuhva (Betel quid) consumption expressed a non-significant relation with the MN frequency which is against previous study that chronic consumption of areca nut may trigger the synthesis of collagen resulting in oral submucous fibrosis (Alexander *et al*., 2019).

Current findings may indicate dietary habits such as daily fat intake (saturated fats and trans-fats, which should not exceed 10% and 1% of the total daily energy intake respectively; WHO, 2018) and saum (fermented pig fat) were not statistically significant. Other lifestyle habits which were considered, such as frequency of fruit intake and other tobacco products such as sahdah and zarda were statistically not significant for MN count in our study samples. The rate of physical exercise shows significant relation ($p<0.01$) with MN frequency but DNA effects identified as a result of intense physical activity were not due to apoptosis or necrosis (Hartmann *et al*., 1998).

**Table 3.20: General linear regression of MN frequency with potential confounders.**

| | MN | | |
|---|---|---|---|
| | **B** | **Std. Error** | **Sig.** |
| Alcohol | -4.885 | 0.674 | 0.000 |
| Fat intake | -0.268 | 0.572 | 0.64 |
| Saum | 0.191 | 0.602 | 0.752 |
| Salt intake | -3.861 | 0.638 | 0.000 |
| Fruits | -0.055 | 0.616 | 0.929 |
| Water intake | 0.916 | 0.616 | 0.138 |
| Exercise | 1.447 | 0.67 | 0.032 |
| Smoked meat | 3.303 | 0.746 | 0.000 |
| Smoked veg | 6.753 | 0.706 | 0.000 |
| Tuibur | 0.185 | 0.982 | 0.851 |
| Kuhva | -0.52 | 0.935 | 0.579 |
| Zarda | -2.021 | 2.031 | 0.321 |
| Sahdah | 0.233 | 0.957 | 0.808 |

# CHAPTER 4

## SUMMARY AND CONCLUSION

# CHAPTER 4

## SUMMARY AND CONCLUSION

Consumption of tobacco in smoking and smokeless forms has been a habitual custom among the Mizo community since ages where sharing of tobacco were represented as an approval and favoritism within the society. Majority of the smoking population would start smoking at an early age. Without the knowledge of its possible deleterious effects, tobacco consumption was deeply embedded in the Mizo culture. Public awareness regarding the scientific characteristics of *zozial* is still very limited and is a much needed topic to educate the users. To the best of our knowledge, no study has been performed on *zozial* and the present study is a first substantial scientific study on the physico-chemical characterization and evaluation of induced oxidative potential of *zozial* and will contribute to the vital consciousness among the populace of the Mizo society.

With the availability of commercial cigarettes from Indian and Myanmar manufacturers along with *zozial*, smokers from the study population are left with a broad range of smoking tobacco products. The smoker population within Mizoram may be broadly classified into two groups; the commercial cigarette smokers and the *zozial* smokers. The epidemiological data indicated that majority of regular *zozial* smokers belongs to farmer population within rural areas with lesser annual income and lower educational qualifications as compared to commercial cigarette users. Due to the farm working culture of Mizo society, *zozial* is consumed at a continuous rate throughout the day using it as a repellent from mosquitoes and other insects while working, eventually leading to nicotine dependence. Despite the availability of commercial cigarettes, majority of the smokers prefers *zozial* due to regular and easy availability and comparatively lower price which in due course results in the preference of flavor. Due to the absence of the putative ‘protective’ filter in *zozial*, the concentration of the long-lived organic radical species in the particulate phase, known as ‘tar’ (water insoluble species which are relatively non-polar), would be prohibitive.

*Zozial* has relatively low combustibility more akin to 'bidi' forcing smoker to smoke more intensely possibly because of moisture content in tobacco matter and the nature of low-quality rolling paper lacking adequate ventilation. More akin to bidi, while smoking *zozial*, it requires more recurrent and deeper puffs to keep it lit, consequently has a grueling impact on the smoker's lungs than commercial cigarette smokers (Kamboj, 2008). Bidis are reported to produce equal or higher levels of carbon monoxide, nicotine, tar, and other toxic chemicals (Gupta and Asma, 2008). In contrast to other "roll your own" cigarettes that still contain additives and other chemical species (FDA, 2018), most likely the rudimentary processing of tobacco leaves entails lesser nicotine yield which may prompt the *zozial* smoker to smoke rather frequently. Particulate matter generated by tobacco smoke has been reported to alter the iron equilibrium in lungs (Ghio *et al*., 2008). The polycyclic aromatic hydrocarbons (PAHs) in cigarette smoke are proven carcinogens (Stepanov *et al*., 2011).

INAA detects the presence of alkaline earth metals such as Na, Mg, K, Ca, Rb, Cs, Sr, Ba along with Transition elements such as V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Zr, and Rare earth elements such as La, Ce, Nd, Sm, Eu, Tb, Dy, Yb, Hf, Ta, W, Th, and U in the tobacco leaf samples at ppm (μg/g) levels. The most abundant elements analyzed from our tobacco samples were Mg, Al, Si, Cl, K, Ca and Fe. Elements such as Na, Ti, Mn, Cu, Zn, Br, Rb, Sr and Ba were found to be present at minor levels, and Sc, V, Cr, Co, Ni, As, Zr, Sb, Cs, La, Ce, Nd, Sm, Eu, Tb, Dy, Yb, Hf, Ta, W, Th and U at trace levels. Potassium, Calcium, Chlorine, Magnesium and Iron are essential for the growth and development of plants (Wang *et al*., 2013; White and Broadley, 2003; Chen *et al*., 2010; Ducu *et al*., 2010; Konieczyski and Wesoowski, 2012; Guo *et al*., 2015; Abbaspour *et al*., 2014). Fe, Mn, Zn, and Br are considered as essential nutrients for human body (Demoz *et al*., 2016). At significantly low concentrations, REEs have a positive effect on the growth of medicinal plants and negative effects at comparatively high concentrations (Zhang *et al*., 2013)

ICP-OES method was used for the determination of trace element content since it is sensitive, selective, and reliable method for the simultaneous and accurate

determination of various elements in trace levels. Tobacco samples collected from various cultivation sites within Mizoram were quantitatively characterized for their trace elemental contents. The ICP-OES data of collected tobacco samples indicated the presence of trace elements such as As, Cd, Pb, Hg, Ni and Cr in high concentrations. The comparison between of the heavy metal concentrations with their corresponding permissible limits indicated that the mean concentrations of Cadmium and Lead are above their permissible limits while those of Mercury, Nickel and Chromium were within the permissible limits. The presence of these classified carcinogenic heavy metals in high concentrations in *zozial* may indicate the prevalence of high rates of several types of cancer within the state. The latest PBCR data (PBCR, 2014) indicated the high incidence of cancer within Mizoram which may well be correlated with the high prevalence of tobacco consumption by the Mizo population as observed in GATS (2017). The current study delivers a reputable data on the concentration levels of various toxic elements in frequently used *zozial*. Consequently, the current data produced would be helpful for researchers in the common field to evaluate the health effects of *zozial* and its potential implications towards its continuous use that feasibly results in the bioaccumulation of toxic metal species among the *zozial* users.

Tobacco smoking is reported to inhibit the methylation capacity of arsenic and its elimination from the body causing DNA damage in lungs (Hays *et al*., 2006). Arsenic shares many properties with tumor promoters (Schoen *et al*., 2004). It is well characterized that lead accumulation in the brain and central nervous system may cause impaired development of nervous systems (Landrigan *et al*., 1991). Cadmium exposure is related to kidney damage. The precursor of renal lesion is frequently a tubular dysfunction, leading to a devastating irreversible cadmium induced tubular damage (Jarup *et al*., 1998). Cadmium and its compounds were classified as carcinogens by the International Agency for Research on Cancer (IARC, 1993). Mercury is another highly toxic environmental pollutant whose harmful impacts include neurotoxicity, immunosuppression, autism, myocardial infarction, Alzheimer disease, and other pertinent issues (Bernard *et al*., 2002; Clarkson *et al*., 2003). Exposure to mercury, even at extremely low concentration, can cause irreversible

damage to the human central nervous system (Blyth, 1999). Earlier studies have indicated that exposure to mercury, iron, chromium, and cadmium leads to DNA damage and altered intracellular redox status (Stohs and Bagchi, 1995).

In general, tobacco cultivation in Mizoram is carried upon without various anthropogenic activities and applications of chemical fertilizers and pesticides. Soil pH, soluble organic matter (SOM), soil type, chemical contents of soil are known to play an important role in the translocation of heavy metals in plants (Noler *et al*., 2006). So, it is pertinent that soil character of Mizoram plays a significant role in the concentration of heavy metal species on collected tobacco plants. The positive correlations observed between the pH of the soil and all the elements showed that pH of the soil play an important character on the concentration of trace elements in tobacco samples collected within Mizoram.

It is a well established fact that the metal contents in tobacco vary depending on the agro climatic conditions. Tobacco plants are known to accumulate heavy metal ions from the soil through the roots and sequester them predominantly in the leaves (Lougon-Moulin *et al*., 2004; Adamu *et al*., 1989; Bell *et al*., 1992). Other than some important micro and macronutrients, metals such as arsenic, cadmium, chromium, lead and nickel that pose serious health implications are also possibly absorbed from the soil treated with lead-arsenate insecticide, phosphate, tobacco-specific fertilizers, and organic fertilizers (Gutemann *et al*., 1982; WHO, 2002; Wu *et al*., 2020).

Estimated daily intakes (EDI) of all the analyzed heavy metals for zozial users lies below the tolerable daily intake (TDI) and target hazard quotient (THQ) of all heavy metals also shows a value within the acceptable range, the hazard index (HI) and carcinogenic risk (CR) of zozial and its users is significantly high and above the permissible levels with the probability of occurrence of adverse effects due to exposure to zozial. Researchers have reported calculated values such as hazard quotient (HQ) to be a reliable data for evaluation of risk associated with the consumption of metal contaminated vegetables (Rupert *et al*., 2004; Rattan *et al*., 2005).

Although the calculated values of estimated daily intake (EDI) and Target Hazard Quotient (THQ) of *zozial* consumers due to exposure to the detected heavy metals in the tobacco samples, such as As, Cd, Pb, Ni and Cr were lower than tolerable values from JECFA, USEPA, ISIRI and EFSA standards, the calculated Hazard Index (HI) for all the heavy metals on each of the samples showed tremendously high values. The calculated Carcinogenic risk (CR) values of As, Cd, Ni and Cr for *zozial* consumers are above the acceptable range. The HI and CR values exhibit the hazardous nature of chronic use of *zozial*.

The indigenously hand-rolled *zozial* is prepared mostly by women at cottage scale industries using tobacco cultivated within Mizoram. Tobacco cultivation in Mizoram is more or less free from various anthropogenic activities and applications of chemical fertilizers and pesticides which are known to affect its physical and chemical properties along with the smoke quality. The chemical composition of the tobacco leaf strongly affects the organoleptic, pharmacological and toxicological properties of tobacco smoke (Leffingwell and Leffingwell, 1988; Tso, 1990). The curing (sun-curing) and storage process of *zozial* also takes much shorter time as compared to flue-cured tobacco used in most commercial cigarettes and other tobacco products, during which the leaves undergo important chemical changes (Bacon *et al*., 1952; Huber, 1989). It is important to mention here that *zozial*, unlike other commercial cigarettes are packed and marketed without any health warning signs and any other necessary information which are mandatory for marketing any tobacco products.

LC-MS/MS study has indicated the presence of TSNAs such as N'-nitrosonornicotine (NNN), 4-methylnitrosamino-1-(3-pyridyl)-1-butanone (NNK) and its metabolite 4-(methylnitrosamino)-4-(3-pyridyl)-butanal (NNAL) and tobacco alkaloids such as Nicotine, Cotinine and Nornicotine along with nitrogen oxide species in *zozial* samples, Tobacco leaf filler (TLF), Indian and Myanmar commercial cigarette samples in our present study. International Agency for Research on Cancer (IARC) has classified TSNAs as Group 1 human carcinogen (IARC, 2004). TSNAs are product of nitrosation of tobacco alkaloids during the

curing and storage of tobacco leaves. Between the commercial cigarette samples, concentrations of all TSNAs detected in commercial cigarette from Myanmar were significantly of higher those of commercial Indian cigarettes. The concentrations of TSNAs in zozial and TLF are significantly lower than those of the commercial cigarettes. Concentrations of NNK and NNAL from some TLF and *zozial* samples were below the limit of detection of the spectrometer. The higher TSNAs levels in burley tobacco (used for commercial cigarettes) are partly due to the relatively higher levels of TSNAs precursors, such as alkaloids and oxide of nitrogen, that are present in the leaf tissue (Bush *et al*., 2001; Shi *et al*., 2003,2012). The comparatively lower concentrations of TSNAs in all of tobacco leaf fillers and *zozial* samples may reflect the absence of chemical fertilizers during cultivation as levels of alkaloids and nitrosamines were reported to increase with increasing fertilization levels (Chamberlain and Chortyk, 2015). The short duration of curing process of *zozial*, which is mainly around a week, may also be the reason behind the low TSNA concentrations as levels of TSNAs were reported to increase substantially during the fourth to seventh weeks of air-curing (Cui, 1998).

The nicotine and nornicotine concentrations were highest in commercial cigarette followed by tobacco leaf filler and then zozial. As nornicotine is a demethylated derivative of nicotine (Lewis *et al.,* 2010), the lower concentration of it in zozial compared to TLF could presumably be due to their difference in duration of storage. As the analyzed zozial samples were collected earlier than TLF samples, they were provided with more time to form NNN from nornicotine. The storage duration may also reflect on the lower concentration of NNN in TLF as nornicotine reacts with nitrosating agents to form NNN. The concentration of detected alkaloids, TSNAs and nitrogen oxide species were significantly higher in commercial cigarettes than those of tobacco samples collected from Mizoram (zozial and TLF). The concentration of nitrate in commercial cigarette is tenfold higher than that measured in zozial. This significant difference in concentrations of alkaloids, TSNAs and nitrogen oxide species implies the difference in the process and duration of curing and cultivation of tobacco used.

In tobacco, the concentration of nicotine and the level of nitrogen are in close association following similar patterns of accumulation (Weybrew *et al*., 1953). The nicotine concentration increases as nitrogen uptake and accumulation increases (Elliot and Court, 1978; Miner, 1980) indicating that higher nitrogen fertilization leads to higher concentration of nicotine. The organic cultivation (without implementation of chemical fertilizers) of *zozial* tobacco may result in lower nicotine concentration of *zozial* when compared to commercial cigarettes as *zozial* smokers smoke more sticks per day to meet the satisfying dose of their compulsive nicotine craving as compared to commercial cigarette users. This dose of smoking (sticks per day) also plays a significant role in the frequency of DNA damage according to the data reported in this study.

Oxidative stress is a well known factor of DNA damage (Souliotis *et al*., 2019). In the current study, DNA damage among *zozial* consumers and controls was evaluated using different available and relevant approaches. The reactive oxygen species (ROS) that originates as a result of normal cellular metabolism is known to attack DNA base guanine that results in lesioning of 8-OHdG having a mutagenic potential (Karihtala, 2009). Our findings indicate that there is an elevated urinary 8-OHdG concentration among *zozial* consumers in comparison with non smokers (controls). The formation of superoxide anion (free radical ROS) by the addition of extra electron to the molecular oxygen is mediated by antioxidant enzymes such as superoxide dismutase (SOD), which are critical players against overproduction of reactive oxygen species (ROS) (Flora, 2011; Jomova *et al*., 2015). SOD converts superoxide anion into hydrogen peroxide ($H_2O_2$) and molecular oxygen ($O_2$). Our results demonstrated that the SOD activity significantly increase for *zozial* smokers due to heavy metal exposure. This increase in inhibition activity suggests an elevated generation of reactive oxygen species (ROS) in *zozial* consumers when compared with controls. The buccal mucosa cells play a crucial role as the first barricade against foreign belligerence. The present study revealed that there is a significant difference in the frequency of micronucleus (MN) between smokers and non-smokers. Our study reveals that among smoking tobacco users, *zozial* smokers shows elevated concentration of MN compared to commercial cigarette users.

The high concentrations of toxic heavy metals and the absence of cellulose acetate filter in *zozial* may be the underlying motive behind the elevated frequencies of DNA damage among the users. The higher rate of smoking (sticks per day) by *zozial* consumers, when compared with commercial cigarette users, also shows a strong association with elevated incidence of DNA damage. Smoking is known to exacerbate the risk of cancer (Carbone, 1992). Consumption and exposure to these detected toxic metals through smoking in longer period can lead to toxicity due to accumulation nature of these metals in the body. In consideration to the current study, the presence of heavy metals in *zozial* may have numerous, wide range adverse effects on the health of the person exposed to it. However, a further investigation is required in order to establish a firm stance on the cytotoxic, mutagenic and carcinogenic potential of *zozial*.

The indigenous filter-less hand-rolled *zozial* is a more preferred smoking tobacco product by the local population, residing mainly at rural areas, due to its abundance and cheaper in terms of cost. The smoking community of Mizoram has a limited knowledge and awareness with respect to the health risks associated with *zozial* consumption. The marketing of *zozial* taking place in a cottage scale industry in Mizoram do not display any health warnings. In Mizoram, sharing of tobacco is viewed as a sign of favoritism within the society and is still a very common practice among the Mizo population. *Zozial* smoking is considered to putatively pose a much higher health risk due to the potential synergistic effects of various xenobiotic compounds that are potentially hazardous to ingest. It is pertinent to mention here that the present study is the first scientific study on the characterization and evaluation of oxidative potential of *zozial* that will help in understanding and a much needed awareness among the *Zozial* smokers within the community. Along with this, a proper packaging with appropriate labeling bearing health warnings of potential toxicity risks as a result of the heavy metal content of *zozial* would be genuinely recommended. As a result, there is an urgent need for proper and scientific monitoring of these toxic metal residues in the filter-less *zozial*.

**ADDENDUM** (Answers for the questions asked during PhD viva)

**Summary**:

Tobacco smoke and ash may well be potential sources of metal bioaccumulation in smokers instigating adverse human health effects through direct tobacco use (Verma *et al*., 2010). Cigarette ash retains around 71% - 86% elemental concentrations in the tobacco matter, while tobacco smoke (aerosol of gas phase and particulate phase) contains 14% - 29% of the volatilized elemental contents (Inga *et al*.,2018). For example, in case of arsenic, only 28%-32% remains in the ash (Inga *et al*.,2018) while the remaining arsenic primarily volatilized to inorganic arsenic (mostly arsenate and arsenite) and concomitantly transported through smoke (Taebunpakul *et al*., 2011). Inorganic arsenic has been reported to produce methylated species such as methylarsonic acid and dimethylarsinic acid in the liver after absorption through the gastrointestinal tract (Del Razo *et al*., 1997; Hughes *et al*., 2003). Importantly, during smoking, ~ 40%-60% of inhaled cadmium is directly absorbed into the blood (Kazi *et al*., 2009). Smokers were found to contain elevated levels of lead in their blood than non-smokers (Chiba and Masironi, 1992).

With an average annual rainfall of 2500 mm to 3000 mm over an undulating hilly terrain, Mizoram soil is naturally acidic leading to soil mineral leaching. Soil pH plays an important role in heavy metal absorption by plants (Amini *et al*., 2005) and the topography is known to affect soil pH (Moore *et al*., 1993). Low soil pH increases the solubility and bioavailability of heavy metals thereby increasing metal absorption (Fässler *et al*., 2010). Since the tobacco samples were collected from different cultivation sites across Mizoram state with varying elevation in landscapes, correspondingly the solubility and bioavailability of heavy metals also vary that entails a wide range of heavy metals concentrations in the analyzed samples leading to the calculated standard deviations (Tables - 3.1 and 3.2). The limited sample size is due to the less number of available large scale tobacco cultivation sites within the sampling area which correspondingly contributes to the estimated standard deviation values.

Retention of particulate matters (mean diameter < 1 μm) from cigarette smoke exposes the lungs at high rate of deposition (Baker, 2000) and results in lung injury (e.g., chronic obstructive pulmonary disease [COPD] and cancer) (Ghio et al., 2008). Pyrolysis of tobacco occurs at the combustion zone (lighted tip of cigarette with temperature reaching 800-950°C) after which incomplete combustion occurs due to a rapid drop in temperature (200-600°C) and lack of oxygen generating a complex aerosol. This includes toxic volatile organic compounds (VOCs) and non-volatile, non-polar, condensed tar phase. Due to the absence of filter in zozial, more tar is produced. More akin to another unfiltered 'cigarette' i.e., bidis, imperforated nature of rolling paper used in zozial also requires deeper and more frequent puffs to keep it lit as tobacco matter extinguish more easily which may be consequently gruelling on the smoker's lungs as compared to commercial cigarette smokers (Kamboj, 2008). This may be one of the important factors that would have caused the observed high frequency of micronuclei in zozial users compared to the cigarette users.

With the bibliographic details collected from the voluntary participants for the evaluation of oxidative potential of zozial, higher mean age of zozial consumers than that of controls was observed among the studied population. This is because majority of zozial smokers started smoking at a very young age (~ 15 years of age) expanding the age gap between the youngest and oldest smoker resulting in the calculated mean; during sampling for controls, an unforeseen event of Covid-19 pandemic disrupted the sampling process, limiting the number of samples especially for controls. It is important to mention here that the provided data does not imply a possible false observation that zozial smokers live longer than non-smokers and/or commercial cigarette smokers, despite of observed elevated oxidative stress levels. The extent of DNA damage observed in our data for control population suggested that natural biological DNA aberrations can occur (baseline) due to factors such as ageing, lifestyle and dietary habits but significantly higher levels among the smokers as continuous exposure to tobacco smoke indicates elevated concentrations of DNA damage thereby increasing Oxidative stress.

Due to the organic nature of cultivation and comparatively shorter duration of 'curing' for the tobacco produced in Mizoram for the production of zozial, zozial contains significantly lower concentrations of TSNAs (NNN, NNK and NNAL), tobacco alkaloids (nicotine, cotinine and nornicotine) along with nitrogen oxide species ($NO_2^-$ , $NO_3^-$). The lower nicotine content in zozial tobacco matter as well as the smoke pH (possibly acidic due to the lack of additives such as ammonium salts) which may deliver considerable proportions of nicotine in the 'ionized' form would induce zozial users to smoke zozial sticks frequently in order to meet the satitiate their compulsive nicotine craving as compared to commercial cigarette users. This higher dose (number of sticks per day) among zozial users plays a significant role in inducing significant oxidative stress levels yet considerable oxidative stress levels found in commercial cigarette users when compared to non-smokers. Despite the significantly higher concentrations of toxic heavy elements in zozial, the concentrations of TSNAs (Group 1 carcinogens), are comparatively lower in the case of zozial samples on comparison with commercial cigarette samples. The oxidative stress studies further suggested that high frequencies of DNA damage in both smoking groups (zozial and commercial cigarettes) when compared to controls. This illustrates that smoking of any form is deleterious and exacerbate adverse health effects on the exposed population. It has been proven through voluminous research studies that tobacco smoking in any form (including passive smoking) is injurious to human health. ***Therefore, the findings of this research study on the indigenous tobacco product, i.e., zozial, do not, in any way, promote nor endorse the smoking any kind of tobacco products in general, commercial cigarettes over zozial or vice versa, in particular***.

## APPENDICE

*Questionnaire for Zozial consumption in Mizoram*

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**PERSONAL INFORMATION**:

**Hming** (*Name)*: _____

**Mipa/Hmeichhia**(*Gender*):_____**Kum**(*Age):*_____

**Nupui/pasal nei/neilo** (*Marital status):*_____ **Rihzawng** *(Weight):*_____

**Lehkha zir chen** (*Education*):_____ **Eizawnna**(*Occupation)*:_____

**Kum khat/Thla khat chhung a sum lakluh zat** (*Annual/Monthly Income*):_____

**Address**: _____ **Mobile No**: _____

**Zozial i zu em?** (*Do you consume zozial?):* Aw (*Yes*) [ ] Aih (*No*) [ ]

**Engtik atangin nge zozial i zuk tan?** (*How old were you when you first tried zozial smoking, even one or two puffs?*)

Below 10 years [ ] 10-15 years [ ] 15-20 years [ ]

20-25 years [ ] 25 years and above [ ]

**Nikhat ah engzat nge i zuk thin tlangpui?** *(Average number of Zozial sticks per day)*:

2 atanga 5 inkar (*2 to 5 per day*) [ ]

6 atanga 10 inkar (*6 to 10 per day*) [ ]

11 atanga 20 inkar (*11 to 20 per day*) [ ]

20 aia tam (*More than 20 per day*) [ ]

**Eng ang hunah nge i zuk chak thin?** (*Under what circumstance do you feel like having zozial (like…: under stress / jovial / to celebrate / during social gathering / while with a friend / etc.*) :_____

**I nghei tawh anih chuan, engtik atangin?** *(If quit already, since when?)*: _____

**Thla khat liam ta ah khan vaihlo atanga thil siam hrim hrim chak huam huam i nei em?** (*During the past 30 days, have you had a strong craving or felt like you really needed to use a tobacco product of any kind (such as smoking a cigarette or using chewing tobacco, snuff, dip, or snus?*)

Aw (*Yes*) [ ] Aih (*No*) [ ]

**Engvangin nge cigarette dang aia zozial i thlan?** *(Why do you prefer zozial over other cigarettes?)*_____

**Vaihlo atang a siam thil dang tih I nei em?** (*Do you consume other tobacco products?):*

Aw (Yes) [ ] Aih (No) [ ]

Chungte chu*(Type)*: Branded cigarettes [ ]; Tuibur [ ]; Supari [ ];
Kuhva *(Pan/Beetle nut)* [ ]; Zarda (*Zarda Pan)* [ ]; Gutkha/Tiranga [ ];
Sahdah/Khaini (*Oral snuff*) [ ]; Adangte *(Others)* [ ]

## FREQUENCY OF CONSUMPTION OF OTHER TOBACCO PRODUCTS IN ADDITION TO ZOZIAL:

**Vaihlo atanga thil siam ho ah hian eng ber in nge i vaihlo chak na chhawk ber a i hriat?** [*Among all tobacco products (smoke / smokeless forms) which one gave you the most satisfaction that you feel like consuming to have that satisfaction feeling?*]

_____

**I zing thawh hlim atanga engtik hun ah nge vaihlo i chak thin?** (*How soon after you wake up do you want to use a tobacco product?*)

Within 5 minutes [ ] From 6 to 30 minutes [ ]

From more than 30 minutes to 1 hour [ ] Between 1 to 24 hours [ ]

I rarely want to use tobacco [ ]

**He thu "Reilo te pawh vaihlo tello a awm hi harsa ka ti" tih hi nangmah ah enge a dik dan?** (*How true is this statement for you "I feel restless and irritable when I don't use tobacco for a while."*)

A dik lem lo (*Not at all true*) [ ]; A chang chuan a dik ve tho (*Sometimes true*) [ ]
A ni ve viau mai (*Often true*) [ ]; A dik reng (*Always true*) [ ]

**Vaihlo atang a siam thil chi hrang hrang ah enge i thlan ber ang?** (*If you are offered to choose, which tobacco product do you feel like having it (that includes zozial/branded cigarettes/ tuibur/ sadah/ gutkha/ zarda/ kuhva/etc.*): _____

**Zu i in em?** (*Do you consume alcohol?):* Aw (*Yes*) [ ] Aih (*No*) [ ]

Engtianga zing in nge I in? (*How often do you drink?*) :_____

Engtik atangin nge I in tan? (*When did you start taking alcohol?)* : _____

Eng zu nge? (*Typel*): Mizo siam *(Local)* [ ] Hnamdang siam (*Branded)* [ ]

I nghei tawh anih chuan, engtik atangin? *(If quit already, since when?)*: _____

**Sa i ei em?** (*Do you consume non-veg?):* Aw (*Yes*) [ ] Aih (*No*) [ ]

Aw*(If yes):* Ei nasa (*Heavy)* [ ]; Ei nasa lo(*Average)* [ ]; Ei tlem(*Little)* [ ]

**Eng sa nge i ei thin ?** (*Item consumed)*:

Vawksa (*Pork)* [ ]; Bawngsa (*Beef)* [ ]; Kelsa (*Mutton)* [ ]; Arsa (*Chicken)* [ ]

Sangha/Chakai/Chengkawl/Kaikuang (*Sea food)* [ ]; Adang *(Others)* [ ]

**Sa rep i ei ngai em?** (*Do you consume smoked meat?):*

Ei nasa (*Heavy*) [ ]; Ei nasa lo (*Average*) [ ]; Ei tlem (*Little*) [ ]; Ei ngailo (*Never*) [ ]

**Thlai rep i ei ngai em?** (*Do you consume smoked vegetables?*):

Ei nasa (*Heavy*) [ ]; Ei nasa lo (*Average*) [ ]; Ei tlem (*Little*) [ ]; Ei ngailo (*Never*) [ ]

**Thau/Mawm i ei thin em?** (*Fat intake*):

Ei nasa (*Heavy*) [ ]; Ei nasa lo (*Average*) [ ]; Ei tlem (*Little*) [ ]; Ei ngailo (*Never*) [ ]

**Sa um i ei em?** (*Do you consume'Sa Um'?):*

Ei nasa (*Heavy*) [ ]; Ei nasa lo (*Average*) [ ]; Ei tlem (*Little*) [ ]; Ei ngailo (*Never*) [ ]

**Chi i ei nasa em?** (*Do you take extra salt?):*

Ei nasa (*Heavy*) [ ]; Ei nasa lo (*Average*) [ ]; Ei tlem (*Little*) [ ]; Ei ngailo (*Never*) [ ]

**Thei i ei thin em?** *(Do you take fruits?)*

Ei nasa (*Heavy*) [ ]; Ei nasa lo (*Average*) [ ]; Ei tlem (*Little*) [ ]; Ei ngailo (*Never*) [ ]

**Ni khat ah tui engzat nge i in?** *(What is your daily dosage of water intake?)*

In nasa (*Heavy*) [ ] In nasa lo (*Average*) [ ] In tlem (*Little*) [ ]

**Exercise i la ngai em?** (*How often do you exercise?*)

Ngai lo (*Never*) [ ]; Nasa lo (*Average*) [ ]; Tlem (*Little*) [ ]; Nasa (Heavy) [ ]

**I hna a hahthlak viau em, zan lam ah hna i thawk em(night duty)?** (*Is your job stressful or do you perform shift work (night duty)?)*:

Aw (*Yes*) [ ] Aih (*No*) [ ]

**Natna/Damlohna i nei em?** (*Do you have any diseases?):*

Aw (*Yes*) [ ] Aih (*No*) [ ]

I neih chuan, eng natna nge? (*If yes, what type of disease?):* _____

**Khawngaih takin he zirchianna hi i ngaihdan han sawi teh** (*Please give your opinion about this zozial / Tobacco use survey.*)

_____

_____

_____

**FAMILY INFORMATION:**

**In chhungkua ah Cancer vei an awm em?** *(Anyone having cancer in your family)*:

_____

_____

_____

**In chhungkua ah natna dang nei an awm em?** *(Any family members having other disease in your family?)*:

_____

_____

_____

**REMTIHNA *(Consent):***

Heng a chunga thu te hi ka hriatpui a, ka biological sample hi zir chian atan pek ka remti thlap e.

*(The information provided above was given with my full consent and I do not have any objection in providing my biological sample for research purposes. I have read and understood the consent information).*

Hmun*(Place*): Signature:

Date: Hming (*Name)*:

## KA LAWM E

## *(THANK YOU VERY MUCH FOR YOUR HELP)*

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## (A) Journals:

1. **Lalrammawia K**, Buragohain A, Kakki B, Zote L, Marak NK, Lalrinhlupuii, Malsawmtluangi, Lalmuanpuii R, Kumar NS, Jahau L, Sudarshan M, Chaligava O, Yushin N, Grozdov D, Nekhoroshkov P, Vergel K, Zinicovscaia I, Muthukumaran RB. Determination of Multi Elements in Tobacco Plant of Northeast India by Neutron Activation Analysis and Atomic Absorption Spectrometry. Biol Trace Elem Res. 2021 Nov 24. doi: 10.1007/s12011-021-03040-2. Online ahead of print.PMID: 34820780

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2. **K.Lalrammawia**, Rajendra Bose Muthukumaran. Heavy Metal Analysis of Tobacco Leaves Collected from 'Organic' tobacco cultivated in Mizoram. *Proceedings of Recent Advances in Biotechnology*, $9^{th}$-$10^{th}$ November, 2017, Department of Biotechnology, School of Life Sciences, Mizoram University, Aizawl, Mizoram, India.

3. **K.Lalrammawia**, M. Sudarshan, Rajendra Bose Muthukumaran. Assessment of Exposure to Tobacco Smoke: A Biomarker Study. *Proceedings of Chemistry & Environmental Sustainability (ICCES – 2019),* $19^{th}$ – $22^{nd}$ February, 2019, Department of Chemistry, School of Physical Sciences, Mizoram University, Aizawl, Mizoram, India.

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# Determination of Multi Elements in Tobacco Plant of Northeast India by Neutron Activation Analysis and Atomic Absorption Spectrometry

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## Abstract

Even when cultivated in uncontaminated soils, tobacco plant has higher propensity to extract and accumulate trace elements. The concentrations (mass fractions) of essential elements (K, Ca, Mg, Na, Cl, Mn, Fe, Cu, and Zn) and 28 non-essential elements in tobacco plant (leaves, stem, and root) of Northeast India and their respective soils were quantitatively measured. Hg mass fraction in all samples analyzed were found to be < 10 mg/kg. The heavy element mass fractions of tobacco are weakly correlated to different soil parameters. The bioconcentration factor values indicated that Cd (7) is selectively absorbed and translocated in the tobacco leaves compared to Zn (1.7), Cu (1.5), Ni (0.12), and Pb (0.1). Under acidic soil conditions, tobacco plant efficiently absorbed and translocated $Cl^-$ ion with great ease, whereas it may be a very low accumulator of rare-earth elements. The mass fractions of Mn, Cu, Sb, Cs, Rb, and Pb are very similar to the "reference plant," whereas significantly higher mass fractions of Al, Sc, Ti, Zr, Hf, Ta, Th, and U are present in the roots of tobacco plant relative to the "reference plant." Principal component analysis has revealed that Northeast Indian tobacco can be clearly differentiated from other varieties of tobaccos used in different countries because of their element profiles.

**Keywords** Bioconcentration factor · Translocation factor · Heavy elements · Chloride · Principal component analysis

## Introduction

Despite the cumulative evidence on the adverse health effects of tobacco, its consumption has not reduced [1]; besides, it is one of the major non-edible cash crops of South Asian countries. The propensity of tobacco plant to readily absorb heavy elements from the soil and sequestering them in their leaves has been well documented [2–5]. Elemental profile of tobacco leaf can be influenced by various factors, viz., soil pH, soil type, plant genotype, stalk position, soil, and leaf residues, besides the application of pesticides/fungicides containing metals and soil amendments such as fertilizers, municipal sludge, or farm manure [3, 6–10]. Heavy elements are natural constituents of the Earth's crust [4, 5,

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11]. By virtue of wet rainy monsoon agro-climatic conditions, soil acidification is a naturally occurring phenomenon in Mizoram which is attributed to the leaching of mineral elements. Thus, mobility of heavy elements is a major detrimental factor in acidic soil. Depending on the edaphic conditions of soil, the chemical forms of heavy elements in soil can vary and thereby influence the efficacy of absorption and translocation by the plant [12, 13].

With the knowledge of elemental distributions in soil, it is possible to assess the bioaccumulation and translocation potential of plants [14, 15]. Plants which are known to tolerate and absorb heavy element species can be distinguished as excluders, indicators, accumulators, and hyperaccumulators depending upon the ability to absorb and transport heavy elements to the aerial parts through roots from the soil ranging from modest concentrations (mass fraction) (< 1 mg/kg) to substantially high mass fraction levels (> 1000–10,000 mg/kg) [10, 14]. Tobacco plant has considerable ability to absorb, translocate, and accumulate Cd preferentially even in the presence of high mass fraction of Zn, Cu, and Ni species in the soil; nevertheless, it is not considered a hyperaccumulator plant species for Cd [15, 16].

In India, tobacco products are consumed on a fairly high rate and the consumption frequency has been reported to be even higher among the Northeast Indian (NEI) populace, in particular [1]. In Mizoram, similar to other NEI states, shifting cultivation has been a common cultural practice which entails "organic" cultivation methods in the hilly terrain [17]. As a result, various agricultural practices causing the incremental deposition of heavy metals in the soil are not applied. Tobacco (*Nicotiana tabacum*) plant is organically cultivated in Mizoram, mainly for the preparation of an indigenous smoking tobacco product called "*zozial*." "Zozial" is, essentially, a hand-rolled nonfilter cigarette, available in the NEI market. Tobacco filler of zozial has a distinctive processing method, where matured tobacco leaves are harvested by priming method, and thoroughly squashed with bare foot on a bamboo mat. Then, under direct sunlight during cooler winter months, sun-curing is practiced, however, only for a very short duration (~ 10–15 days) until the semi-dry tobacco leaves turn dark brown with the loss of all chlorophyll content. Subsequently, it is made into coarse flakes using wooden mortar and pestle. Finally, it is rolled with bare hands by using "cheap" cigarette paper, mostly by women.

Major sensitive and robust analytical techniques have been applied to determine the heavy element profiles in different types of tobacco (hookah tobacco, bidi tobacco, cigarette tobacco) and in other cigarette components including tobacco filler, filter, smoke, and ash [3, 18–23]. Instrumental neutron activation analysis (INAA) in combination with atomic absorption spectrometry (AAS) provides simultaneous multi-elemental determination with high accuracy and low detection limits, and is rather free from matrix interferences [23, 24]. It is important to assess the elemental distribution in tobacco cultivated in the NEI region, since NEI region has considerably high incidence of cancers compared to the Indian national average due to high tobacco consumption [25]. Even though a wide range of quantitative analyses on the mineral and non-essential elemental distributions in the different types of tobacco grown in different geographical regions of India have been performed [19, 22, 26], to the best of our knowledge, the elemental compositions in the tobacco grown under "shifting" cultivation conditions of NEI and their corresponding soil have not been reported. It is noteworthy that this is the first preliminary scientific study on the indigenously cured tobacco cultivated in the Mizoram state of NEI. The main objective of this preliminary study is to determine trace elements' distributions and mass fractions, using INAA and AAS methods, in tobacco plant grown and in the respective soil of NEI State of Mizoram where this crop is cultivated. Besides, the extent of accumulation of various elements including rare-earth elements in tobacco plants has also been explored.

## Materials and Methods

### Sample Collection

Samples were collected during December 2019–February 2020 from 8 different cultivation sites from North and East parts of Mizoram (Supplementary Table S1), covering 4 districts. Two to three sets each of 5–10 matured tobacco leaves of lower priming (lug leaves) from each tobacco plant were collected from each sampling site. The roots and stems from the same tobacco plants were also collected. All the samples were packed separately using clean polyethylene bags. The surface soil near the roots (15–30 cm in depth) was also collected from each sampling site. Soil samples were sieved and packed in a fresh polyethylene bags. All samples were carefully labelled and stored in a clean and dry area prior to analysis.

## Soil Parameters

#### *Available Nitrogen*

Alkaline permanganate method was used to determine the available nitrogen in soil samples [27]. Twenty grams of dry soil sample was distilled at a constant rate in a distillation assembly with 100 ml of 0.32% $KMnO_4$ and 100 ml of 2.5% NaOH. In a 250-mL conical flask containing 20 ml of 4% boric acid and a few drops of mixed indicator (0.5 g bromocresol and 0.1 g methyl red dissolved in 100 ml of

95% ethanol), the released ammonia was collected. After 30 min, the distillate was collected and subsequently titrated with 0.01 N standard $H_2SO_4$ solutions using methyl orange as an indicator until the color turns to pink. Similarly, blank reading was taken for the solution without soil.

### *Available Phosphorus*

The Bray and Kurtz method was used to determine available phosphorus in soil sample [28]. In a 250-ml conical flask, 5 g dry soil sample mixed with Bray's extractant was taken, concomitantly swirled for 5 min, and filtered using Whatmann filter paper No. 42. In a 25-ml volumetric flask, 5 ml of aliquot was transferred, mixed with 5 ml of 1.5% Bray's reagent and diluted with distilled water up to 22 ml, and immediately, 1 ml dil. $SnCl_2$ solution was added and mixed well and the final volume was brought up to 25 ml. Using a spectrophotometer, intensity of blue color obtained was measured at 660 nm. Blank reading without soil sample was also measured under same conditions. Phosphorous concentration was estimated using the standard curve.

### *Available Potassium*

Neutral ammonium acetate method was used to analyze available potassium in soil sample [29]. In a 250-ml conical flask, 5 g of dry soil sample and 25 ml neutral ammonium acetate were taken. Immediately after mixing, the soil solution was filtered using Whatmann filter paper No. 42. First, few milliliters of filtrate was rejected, and then, the potassium concentration in the solution was obtained using a flame photometer. Blank reading without soil sample was also measured under same conditions. Standard curve was plotted by taking the readings of the flame photometer against different concentrations (2, 5, 10, 15, and 20 ppm) of potassium chloride solution (1000 ppm prepared by dissolving 1.907 g potassium chloride in 1 l distilled water).

### SOM

SOM in soil was analyzed using rapid titration method by Walkley and Black [30]. In a 500-ml conical flask, 2 g of dry soil was taken. Ten milliliters of 1 N $K_2Cr_2O_7$ solution was added to the soil sample and mixed well, 1.25% $Ag_2SO_4$ was added to 20 ml conc. $H_2SO_4$, and then, the flask was swirled for 20 to 30 times. It was kept undisturbed for 30 min. After 30 min, 200 ml distilled water was added to the flask; then, 10 ml 85% $H_3PO_4$ and 15–20 drops of diphenylamine indicator were added. The solution was then titrated with N/2 ferrous ammonium sulfate solution until the color changes from violet to bright green and the measured reading was noted. The blank titration without soil was performed in similar manner.

### Soil pH

Soil pH was analyzed by taking 50 g air-dried soil sample into a 100-ml beaker. To this, 50 ml deionized water was added, left undisturbed for 12 h, and then mixed well and allowed to stand for 30 min. The suspension was swirled again after 1 h. Subsequently, pH of the solution was taken using a pH meter (Eutech-510).

### Instrumental Neutron Activation Analysis (INAA)

For NAA, all samples were dried at 105 °C until the constant weight and homogenized. Plant material was pelletized. Then, samples (about 300 mg of plant material and 100 mg of soil) were packed in polyethylene bags for short-term irradiation and in aluminum bags for long-term irradiation. Elemental profile of samples was determined using NAA at the installation REGATA of the pulsed fast reactor IBR-2 of the Frank Laboratory of Neutron Physics (JINR, Dubna, Russia). Two procedures of samples irradiation were applied. To determine elements (Mg, Al, Si, Cl, Ca, Ti, V, Mn, and Dy) with short-lived radionuclides (Supplementary Table S2), samples were irradiated at a thermal neutron flux of $1.6\times10^{12}$ ncm$^{-2}$ s$^{-1}$ (soils for 1 min and plant samples for 3 min) and measured for 15 min. To determine elements (Na, K, Sc, Cr, Fe, Co, Ni, Zn, As, Br, Rb, Sr, Zr, Sb, Cs, Ba, La, Ce, Nd, Sm, Eu, Tb, Yb, Hf, Ta, W, Th, and U) with long-lived radionuclides (Supplementary Table S2), samples were irradiated for 4 days at a neutron flux $3.31\times10^{11}$ ncm$^{-2}$ s$^{-1}$, re-packed, and measured twice using HP germanium detectors after 4 and 20 days of decay, respectively. The NAA data processing and determination of element mass fractions were performed using Genie2000 and software developed in JINR. The quality control of NAA results was ensured by simultaneous analysis of the examined samples and standard reference materials: NIST 1633b (Constituent Elements in Coal Fly Ash), NIST 2709 (San Joaquin Soil), NIST 1547 (Peach Leaves), NIST 1575a (Trace Elements in Pine Needles), and NIST 1632c (Trace Elements in Coal). The measured mass fractions were in good agreement with the recommended values [31, 32].

### Atomic Absorption Spectrometry (AAS)

For AAS analysis, 0.2 g of samples was placed in a Teflon vessel and treated with 3 ml of concentrated $HNO_3$ and 1 ml of $H_2O_2$ (plant material) and 8 ml of $HNO_3$ and 2 ml of HF (soil samples). Teflon vessels were introduced in a microwave digestion system (Mars; CEM, USA) for complete digestion. Digestion was performed in two steps: (1) ramp: temperature 260 °C, time 20 min, power 290–1800 W, and pressure 20 bar; (2) hold: temperature 260 °C, hold time 15 min, power 1800 W, and pressure 20 bar. Digests were

quantitatively transferred to 50-ml calibrated flasks and made up to the volume with bi-distilled water. The reagents used for this study were of analytical grade from Merck (Darmstadt, Germany).

The Pb, Cd, Cu, and Hg mass fractions in the experimental solutions were measured using an atomic absorption spectrometer (Thermo Scientific iCE 3400 series, USA) with electrothermal atomization. The calibration solutions were prepared from a 1 g/L stock solution (AAS standard solution; Merck, Germany).

### Statistical Analysis

Pearson's correlation coefficients were calculated using SPSS (V16.0) statistical software to explore the relationships between tobacco samples and the corresponding soil by applying the confidence levels of $p < 0.05$ and $p < 0.01$.

Multivariate statistical analyses, viz., principal component analysis (PCA) and hierarchical cluster analysis (HCA), were performed, after autoscaling, for a data matrix comprising the experimentally determined element profiles and the published data on tobacco matter (vide infra) in order to assess the correlations between different elements present in tobacco matter. Several iterations of PCA were computed using different elements' profile as different subsets of selected matrices in order to discern tobacco samples belonging to various regions. The statistical data processing was performed using "factoextra" package in the R version 4.0.3 for these exploratory analyses [33]. After preliminary iterations, NEI tobacco sample IND-T5 was considered an outlier. IND-T5 exhibited significantly higher values of mass fraction for various elements than the rest of the tobacco samples considered. Even though no statistical analysis was applied to evaluate the outlier among the samples, yet IND-T5 was omitted from the data set.

## Results

The measured elemental mass fractions in the tobacco plant, viz., tobacco leaf, stem, and roots, and the corresponding soil samples are summarized in Table 1. Basic soil properties were determined and soil pH values ranged from strongly acidic (pH = 4.4) to marginally acidic (pH = 6.9) with a mean soil pH = 5.3 (Supplementary Table S3). In terms of grain size distributions, the soil may be classified as sandy soil to silt loam soil [34]. Besides, essential mineral elements and non-essential trace elements including the rare-earth elements (REEs) are widely distributed in soil. In the acidic soil of Mizoram, the mean elemental mass fractions are present in the following order: Si > Al > Fe > Mg > K > Ti > Na > Ca > Mn > Ba > Zr > Rb > Cr > V > Cl > Zn > Ce > Sr > Ni > La > Nd > Co > Th > Cu > Sc > Pb > As > Hf > Br > Sm > Cs > Dy > Yb > U > W > Eu > Ta > Tb > Sb > Cd. Besides, Hg content was below the detection limit (< 10 mg/kg) in all soil samples. Among the studied elements determined in the soil samples, Cl exhibited the highest coefficient of variance, followed by Ca, Cu, and Br, respectively, whereas Ti, Pb, and REEs had the lowest coefficient of variance.

The observed ranges of elemental mass fractions in tobacco leaf samples show that most of the elements including few macro elements, viz., K, Ca, Mg, Ba, V, and Sr, vary by a factor of ≤ 2.5, besides As, Co, Cr, Cs, Si, Sb, Cd, Pb, and Ti, along with many REEs varied within a moderate range by a factor of ≤ 4.5, while few elements, viz., Sc, Fe, Mn, Cu, Zn, Th, Na, U, Ta, Al, Br, and Ni, vary by two orders of magnitude or more. It is noteworthy that mass fractions of Cl in tobacco leaves vary substantially (Table 1).

The tobacco plant, under strongly acidic soil and natural rhizosphere conditions, exhibited lower accumulative capacity with mean mass fraction levels for Al: soil (72,175 mg/kg) → root (2681 mg/kg) → stem (1379 mg/kg) → leaves (1446 mg/kg) and for Fe: soil (41,788 mg/kg) → root (1798 mg/kg) → stem (1051 mg/kg) → leaves (1184 mg/kg). In contrast, tobacco plant exhibited higher accumulative capacity for Cd: soil (0.05 mg/kg) → roots (0.07 mg/kg) → stem (0.09 mg/kg) → leaves (0.4 mg/kg) when compared with Zn: soil (88 mg/kg) → roots (43 mg/kg) → stem (55 mg/kg) → leaves (150 mg/kg) and with Cu: soil (14 mg/kg) → roots (8 mg/kg) → stem (8 mg/kg) → leaves (19 mg/kg). The order of mean bioconcentration for tobacco leaf is Cl > > > Ca > Br > Cd > > K > Sr > Zn and the order of translocation is Cd > Zn > Mn > Mg > Br > Ca > Cl > Si > Cu > Ba > W > Sr > Pb > K > Sb > Ni > Co. Both mean bioconcentration factor (BCF) and mean translocation factor (TF) for Al, V, Th, Sc, and Cs are < 0.55.

Pearson's correlation shows a weak positive correlation between pH of the soil and mass fraction of elements such as K, Ni, Zr, and Eu indicating that as pH of the soil increases, the mass fraction of these elements increases and vice versa (Supplementary Table S4); all the other elemental mass fractions show weak negative correlation with pH indicating that the pH of the soil is inversely proportional to the mass fraction of the elements. A significant positive correlation exists between N (in soil) and Cl, while P exhibits a significant positive correlation (in soil) with Na and Cs ($p < 0.01$). Significant positive correlation was also found to exist between N (in soil) and Si, Ca, Cr, and Dy while P (soil), Al, and Sb also exhibit a significant negative correlation ($p < 0.05$). A significant negative correlation ($p < 0.05$) was also found between K (in soil) and Tb. The mass fraction of elements such as Na, Al, Sc, V, Zn, Cs, Ta, W, and Th shows significant negative correlation with SOM at $p < 0.05$ level. However, there is no significant correlation between the heavy element mass fractions in tobacco leaves and in the soil (Supplementary Table S4).

**Table 1** Range and mean of element concentrations (mass fractions) in tobacco plants (leaves roots and stems) and the corresponding soils

| Elements | Soils ($n=8$) | | Roots ($n=8$) | | Stems ($n=8$) | | Leaves ($n=8$) | |
|---|---|---|---|---|---|---|---|---|
| | Range (mg/kg) | Mean ± SD | Range (mg/kg) | Mean ± SD | Range (mg/kg) | Mean ± SD | Range (mg/kg) | Mean ± SD |
| Na* | 2730–5680 | 3911 ± 1283 | 290–2500 | 846 ± 863 | 78–1890 | 560 ± 680 | 129–753 | 411 ± 267 |
| Mg* | 17,000–30,200 | 21,900 ± 4900 | 990–4060 | 2524 ± 853 | 1180–6100 | 3054 ± 1732 | 4900–11,600 | 8563 ± 2117 |
| Al* | 59,000–103,000 | 72,175 ± 15,108 | 944–2940 | 2681 ± 2016 | 421–3780 | 1379 ± 1104 | 599–3140 | 1446 ± 829 |
| Si* | 40,400–395,000 | 339,500 ± 68,197 | 4950–29,700 | 14,306 ± 7071 | 6100–20,100 | 11,907 ± 5015 | 15,900–64,600 | 37,500 ± 15,303 |
| Cl* | 43–275 | 98 ± 76 | 1360–14,000 | 6073 ± 4955 | 776–26,300 | 11,666 ± 10,132 | 643–42,100 | 16,927 ± 15,475 |
| K* | 11,800–25,500 | 18,600 ± 4530 | 20,200–64,000 | 33,725 ± 14,866 | 25,600–117,000 | 49,900 ± 29,717 | 33,400–64,000 | 49,050 ± 11,310 |
| Ca* | 633–2870 | 1885 ± 1052 | 6080–14,100 | 9248 ± 3228 | 3930–13,500 | 7517 ± 3604 | 18,600–35,900 | 26,050 ± 7336 |
| Sc* | 10–16 | 14 ± 2 | 0.3–2 | 0.7 ± 0.5 | 0.1–1 | 0.3 ± 0.2 | 0.1–0.8 | 0.3 ± 0.2 |
| Ti* | 3670–5620 | 4864 ± 623 | 49–259 | 186 ± 128 | 31–248 | 108 ± 66 | 61–172 | 114 ± 35 |
| V* | 80–149 | 104 ± 24 | 1–10 | 4 ± 3 | 0.6–6 | 2 ± 1.7 | 1–2 | 2 ± 1 |
| Cr* | 75–142 | 105 ± 20 | 3–12 | 7 ± 4 | 2–10 | 4 ± 3 | 2–8 | 4 ± 2 |
| Mn* | 503–1700 | 1047 ± 387 | 24–100 | 63 ± 28 | 17–91 | 46 ± 26 | 107–681 | 214 ± 190 |
| Fe* | 31,500–54,400 | 41,788 ± 7463 | 713–4490 | 1798 ± 1235 | 870–3660 | 1051 ± 1091 | 442–3390 | 1184 ± 951 |
| Co* | 15–23 | 20 ± 7 | 1–2 | 1 ± 0.5 | 0.5–1 | 0.7 ± 0.3 | 1–4 | 1.3 ± 1 |
| Ni* | 36–62 | 49 ± 8 | 2–6 | 5 ± 2 | 3–8 | 4 ± 2 | 2–10 | 6 ± 2 |
| Zn* | 58–124 | 88 ± 25 | 17–115 | 43 ± 32 | 26–155 | 55 ± 44 | 42–265 | 150 ± 71 |
| As* | 6–12 | 9 ± 2 | 0.2–1 | 0.4 ± 0.3 | 0.3–1 | 0.3 ± 0.2 | 0.2–1 | 0.4 ± 0.2 |
| Br* | 2–15 | 8 ± 4 | 5–40 | 21 ± 13 | 12–98 | 42 ± 33 | 25–129 | 62 ± 35 |
| Rb* | 89–171 | 134 ± 29 | 11–31 | 18 ± 8 | 8–56 | 20 ± 15 | 7–31 | 21 ± 10 |
| Sr* | 45–90 | 69 ± 14 | 54–146 | 91 ± 31 | 68–130 | 79 ± 26 | 130–221 | 173 ± 34 |
| Zr* | 160–170 | 225 ± 41 | 3–27 | 11 ± 8 | 7–17 | 7 ± 5 | 4–13 | 7 ± 3 |
| Sb* | 0.4–0.7 | 0.6 ± 0.1 | 0.003–0.2 | 0.07 ± 0.04 | 0.05–0.2 | 0.06 ± 0.07 | 0.05–0.2 | 0.1 ± 0.06 |
| Cs* | 4–9 | 7 ± 2 | 0.2–1 | 0.4 ± 0.2 | 0.2–0.4 | 0.2 ± 0.1 | 0.1–0.3 | 0.2 ± 0.1 |
| Ba* | 338–520 | 446 ± 73 | 54–126 | 78 ± 32 | 61–165 | 98 ± 44 | 107–241 | 159 ± 45 |
| La* | 32–44 | 41 ± 4 | 1–5 | 2 ± 1 | 1–3 | 1 ± 0.8 | 2–5 | 3 ± 1 |
| Ce* | 64–91 | 84 ± 9 | 2–10 | 4 ± 3 | 2–6 | 2 ± 1.7 | 2–7 | 3 ± 2 |
| Nd* | 23–42 | 34 ± 6 | 1–4 | 2 ± 1 | 1–2 | 1 ± 0.2 | 1–4 | 2 ± 1 |
| Sm* | 5–8 | 7 ± 1 | 0.1–1 | 0.4 ± 0.3 | 0.3–0.5 | 0.2 ± 0.1 | 0.3–0.8 | 0.4 ± 0.2 |
| Eu* | 0.7–1.4 | 1 ± 0.2 | 0.03–0.2 | 0.07 ± 0.05 | 0.04–0.1 | 0.04 ± 0.03 | 0.04–0.2 | 0.07 ± 0.04 |
| Tb* | 0.6–1 | 1 ± 0.2 | 0.02–0.1 | 0.05 ± 0.04 | 0.03–0.06 | 0.03 ± 0.02 | 0.02–0.1 | 0.04 ± 0.02 |
| Dy* | 3–7 | 5 ± 1 | 0.2–0.7 | 0.4 ± 0.2 | 0.2–0.4 | 0.3 ± 0.1 | 0.3–0.8 | 0.5 ± 0.2 |
| Yb* | 2–4 | 3 ± 0.4 | 0.07–0.5 | 0.2 ± 0.1 | 0.1–0.3 | 0.1 ± 0.07 | 0.3–0.8 | 0.1 ± 0.07 |
| Hf* | 6–9 | 8 ± 1 | 0.09–1 | 0.4 ± 0.3 | 0.3–0.6 | 0.24 ± 0.19 | 0.2–0.5 | 0.3 ± 0.1 |
| Ta* | 1–1.3 | 1 ± 0.1 | 0.02–0.1 | 0.06 ± 0.04 | 0.04–0.1 | 0.03 ± 0.03 | 0.01–0.07 | 0.03 ± 0.02 |
| W* | 1.8–2.3 | 2 ± 0.2 | 0.1–0.2 | 0.2 ± 0.06 | 0.2–0.4 | 0.2 ± 0.1 | 0.2–0.4 | 0.3 ± 0.08 |
| Th* | 15–18 | 16 ± 1 | 0.3–2 | 0.8 ± 0.6 | 0.5–1 | 0.4 ± 0.3 | 0.2–1 | 0.4 ± 0.3 |
| U* | 2.7–3.2 | 3 ± 0.2 | 0.05–0.4 | 0.2 ± 0.1 | 0.1–0.2 | 0.08 ± 0.06 | 0.04–0.2 | 0.1 ± 0.06 |
| Cd† | 0.02–0.08 | 0.05 ± 0.03 | 0.04–0.1 | 0.07 ± 0.03 | 0.03–0.2 | 0.09 ± 0.06 | 0.2–0.8 | 0.4 ± 0.2 |
| Cu† | 8–22 | 14 ± 5 | 5–12 | 8 ± 3 | 3–15 | 8 ± 5 | 7–43 | 19 ± 16 |
| Pb† | 9–13 | 11 ± 2 | 0.3–2 | 0.8 ± 0.5 | 0.3–2 | 0.7 ± 0.6 | 0.6–2 | 1 ± 0.5 |

*NAA data

†AAS data

## Discussion

Essential nutrients play a major role in the physiological developments of plants [35]. Elements that are naturally required in higher concentration (> 1 ppm) are considered macronutrients, while those elements normally required in lower concentration (< 1 ppm) are known as micro-elements [36]. N, K, Ca, Mg, P, S, and Si are classified as macronutrients and B, Na, Cl, Mn, Fe, Ni, Cu, Zn, and Mo are classified as micronutrients [37]. N and S play a key role in the plant growth and development. Both elements serve as major constituents of biological macromolecules and other co-enzymes. P helps in providing requisite structural integrity for the cellular membranes, DNA, and RNA; besides, it

is also involved in the covalent regulation of enzyme activities and energy transduction [37]. While Si is a non-essential yet beneficial element, it also serves as a part of defense mechanism(s) against microbial pathogens and other abiotic stress factors such as drought. In addition, Si as a cofactor is involved in photosynthesis, plant growth, sturdiness, and leaf transpiration. K, Ca, Mg, Na, and Cl in their ionic form are critical during the regulation of stomata, and cellular osmotic potentials. Zn is an essential micronutrient and it is involved in the chlorophyll synthesis and cellular signal transduction, as well as DNA transcription and gene expression. Zn as a Lewis acid acts as the metal cofactor for many enzymes, while acting as a structural cofactor of enzymes such as superoxide dismutase (SOD). In plants, SOD with metal cofactor functions as antioxidant enzyme to counteract reactive oxygen species (ROS).

Three major classes of SOD such as Fe SOD, present in the chloroplast, Mn SOD in the mitochondrial matrix, and Cu–Zn SOD in the chloroplast and cytosol have been studied in plants [37–39]. Mn, Fe, Ni, Cu, and Mo are the essential transition elements and can exist in more than one oxidation state and thus participate in a wide range of enzymatic redox reactions aiding the vital metabolic processes, while Fe and Cu also play a major role in electron transfer proteins. These micronutrient elements play important roles in diverse physiological processes such as photosynthesis and nitrogen fixation, in addition to various anabolic and catabolic processes. Indeed, Mg, Mn, Ca, and Cl play major roles, where Mg is the part of light harvesting pigment chlorophyll, while Mn and Ca are the metal cofactors of oxygen evolving complex with chloride ion as a cofactor assisting the proton transport during the evolution of oxygen by virtue of oxidation of water at the oxygen evolving complex [37–39]. Co functions as a metal cofactor in vitamin B12 albeit plants do not require Co for their metabolic processes. Similarly, elements such as Al, As, Cd, Cr, Se, Ba, and Pb can be accumulated and compartmentalized in plant tissues by sequestration in vacuoles, yet they are not known to be beneficial for plants, and therefore, they are considered non-essential elements. Tobacco is known to accumulate non-essential elements, mostly, in the aerial parts of the plant [37, 38].

According to Markert [40, 41], the elemental compositions naturally present in plants from uncontaminated environment could be considered a reference data, viz., "reference plant" (RP). Interestingly, plant species can be characterized by the elemental compositions corresponding to the accumulation or exclusion patterns of different elements in comparison with RP. Thus, RP values can be applied to assess the "chemical fingerprint" [41] for plantations grown in any kind of soil. Supplementary Table S5 presents the relative deviation in concentrations of elements in tobacco from the "reference plant." Fig. 1 depicts the relative mass fractions of various inorganic elements determined in NEI tobacco plant with respect to RP. Surprisingly, Mn, Cu, Sb, Cs, Rb, and Pb levels of NEI tobacco were very similar to the RP values, especially in the leaves. Moreover, relative mass fractions of Cd, Cl, Cu, Zn, and Mg were considerably higher in the leaves than in the roots when compared to RP

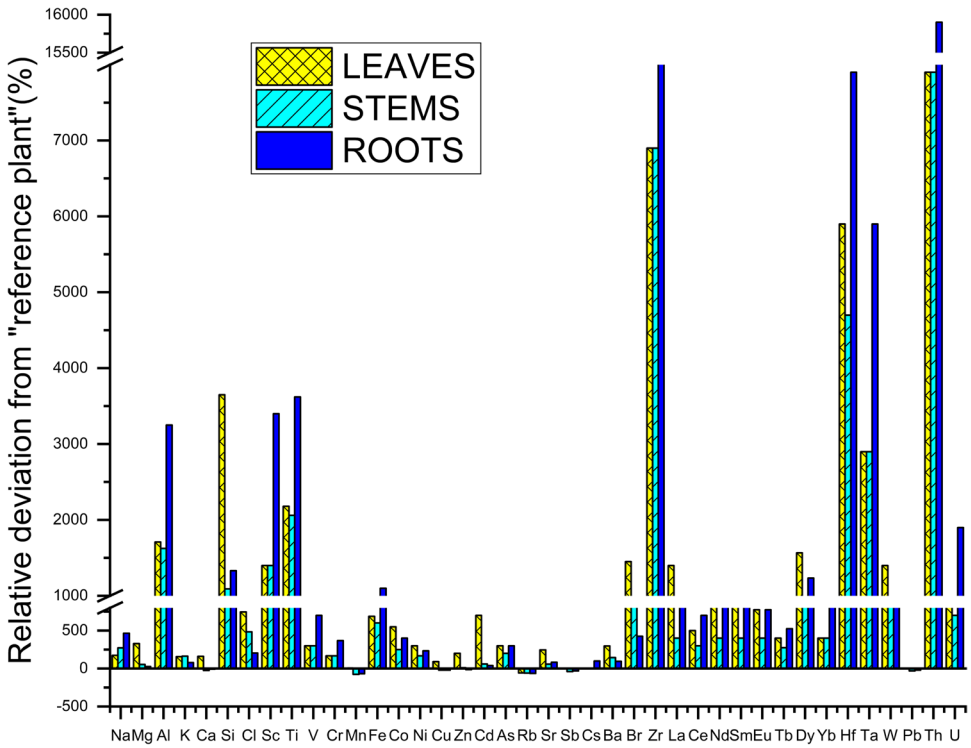


**Fig. 1** Indicator "fingerprints" of element concentration (mass fraction) in tobacco plants expressed as the relative deviation from the "Reference Plant" [40]. The figure is generated

and evidently the determined mass fractions of Cd and Cl in NEI tobacco leaves were at least 7-fold higher than in RP (Fig. 1). Few elements were exhibiting values below 0 (negative values) for the relative mass fraction in comparison with RP values. On the other hand, tobacco plant seems unable to restrict the uptake of higher levels of Al, Ti, Cr, Co, Ni, As, Ba, and Zr compared to RP which suggests that the analysis of tobacco leaves and roots may be applied to monitor the mobility of these elements in soil based on the edaphic conditions, accordingly. Besides, relative deviations in higher rare-earth element (HREE) mass fraction of tobacco plants in comparison with RP were exhibiting an interesting trend, viz., the relative mass fractions of HREEs in roots were about 2 times higher than those found in the leaves which are, on average, much higher than those found in RP (Fig. 1). However, the relative deviations in mass fraction of lower rare-earth elements (LREEs) were relatively higher in leaves and roots which are on average at least 5 times higher than RP values.

The present study showed the mean values for Cd, Ni, and Pb mass fractions in Mizoram soil do not exceed the permissible limits of agricultural soil of Indian Standards [11]. The mean values of Cd, Co, Cr, Cu, Ni, Pb, and Zn were within the limit of Indian natural soil background values (Table 2). These values were considerably less than the soil guideline values of Poland, whereas the mean values of Fe and Mn of Mizoram soil exceed Indian natural soil background limits [11]. In addition, the mean soil mass fractions of Cd, Co, Cu, Ni, Pb, and Zn were significantly less than the guideline values of natural soil stipulated by Canada, China, and the Czech Republic as well as the Swedish limits in polluted soil [8, 11]. It is apparent that acidic soil at tobacco cultivation sites in NEI was enriched with Fe and Al.

The determined elemental composition of soil samples depends on the prevailing natural geochemical background features of the soil in the region [24]. On the other hand, studies have shown that the heavy elements in soil are distributed in different chemical forms that can exhibit a variation in the degree of mobility and bioavailability [14]. They are essentially available in soil fractions as an inorganic form (soluble and insoluble forms and also present as exchangeable fractions adsorbed on the surface of mineralized metal oxides in soil). In addition, heavy elements can also bound to humic acids as the organometallic coordination complex forms exhibiting diverse dissolubility and mobility [9]. Tobacco can differentially uptake various heavy elements through roots and translocate and sequester them in leaves [3, 15]. Moreover, tobacco plant can absorb heavy elements, possibly either from the soil, or from amended fertilizers/manure products, or else from the applied pesticides/fungicides [6, 42]. Trace elements levels in tobacco can be higher when grown in soil containing high mass fractions of heavy elements or also possibly when grown in contaminated soil [7]. The uptake of heavy metals in tobacco plant is also the manifestation of agricultural practices such as topping and de-suckering for enhancing leaf attributes and nicotine yield, which enables the tobacco plant to absorb not only the nutrient elements but also the heavy elements, vigorously from the soil where it grows [12, 43].

The mass fraction ranges of most elements (in ppm) present in NEI tobacco leaves, viz., Sb, Cd, As, Th, V, Pb, and Co along with REEs, vary within a narrow range by a factor of $\leq 4.5$. Elemental mass fraction range of Cr, Ni, and Zr exhibits moderate variation. The mass fraction ranges of elements such as Cu, Br, Ti, Zn, and Mn display large variations whereas mass fractions of Al, Fe, and Mg show a greater dispersion of mass fractions. However, the range of mass fraction of Ca, K, Cl, and Si in NEI tobacco leaves varies substantially (Table 1).

Although the uptake of trace elements from soil and the concomitant accumulation in leaves by the tobacco plant are a well-known fact, however, the distribution of heavy elements varies among the leaves. Higher mass fractions of metal species were observed at first priming leaves than at other priming [12]. This variation in bioaccumulation of elements at different primings is indicative of the low mobility of elements through the stalk. In the present study, the determined elemental distributions of tobacco leaves are at higher mass fraction levels most likely due to its location (bottom) at the plant, which is consistent with the previous studies [12]. Bioaccumulation of the essential and non-essential elements in the plant tissues depends on the uptake capacity of plant species as well as the bioavailability of the metals [7, 8]. The bioavailability of various elements is

**Table 2** Comparison of heavy elements mass fractions in soils of the study area with permissible limits for Indian soils

| | Cr (mg/kg) | Mn (mg/kg) | Fe (mg/kg) | Co (mg/kg) | Ni (mg/kg) | Cu (mg/kg) | Zn (mg/kg) | Cd (mg/kg) | Pb (mg/kg) | References |
|---|---|---|---|---|---|---|---|---|---|---|
| Indian natural soil background | 114 | 209 | 32,015 | 15.2 | 27.7 | 56.5 | 22.1 | - | 13.1 | [11] |
| Indian limit for soil | - | - | - | - | 75–150 | 135–270 | 300–600 | 3–6 | 250–500 | |
| Minima (present study) | 75 | 503 | 31,500 | 15 | 36 | 8 | 58 | 0.02 | 9 | Present study |
| Maxima (present study) | 142 | 1700 | 54,400 | 23 | 62 | 22 | 124 | 0.08 | 13 | |
| Mean (present study) | 105 | 1047 | 41,788 | 20 | 49 | 14 | 88 | 0.05 | 11 | |

related to the physico-chemical and rhizosphere attributes of soil; concomitantly, the influence of soil properties on the heavy elements is also varied significantly between the sites [44]. Since, all elements detected in soil are also detected at various parts of tobacco plant which reflects the robust accumulation propensity of tobacco plant [3].

Bioconcentration factor (BCF) is the ability of a plant to enrich the elemental species in plant tissues by extracting from the soil and it is estimated as plant tissue-to-soil quotient. Similarly, the elemental translocation factor (TF) depends on the partition of elemental distributions between aerial plant tissues (leaf/stem) and in roots as it is calculated as the plant's shoot (leaf or stem)-to-root quotient [3, 15]. Owing to undulating hilly terrains in Mizoram, the negligible water stagnation and lower water holding capacity of soil also give rise to acidic soil conditions. As a consequence, acidic soil provides a conducive environment for the higher mobility of $Cl^-$ ions. The higher BCF and high TF of Cl suggest that $Cl^-$ ion can be absorbed and translocated by tobacco plant with great ease, in acidic soil [43]. All REEs have BCF values in leaves, stem, and roots < 0.1, which suggests that as a low accumulator of REEs, tobacco plant restricts the translocation of REEs into the aerial parts, significantly (Table 3). The lower TF value for REEs in acidic soil is also indicative of higher adsorption capacity of soil colloidal solutions [6, 14, 42]

Various factors such as edaphic characteristics, agro-climatic conditions, agricultural practices, and plant cultivars can modulate the efficacy of the absorption and translocation of heavy elements in tobacco plants [3, 12]. The analysis of the partitioning of heavy elements between plant tissues suggests that it is more likely that Cd is preferentially and effectively absorbed and translocated in leaves. However, it was reported that under soil acidification conditions, Zn was more efficiently and strongly mobilized than Cd or Cu in soil solutions. Nevertheless, in acidified soil with fertilizer amendments, Cd was absorbed and translocated in tobacco with better efficacy than Zn [7]. In contrast, when low pH soil was treated with fertilizers and subsequently tobacco was grown, it was found that similar to Cd, Zn was also exhibiting comparable BCF and TF values [3].

It is also noteworthy that for the accumulation of Cd, the absorption and translocation attributes of tobacco can vary significantly between plant varieties, viz., *N. rustica* and *N. rotundifolia* were primarily high root and low foliar accumulators, while *N. tabacum* (Burley and Kentucky cultivars) was shown to be a high leaf and moderate root accumulator [10]. When tobacco was grown in pots containing marginally alkaline soil amended with fertilizers and spiked with different concentrations of Cd solutions, it was demonstrated that in a concentration-dependent manner, Cd was selectively enriched than Zn, Cu, or Ni with higher Cd BCF values. However, beyond critical mass fraction, at higher contamination levels, Cd uptake was retarded [16]. Moreover, tobacco plant has a greater propensity for higher accumulation when exposed to higher heavy element mass fractions in contaminated soil, while when exposed to lower mass fraction levels of heavy elements present in fields, it exhibit relatively lower accumulation propensity [2].

**Table 3** Bioconcentration factors and translocation factors of ions in tobacco plant

| Elements | BCF (L) | TF (L) | BCF (ST) | TF (ST) | BCF (R) |
|---|---|---|---|---|---|
| Na | 0.1 | 0.5 | 0.14 | 0.7 | 0.2 |
| Mg | 0.4 | 3.4 | 0.14 | 1.2 | 0.1 |
| Al | 0.02 | 0.54 | 0.02 | 0.5 | 0.04 |
| Si | 0.1 | 2.6 | 0.04 | 0.8 | 0.04 |
| Cl | 172 | 2.8 | 119 | 1.9 | 62 |
| K | 2.6 | 1.5 | 2.7 | 1.5 | 1.8 |
| Ca | 14 | 2.9 | 4.0 | 0.8 | 5 |
| Sc | 0.03 | 0.5 | 0.03 | 0.50 | 0.05 |
| Ti | 0.02 | 0.61 | 0.02 | 0.6 | 0.04 |
| V | 0.02 | 0.54 | 0.02 | 0.53 | 0.04 |
| Cr | 0.04 | 0.64 | 0.04 | 0.6 | 0.06 |
| Mn | 0.2 | 3.4 | 0.04 | 0.7 | 0.06 |
| Fe | 0.03 | 0.7 | 0.03 | 0.60 | 0.04 |
| Co | 0.07 | 1.1 | 0.03 | 0.60 | 0.06 |
| Ni | 0.1 | 1.2 | 0.1 | 0.9 | 0.10 |
| Cu | 1.5 | 2.5 | 0.63 | 1.1 | 0.6 |
| Zn | 1.7 | 3.5 | 0.62 | 1.3 | 0.5 |
| As | 0.04 | 0.9 | 0.04 | 0.8 | 0.05 |
| Br | 8 | 2.9 | 5.5 | 2.0 | 2.8 |
| Rb | 0.2 | 1.2 | 0.15 | 1.1 | 0.13 |
| Sr | 2.5 | 1.9 | 1.2 | 0.9 | 1.3 |
| Zr | 0.03 | 0.64 | 0.03 | 0.6 | 0.05 |
| Sb | 0.2 | 1.4 | 0.11 | 0.9 | 0.13 |
| Cs | 0.02 | 0.5 | 0.03 | 0.50 | 0.05 |
| Ba | 0.4 | 2.1 | 0.22 | 1.3 | 0.2 |
| La | 0.1 | 1.3 | 0.03 | 0.55 | 0.06 |
| Ce | 0.04 | 0.80 | 0.03 | 0.53 | 0.05 |
| Nd | 0.05 | 1.10 | 0.03 | 0.65 | 0.05 |
| Sm | 0.06 | 1 | 0.03 | 0.55 | 0.06 |
| Eu | 0.06 | 1 | 0.03 | 0.56 | 0.06 |
| Tb | 0.05 | 0.8 | 0.03 | 0.6 | 0.06 |
| Dy | 0.10 | 1.3 | 0.07 | 0.9 | 0.07 |
| Yb | 0.03 | 0.5 | 0.04 | 0.56 | 0.06 |
| Hf | 0.04 | 0.7 | 0.03 | 0.55 | 0.06 |
| Ta | 0.03 | 0.5 | 0.03 | 0.52 | 0.05 |
| W | 0.14 | 1.9 | 0.10 | 1.4 | 0.07 |
| Th | 0.03 | 0.5 | 0.03 | 0.53 | 0.05 |
| U | 0.03 | 0.6 | 0.03 | 0.5 | 0.06 |
| Cd | 7 | 5.6 | 1.7 | 1.3 | 1.3 |
| Pb | 0.1 | 1.5 | 0.06 | 0.9 | 0.07 |

*BCF*, bioconcentration factor; *TF*, translocation factor; *L*, leaf; *ST*, stem; *R*, root

In the present study, under strongly acidic soil and natural rhizosphere conditions, without any form of soil amendments as practiced during shifting cultivation, it was found that in soil, Cd and Zn mass fractions were lower compared with those levels present in plant tissues reflecting tobacco plants' ability to absorb, translocate, and sequester nutrient and non-essential elements, respectively. Nevertheless, in the present study, the mean BCF and TF of Cd in tobacco leaves were considerably higher than those values of Zn, reflecting tobacco plants' selective bioaccumulative capacity that is commensurate with previous studies [15, 16]. Alternatively, the BCF and TF of Cd were calculated based on the mass fraction of the extractable form of Cd arising from soil and biomatrices using the AAS method. However, BCF and TF of Zn (also Ni) were calculated based on the total elemental concentration (soluble and insoluble forms) arising from soil and biomatrices of tobacco plant. This variation in the measured Cd and Zn physico-chemical forms may have contributed to the proportionate reduction in the observed BCF and TF values of Zn. Nevertheless, in acidic soil, the mobility of heavy elements is significantly higher which may have resulted in better uptake and bioaccumulation of heavy elements in the tobacco plant [12, 13].

Unlike tea plant (*Camellia sinensis*), tobacco plant is not considered an aluminum accumulator as excess soluble aluminum normally present in acidic soil often restricts plant growth since aluminum at high mass fraction interferes with calcium homeostasis [45]. There are few plants which normally grow in acidic soil and are known to tolerate aluminum with the development of intrinsic cellular tolerance [46]. In the present study, the calculated BCF ($0.02_{Leaf}$; $0.02_{Stem}$; $0.04_{Root}$) and TF ($0.54_{Leaf}$; $0.52_{Stem}$) values for Al suggest that tobacco possibly exhibits an exclusion mechanism with the sequestration of higher aluminum ($Al^{3+}$) ion mass fraction at the roots. Besides, more akin to Bangladeshi tobacco (2.7–3.7 mg/kg) [47], the tobacco leaves also contained comparable Al mass fractions (0.6–3.14 mg/kg). However, the As mass fraction in the tobacco plant is comparably distributed in leaves ($0.4_{mean}$), stem ($0.3_{mean}$), and roots ($0.4_{mean}$) with moderate TF values ($0.9_{Leaf}$; $0.8_{Stem}$). The arsenic mass fraction in tobacco plants is normally $\leq 3$ mg/kg, when it is grown on soil that are not contaminated with arsenic compounds [9, 48] that is consistent with the observed As mass fraction in NEI tobacco. Importantly, acidic soil is rich in Fe and Mn where the mobility and bioavailability of As can be considerably attenuated due to the prevailing soil colloidal solution conditions [48], which may have caused relatively lesser accumulation of As species in the tobacco plant as observed in this study.

Table 4 presents the comparison between elemental distributions of processed tobacco or tobacco filler of smoking tobacco products (cigarettes or bidis) available in countries of Asia, Africa, Europe, North America, and South America. The NEI tobacco leaves are substantially enriched with Mg, K, Ca, and Cl mass fractions as these essential minerals' mass fraction ranges are varying by two orders of magnitude or even more. It is a well-known fact that although the bioavailability of essential plant nutrients such as Ca, Mg, and Mo would decline when the pH of soil decreases below neutral pH conditions [49], nevertheless, higher Cl absorption by tobacco plants in acidic soil would also stimulate higher absorption and translocation of Mg and Ca [7, 43]. Importantly, when K mass fraction is higher in the tobacco leaf, most likely $K^+$ ion favorably interacts with organic acids such as malic acid, citric acid, and oxalic acid, eliciting better combustion attributes of processed tobacco matter since organic acids concurrently with $K^+$ cation aid favorable burning conditions of tobacco matter [43]. However, higher $Cl^-$ and $Br^-$ mass fraction would facilitate most probably electrostatic interaction of inorganic anions with $K^+$ ion, which is considered unfavorable interactions. These unfavorable inorganic interactions would render undesirable poor burning qualities to tobacco leaves as less $K^+$ ions would be available for interactions with organic acids. Besides, $Cl^-$ would also retain higher moisture content in tobacco leaf matter [43, 50].

The mass fraction range of Cr (2–8 mg/kg) is higher than previously reported for Indian cigarettes, while it is comparable to the mass fraction levels of Indian bidis, Pakistan cigarettes, and Turkish oriental tobacco. However, it is 3.5 times lesser than in Algerian tobacco and 8 times lesser than in Brazilian tobaccos. Although the mass fraction range of As (0.2–1 mg/kg) is comparatively higher than previously reported for Indian cigarettes and bidis, yet it is similar to that found in the UK and US cigarettes, whereas it is significantly lower than that found in Pakistan tobacco products and Chinese cigarettes. Cu mass fraction (7–43 mg/kg) in NEI tobacco leaves was found to be about 2 times higher than that in Indian cigarettes and about 11 times higher than that in US cigarettes, albeit about 7 times lesser than that found in Ghanian tobacco. The determined mass fraction levels of Co (1–4 mg/kg) and Ni (2–10 mg/kg) in tobacco samples were comparatively higher than those ranges reported in the literature. Ni levels were found to be 10 times greater than those in US cigarettes, 4 times higher than those in UK cigarettes, and 2 times higher than those in Chinese cigarettes as well, albeit the observed Ni mass fraction was comparable to those levels found in Indian smoking tobacco products. In the case of Co, it is about 2 times higher than that in Brazilian tobacco and 10 times greater than that in Swiss tobacco, albeit comparable to Co mass fraction found in Chinese cigarettes.

The mass fraction range of Cd is similar to those levels found in Indian bidis and Pakistan bidis as well as Indian and UK cigarettes. However, determined Cd levels were 2 times lower than those in Swiss tobacco products, besides 7 times

**Table 4** Range of element concentrations (mass fractions) in tobacco available at different countries

| Elements | India | | | Pakistan | | Turkey | China | Brazil | Algeria | Ghana | UK | Poland | Switzer-land | USA | Mexico |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Northeast India orien-tal Toba | Beedi | Ciga | Beedi | Ciga | Oriental Toba | Ciga | Brazilian Toba | Toba | Toba. Leaf | Ciga | Ciga | Raw Toba | Ciga | Ciga |
| | | (mg/kg) | (mg/kg) | (mg/kg) | (mg/kg) | (mg/kg) | (mg/kg) | (mg/kg) | (mg/kg) | (mg/kg) | (mg/kg) | (mg/kg) | (mg/kg) | (mg/kg) | (mg/kg) |
| Cl | 643–42,100 | - | - | - | - | - | - | - | - | 5448–7287 | - | 3700–4684 | 3600–16,400 | 2900–6900 | 1500–7360 |
| K | 33,400–64,000 | 6170–9740 | 21,234–25,479 | - | 1300–3700 | 23,000–31,000 | - | - | 29,200–37,500 | 24,014–41,518 | - | 28,717–38,142 | 17,000–62,000 | 17,300–29,800 | 18,300–40,300 |
| Ca | 18,600–35,900 | 31,800–40,200 | 19,392–24,459 | - | - | - | - | - | 15,000–37,700 | 14,137–18,769 | - | 21,148–25,603 | 15,000–40,000 | 13,900–19,600 | - |
| Mn | 107–681 | < 0.001–120 | 81.3–189.4 | 53–140 | 93–165 | 58–73 | - | - | - | 491–663 | 151–174 | 167–246 | 40–790 | 118–142 | 108–244 |
| Fe | 442–3390 | 1140–3370 | 325–556 | 440–2600 | 712–2668 | 900–1200 | - | 520–1180 | 384–823 | 1572–2902 | 264–576 | 464–679 | - | 325–520 | 420–680 |
| Cu | 7–43 | 13.4–19.6 | 9.01–19.2 | 11–14 | 7.3–27 | 0.8–1.5 | - | - | - | 112–297 | 11.7–16.2 | 13–18 | 8–23 | 3.5–4 | - |
| Zn | 42–265 | 18–25 | 26–35.4 | 31–46 | 8.1–21.5 | 15–54 | - | 31–44 | 28–68 | - | 26–40 | 38–52 | 30–79 | 15–18 | 14–56 |
| Al | 599–3140 | - | - | 360–2100 | 360–1420 | - | - | - | - | 774–913 | - | - | 200–2300 | 699–1200 | 237–1106 |
| Cr | 2–8 | 4.3–6.8 | 2.8–5 | 1.7–5.4 | 2.9–6.2 | 4.4–10.3 | 0–1 | 6.0–61 | 2.4–29 | 0.32–0.7 | 1.3–3.1 | < 0.001–3.5 | - | 0.5–1.3 | 0.8–2.4 |
| Co | 1–4 | 0.2–0.9 | 0.3–2.2 | 0.5–1.3 | 0.41–1.8 | 0.6–0.9 | 3.3–3.9 | 0.7–2 | 0.5–0.8 | 0.9–1.6 | - | - | ~ 0.3 | < 0.01–0.9 | 0.3–0.6 |
| Ni | 2–10 | 2.8–11.8 | 7.2–10.2 | 3.6–4.5 | 2.2–5.1 | 1.6–6.7 | 4.2–4.8 | 0.6–2.5 | - | - | 1.1–2.7 | 1.4–3.2 | - | 1.1–1.2 | - |
| As | 0.2–1 | 0.1–0.2 | 0.04–0.15 | 0.7–1.5 | 0.25–2.7 | 0.7–1.6 | 0.3–3.3 | 0.05–0.13 | 2.2–6.4 | 0.1–0.3 | 0.2–0.7 | 0.3–3.3 | 0.3–0.7 | 0.25–0.25 | 0.5–3.2 |
| Sb | 0.05–0.2 | 0.1–0.3 | 0.5–0.6 | - | 0.03–0.22 | 0.05–0.2 | - | 0.01–0.15 | 0.04–0.35 | 0.01–0.04 | - | - | 0.05–0.3 | 0.04–0.05 | 0.06–0.43 |
| Cd | 0.2–0.8 | 0.4–0.7 | 0.5–0.7 | 0.27–0.5 | 0.3–1.8 | 0.4–1.6 | 2.0–5.4 | - | - | 0.3–0.7 | 0.5–0.8 | - | 1.1–2 | 0.017–0.017 | 0.2–3.07 |
| Pb | 0.6–2 | 2.6–3.8 | 0.2–7.4 | 0.8–2.0 | 0.6–1.9 | 2.6–4.5 | 1.2–6.5 | 0.2–0.4 | - | - | 0.4–0.9 | 0.31–41 | - | 0.604–0.607 | - |
| Hg | < 0.001 | 0.11–0.16 | 0.007–0.33 | - | 0.02–0.08 | 0.02–0.08 | 0.14–0.15 | - | - | 0.001–0.03 | - | - | 0.02–0.06 | 0.02–0.021 | - |
| References | Present study | [22, 62, 63] | [22, 57, 62] | [58] | [58, 64] | [65, 66] | [59, 67] | [20, 68] | [69] | [70] | [61] | [20] | [71] | [21, 72] | [73, 74] |

Note: < 0.001—below detection limit

lower than those in Chinese cigarettes. The mass fraction of Pb determined in the present study is comparable to Pakistan cigarettes and Turkish oriental tobacco, while comparatively lower than found in Chinese (3 times) and Indian (3.5 times) cigarettes, whereas it is 19.5 times lower than that in Polish cigarettes. By virtue of chronic tobacco use, the persistent exposure to Cd and Pb entails detrimental effects on human health as the IARC has classified Cd as a group 1 carcinogen and Pb has been elevated from the category 2B to 2A [51, 52]. Besides, the exposure to Pb also imposes significant risks for developing impaired reproductive capacity [53].

The determined Sb (0.05–0.2 mg/kg) mass fraction level was similar to those ranges found in the literature. The mass fraction of Mn (107–681 mg/kg), Fe (442–3390 mg/kg), Al (599–3140 mg/kg) and Cl (643–42,100 mg/kg) was considerably higher than the mass fraction range found in the literature; however, the determined Mn mass fraction is comparable to the mass fraction of Mn found in Ghanaian and Swiss tobaccos. In fact, the determined Zn (42–265 mg/kg) mass fraction was substantially higher than the previously reported literature values. Considerably higher mass fraction of Zn, Mn, Al, and Fe observed in tobaccos was most likely caused by the edaphic and biogeochemical conditions of soil in Mizoram's undulating hilly terrain [24].

The observed Ti (61–172 mg/kg) and Si (15,900–64,600 mg/kg) mass fractions along with higher mass fraction of Al (599–3140 mg/kg) in tobacco leaves are significant as these elements are relatively non-volatile yet they can be converted as ultrafine nano-sized particulate species in the tobacco smoke, which can elicit adverse pulmonary inflammatory reactions [18]. When tobacco filler is smoked as a nonfilter hand-rolled cigarette, the smoker could be taking more frequent and stronger puffs, more akin to Indian bidis, in order to keep the hand-rolled cigarette stick alighted. This vigorous and frequent puffing regime by zozial smokers may deposit substantial quantities of ultrafine nano-sized aluminum silicates, silica, and titanium (IV) oxide particles into the alveoli. Concomitantly, this deposition induces a significant pulmonary inflammation response in alveolar tissues. Al, an element with no known physiological function, however is known for its neurotoxic effects, in addition to its inimical effects on bone tissue, kidney, and liver [54]. Exposure to REEs, perhaps, through tobacco smoke may further exacerbate the human health [6, 42].

Similar to Turkish tobaccos, NEI tobacco samples also have low nicotine content [55]. However, NEI tobacco also has poor combustion quality, due to high chlorine and moisture content [43, 50]. In addition, as a cost-effective measure, cheap papers are normally used to pack the tobacco filler for "zozial," which most probably lack the additives such as citrate salts for facilitating the combustion process. Consequently, a zozial stick would self-extinguish if not puffed frequently and intensively; as a result, often, it may also require re-lighting [56]. Considerable proportions of heavy elemental species in tobacco can be volatilized. By virtue of the intense and frequent puffing, zozial smokers may get potentially higher loads of volatilized toxic metal species as well as ultrafine aluminum silicate, silica, and titanium oxide nano-sized nano particulate species which are most likely get deposited in their alveolar tissues [18]. The exposure to tobacco smoke entails respiratory ailments not only to the smokers, but also to the non-smoking "passive" smokers. Heavy element species like As, Cd, Cr, Ni, Pb, and Hg may also accumulate in tissues and bio-fluids through exposure to tobacco smoke [18, 51]. Beyond critical concentration levels, bio-accumulated heavy elements in human body entail adverse health effects.

Furthermore, in this study, the PCA method enabled us to evaluate the variations in element concentration of NEI tobacco, in comparison with the published data on different varieties of tobaccos [23, 26, 57–61]. To avoid bias, the element concentration of tobacco samples was randomly chosen to represent different regions of the world. In addition, without too much detail, to obtain a simplified view of relationships between the variables, several iterations were performed by using various subsets of different elements [54, 57].

For the first set of data comprising 21 tobacco samples and 17 elements (K, Ca, Na, Fe, Zn, Ce, Cr, Br, Sr, Rb, Sc, As, Sb, La, Ce, Hf, Th), PCA iterations were performed. The first principal component (PC1) represented 25.8%, while the second principal component (PC2) explained 21% of the total variance. Loadings are represented by vectors which spread out from the origin and vector lengths signify concomitant influence on variance in mass fractions of elements [23]. Due to REEs, K, and Zn, NEI tobaccos are separated from Indian bidis as well as African (Algeria and Egypt) tobacco samples. Indian bidi tobacco is strongly associated with Ca, Na, and Sb. African tobacco samples are strongly related with As and Na. Moreover, in the bi-plot, Br and Cr exhibit relatively low variation in all the samples (Supplementary Fig. S1a).

Furthermore, 41 samples and 13 elements (K, Mg, Cl, Na, Fe, Zn, Mn, V, Sr, Br, Sc, Co, Al) were considered for another subset of PCA iterations. The more dominant component (PC1) depicts 38% of the total variance, while the second component explains 14.5% of the total variance. NEI tobaccos are strongly associated with Al, Mg, Mn, and Zn as they are clearly discerned from the Syrian tobacco and Egyptian cigarettes (Supplementary Fig. S2a). Russian Papirosi cigarette is strongly influenced by Na, and it is weakly influenced by K, and Cl, along the PC2. It is also clearly differentiated from the Egyptian cigarettes and Syrian tobacco. Besides, Syrian tobacco are also strongly influenced by Br, and weakly related to Al, Mg, and Mn. Egyptians cigarettes

(local and imported) are located very close to the origin, very weakly influenced by the loadings, and, therefore, clustered tightly.

Analogous results were obtained with the application of the PCA method, when 8 variables (Fe, Zn, Cu, Mn, Ni, Cr, Cd, and Pb) and 140 samples, including standard tobacco samples' elemental data set, were also used. The two principal components cumulatively explained 54.2% of the variance within the metal mass fractions. The PC1 contains a higher percentage of variances (29.3%), while the PC2 explains (24.9%) of total variance. Fe, Cu, and Ni vectors cluster more tightly together along the negative side of PC1 exerting a strong influence on NEI tobacco. Different brands of Indian cigarettes are rather confined to a narrow region in the exploratory bi-plot. The modest influence of heavy elements on Indian cigarettes suggests that different brands of Indian cigarettes consist of similar element mass fractions. PC2 can separate illicit cigarettes of UK and Pakistani tobacco products (Fig. 2). In addition, Pakistani cigarettes are interspersed with Turkish cigarettes. Moreover, Turkish cigarettes are interspersed with UK cigarettes and tobacco products imported in Pakistan.

More akin to UK cigarettes, different brands of Indian cigarettes, available in both Indian and Turkish markets, are closely clustered in the bi-plot within a smaller area near the origin, most likely due to the use of high-quality FCV tobacco. UK cigarettes and Indian cigarettes are also subtly discerned, along PC2. Mainly accounted by Mn, Cd, and Pb, illicit cigarettes seized in the UK are also scattered, so widely and extensively in the bi-plot in accordance with the previous reports [57]. This is a consequence of large variations in metal mass fractions found in those illicit cigarettes, more likely blending different types of tobacco, perhaps to obscure the originating region [61].

Hierarchical cluster analysis (HCA) was applied, using *k*-means method, to further confirm the clustering pattern obtained using the PCA method. While comparing published literature data on different types of tobaccos, an exploratory evaluation of similarity between their element mass

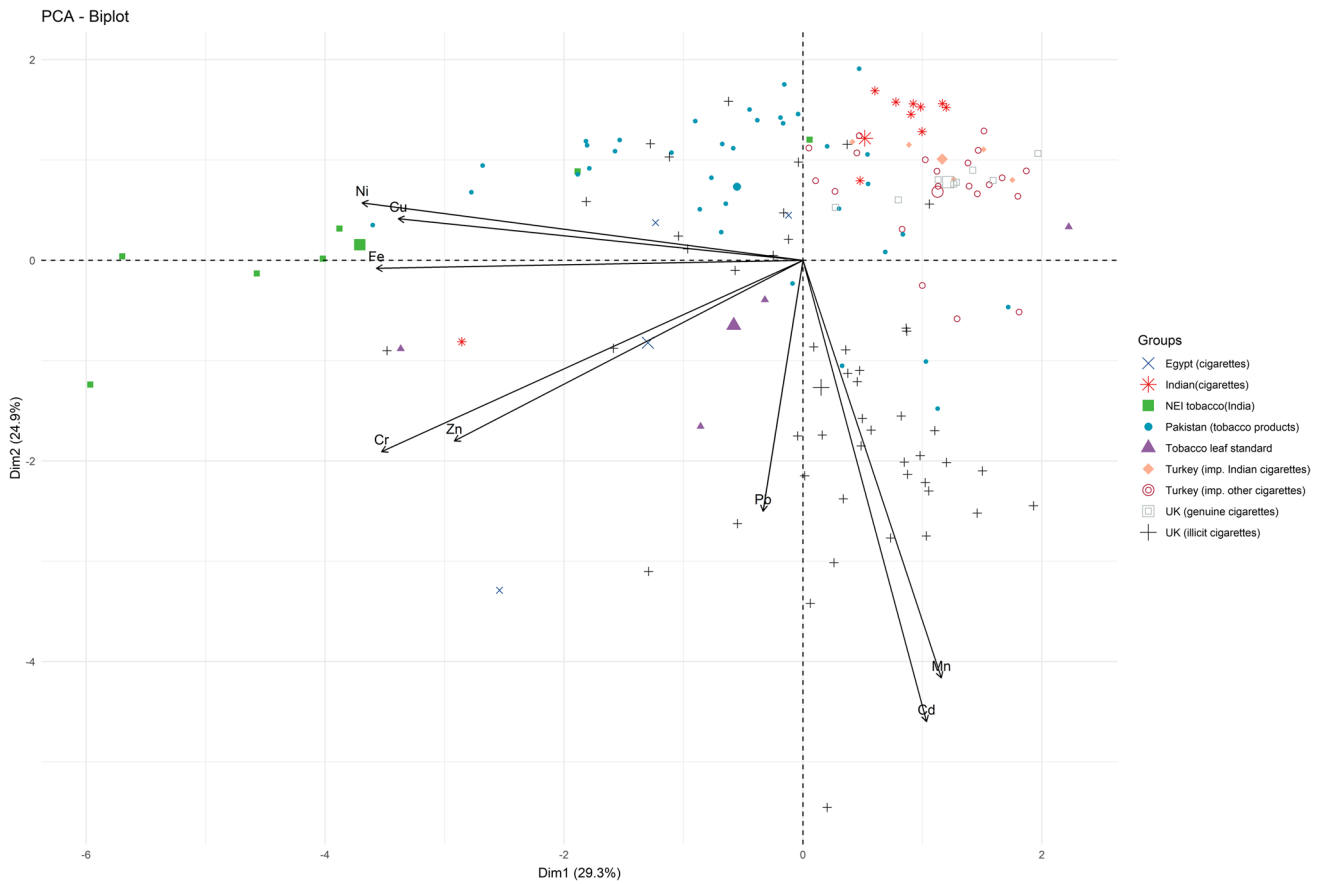


**Fig. 2** Bi-plots of heavy element distributions in tobacco samples of different countries; tobacco samples of NEI (zozial), India (cigarettes), Pakistan (tobacco products), Egypt (cigarettes), Turkey (imported cigarettes from India and others), and UK (genuine and illicit cigarettes); and tobacco leaf standards (oriental and Virginia). The figure and supplementary Figures Fig S1a–Fig S3b are generated using "factoextra" package in the R ver. 4.0.3

fractions was performed [33]. The dendrogram shows that Virginia and oriental tobacco formed two major groups. Despite being a type of oriental tobacco, albeit NEI tobaccos exhibit subtle dissimilarity (Supplementary Fig. S1b), they have been sub-clustered into two varieties of oriental tobacco, viz., oriental tobacco (OTL) and oriental Bassma tobacco (OBTL). These results commensurate with the *k*-means cluster plot, where tobacco samples with similar elemental compositions are grouped into a cluster (Supplementary Fig. S1c).

Thus, the results obtained using HCA confirm the discrimination of tobacco samples according to the type of tobacco and according to their geographical origin. It should be noted that some overlap of sub-clusters can be attributed to the similarities in the mass fractions of macro- and micronutrient elements of the tobacco samples as seen in the tobacco samples of Syria (Supplementary Fig. S2b, and S2c). Interestingly, Indian cigarettes are distributed within a smaller sub-clustering suggesting a more homogeneous distribution of elements at moderate mass fractions, more akin to UK cigarettes, along with standard Polish Virginia tobacco (Fig. S3a and S3b). A similar clustering tendency was also observed for Turkish cigarette samples besides standard Virginia tobacco. However, Pakistani cigarettes are apparently clustered between OTL and VTL. Illicit cigarettes seized in the UK are interspersed with many sub-groups belonging to both Virginia tobacco and oriental tobacco, illustrating the large variations in their element compositions leading to the observed heterogeneity of these cigarettes.

## Conclusion

In summary, under acidic soil conditions and in natural settings, Cd is more selectively absorbed and accumulated than Zn, and Cu, by tobacco plant. In addition, it also exhibited lower translocation propensity for Fe, Al, and Mn and extremely lower translocation potential for the REEs. NEI tobacco also possessed relatively lesser As content which reflects the lower bio-available forms of As in the soil solutions, as the manifestations of higher Fe and Mn mass fractions in acidic soils. NEI tobacco can accumulate higher levels of K and significant levels of Cl. The mass fractions of Mn, Cu, Sb, Cs, Rb, and Pb are very similar to the "reference plant," whereas significantly higher mass fractions of Al, Sc, Ti, Zr, Hf, Ta, Th, and U are present in the roots of tobacco plant relative to the "reference plant." Range of elemental mass fractions in NEI tobacco is comparable to the range of reported values for other internationally available tobacco, except for Zn, Al, Fe, and Cl. By virtue of element compositions, NEI tobacco is also clearly differentiated from other varieties of tobaccos. It is apparent that the geographical identification of tobacco samples may be feasible with the application of pattern recognition methods such as HCA and PCA.

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# Macro-, micro-, and trace element distributions in areca nut, husk, and soil of northeast India

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**Abstract** In areca nut and husk, 14 elements (As, Ca, Cd, Cl, Co, Cu, K, Mg, Mn, Na, Rb, Sb, and Zn) were determined, while 34 elements including rare earth elements were detected in the corresponding soil samples using instrumental neutron activation analysis and atomic absorption spectrometry methods, whereas the concentration levels of Hg in tested samples are negligible, perhaps, below the detection limits. No rare earth elements were detected in edible areca nut. The concentration levels of various essential elements and heavy elements such as As, Cd, and Cu present in areca nut are within the permissible levels, whereas Pb content is relatively higher than FAO/WHO's permissible levels. The order of bioaccumulation index for heavy metals in areca nut was Cd > Sb > Cu > Zn ≥ Mn ≥ Co > Pb ≥ As. Bioaccumulation index values are indicating that areca palm may not be able to accumulate other heavy elements in the edible areca nut, except for Cd. On the basis of pollution indices, Northeast Indian soil may be relatively unpolluted.

**Keywords** Areca nut · Bioaccumulation · Pollution index · Heavy elements · Rare earth elements

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## Introduction

Areca palm (Family Arcaceae) grows normally in the tropical region of Asia and Austronesian islands, with the soil pH ranging from 4.5 to 8.5, over diverse landscape covering a wide range of agroclimatic conditions (Peng et al. 2015; Srimany et al. 2016; Papke et al. 2020). In South Asia, betel quid combined with smokeless tobacco products or consumption of betel quid or chewing only the areca nut (either processed mature, flavoured and granulated, or unprocessed mature and fresh moist) is a cultural practice (Gupta and Ray 2004).

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It is basically consumed by the populace for its' milder stimulatory action as a masticatory substance (Papke et al. 2020; Gupta and Ray 2004). Especially in north-east India (NEI), virtually 60–65% of the population, on a daily basis, consume mature fresh areca nuts (2 areca nuts on average, whereas some 'addicts' consume 5–6 areca nuts on average).

Since lifestyle products are very frequently consumed on daily basis, information on trace element distribution is of utmost importance in evaluating ingestion of essential macro- and microelements as well as in assessing the potential health risks arising from exposure to inessential toxic elements in lifestyle products. Chronic areca nut chewing or betel quid chewing tobacco else betel quid chewing without tobacco is classified (group 1) as carcinogenic mainly on the basis of the carcinogenicity of areca alkaloids (IARC 2004). It is currently unknown if the adverse health effects observed in NEI populace, who consume tobacco products containing trace element species, may be further exacerbated by betel quid/areca nut chewing. Nutritionally essential element content may modulate the health risks arising from exposure to inessential toxic metals as few of these inessential elements exert toxic effects even at very low concentration levels (Al-Rmalli et al. 2011; Gupta et al. 2010; Margiloth et al. 1983).

Heavy metal species ingestion is detrimental to human health. Some of the lifestyle disorders including cancers and diabetes have been linked to elevated exposure to certain toxic elements (Hafeman et al. 2006). Most of the countries impose restrictions on the concentration levels of trace elements such as cadmium (Cd), lead (Pb), and arsenic (As) in food and beverages; however, no such restrictions are imposed for lifestyle products. It is pertinent to determine the elemental composition in the lifestyle products in order to understand their putative bioactivities besides to gain an insight into the impact of the environment on variations in their elemental concentration levels. On the other hand, the elemental distributions in plants depend on many factors, viz., species and maturity of the plant, the oxidation state of an element available in soil, edaphic factors, as well as the nature of agricultural practices of the region where the plant species is cultivated. Indeed, the potency of lifestyle products may be determined by the synergetic actions of organic and inorganic components (Khan et al. 2015) whose concentration levels is modulated by the agroclimatic conditions of the region where it was cultivated (Peng et al. 2015; Barbosa et al. 2014; Gupta et al. 2010; Özcan et al. 2019).

Not all elements present in soil may be transferred to plant, and those elements which are not detected in plant matrix may be considered as non-mobile with respect to bioavailability (Jin et al. 2005; Zeng et al. 2011). It is important to note that mineral macronutrient content, as well as micronutrient distribution, and the inessential trace element composition are influenced by regional environmental factors such as agroclimatic conditions, and soil chemistry and regional edaphic factors albeit anthropological activities including agricultural practices also have a significant influence on the concentrations of some elements (Gupta et al. 2010; Olafisoye et al. 2017). It is also important to note that analytical techniques such as atomic spectrometric methods, X-ray fluorescence methods, and nuclear analytical methods can be employed to authenticate the type of agricultural practices followed for the production of lifestyle products (Mishra et al. 1986; Stephens et al. 2005; Haidu et al. 2017; Valentin and Watling 2013; Pepi and Vaccaro 2018; Özcan et al. 2019).

Previous studies on the distribution of elements in raw or processed areca nut are rather scarce (Zaidi et al. 2002). Most of the earlier studies were focussing on the quantitation of few specific elements such as Cu, As, Cd, and Pb either in processed areca nut or components of betel quid containing areca nut and tobacco using induction coupled plasma-atomic emission spectrometry (ICP-AES) methods. Trace elements As, Pb, and Cd in areca nut may be possibly exerting health risks (Al-Rmalli et al. 2011), whereas Cu present in areca nut may be putatively stimulating collagen synthesis that elicits oral submucous fibrosis due to chronic betel quid chewing (Raja et al. 2007; Alexander et al. 2019; Khan et al. 2015). Besides, trace elements can induce and exacerbate the malignancy of head and neck, gastrointestinal, and cervical cancers (Gupta et al. 1993; Margiloth et al. 1983). Therefore, good analytical measurements of mineral contents are always required on a periodic basis to assess lifestyle products for evaluating any increase or deficiency beyond the critical limits in the amount of macro-, micro-, and trace elements which may cause adverse physiological disturbances in plants as well as humans.

Although many advanced instrumental techniques can be used for the evaluation of the elemental profile in various lifestyle products, instrumental neutron activation analysis (INAA) of lifestyle products allows for the simultaneous determination of multiple elements with accurate quantitation, while it is relatively free from

matrix interferences. When combined with atomic spectrometric methods such as atomic absorption spectrometry (AAS) studies, these quantitative measurements can provide crucial information on the elemental contents in various life style products (Kumara et al. 2005; Monged 2016; Zinicovcaia 2019, 2020a, b). In this study, INAA and AAS methods were employed as reliable, robust, and sensitive analytical tools, for an initial determination of bioavailability of essential mineral elements and possible inessential trace elemental species, simultaneously, in the lifestyle product, unprocessed "fresh and moist" areca nut (endosperm) as well as areca palm husk (pericarp) obtained from the mature ripened areca palm fruit, and the elemental distributions in the corresponding soil samples of areca palm plantations in NEI. It is pertinent to note that no published research work, to the best of our knowledge, on the mineral and inessential elemental distributions at different parts of the areca palm fruit (endosperm and pericarp) has been published yet. In addition, a database has been developed for the relative elemental distribution in monsoon-rain leached acidic soils of NEI, as this preliminary soil survey work may also shed light on the total elemental contents in the soils of NEI.

## Materials and methods

### Sample collection

Samples for analyses were collected during June–July 2019. Soil (15–30 cm in depth) was collected at 6 different directions in very close proximity to the areca palm tree located at selected small household plantations of two states in NEI, viz., villages within Mizoram (5 soil samples), Tripura-Mizoram border village (1 soil sample), and villages in Assam (2 soil samples). From each location, approximately 15 kg soil samples were collected at each plantation site. The collected soil samples were sieved to remove debris and packed using fresh and clean polyethylene bags. Approximately 30 fresh and ripened areca palm fruits were collected from every plantation site where soil samples were obtained. Each areca palm fruits were dehusked using artisanal clippers to extract the husk and nut for the subsequent elemental analysis. Areca nuts and husk samples were packed separately using clean polyethylene bags. All samples were stored in clean and dry area away from direct sunlight prior to the analysis.

### Soil analysis

Soil pH and the SOM content were measured as described (Baruah et al. 2013).

### Neutron activation analysis

For neutron activation analysis (NAA), samples were dried at 40 °C to obtain constant weight and then homogenized. About 0.3 g of husk and nut samples and 0.1 g of soil samples were contained in polyethylene foil bags for short-term irradiation and in aluminium cups for long-term irradiation. Subsequently, sample irradiation was carried out at the installation REGATA of the pulsed fast reactor IBR-2 of the Frank Laboratory of Neutron Physics (JINR, Dubna, Russia). To determine elements with short-lived isotopes (Mg, Al, Si, Cl, Ti, V, Mn, and Dy), soil samples were irradiated for 1 min, while husk and nuts samples for 3 min and concomitantly measured for 15 min. To determine elements with long lived isotopes: Na, K, Sc, Fe, Co, Zn, Ga, As, Rb, Sr, Zr, Sb, Cs, Ba, La, Ce, Sm, Tb, Hf, Ta, W, Th, and U, the cadmium-screened channel 1 was used. Samples were irradiated for 4 days, re-packed, and measured twice using HP germanium detectors after 4 and 20 days of decay, respectively.

The NAA data processing and determination of element concentrations were performed using software developed in JINR.

The quality control of NAA results was ensured by simultaneous analysis of the examined samples and standard reference materials: NIST 1633b (Constituent Elements in Coal Fly Ash), NIST 2709 (San Joaquin Soil), NIST 1547 (Peach Leaves), NIST 1575a (Trace Elements in Pine Needles), and NIST 1632c (Trace Elements in Coal). The measured concentrations were in good agreement with the recommended values.

### Atomic absorption spectrometry

For AAS analysis, 0.2 g of samples were placed in a Teflon vessel and treated with 3 mL of concentrated $HNO_3$ and 1 mL of $H_2O_2$ (husk and nut) and 8 mL of $HNO_3$ and 2 ml of HF (soil samples). Teflon vessels were introduced in a microwave digestion system (Mars; CEM, USA) for complete digestion. Digestion was performed in two steps: (1) ramp: temperature 260 °C, time 20 min, power 290–1800 W, and pressure 20 bar; (2) hold: temperature 260 °C, hold time 15 min,

power 1800 W, and pressure 20 bar. Digests were quantitatively transferred to 50-mL calibrated flasks and made up to the volume with bi-distilled water. The reagents used for this study were of analytical grade from Merck company (Darmstadt, Germany).

The Pb, Cd, Cu, and Hg concentrations in the experimental solutions were measured using an atomic absorption spectrometer (Thermo Scientific iCE 3400 series, USA) with electrothermal atomization. The calibration solutions were prepared from a 1 g/L stock solution (AAS standard solution; Merck, Germany).

Bioaccumulation index:

Bioaccumulation (*BA*) Index (Kabata-Pendias 2011) was calculated for heavy elements as the quotient of concentration of an element in the aerial part of the plant ($C_p$) to the concentration of the element in soil ($C_s$).

$$\mathrm{BA} = \frac{C_\mathrm{P}}{C_\mathrm{S}}$$

Pollution index:

Using the following respective equations, single index ($C_F$, $I_{geo}$) and integrated pollution index (PLI) were calculated.

The contamination factor $C_F$ is defined as:

$$C_{\mathrm{F}=\frac{C_\mathrm{M}}{C_\mathrm{B}}}$$

where $C_M$ is the measured content of the metal at any given site, and $C_B$ is the background level for that element. As background values were taken from the established Continental Crust data (Rudnick and Gao 2003).

Index of geo-accumulation, $I_{geo}$, was calculated using the following equation:

$$I_{\mathrm{geo}=\frac{C_\mathrm{M}}{1.5C_\mathrm{B}}}$$

where $\frac{C_M}{C_B}$ is contamination factor.

The factor of 1.5 is introduced to minimize the effect of possible variations in the background.

The PLI represents the *n* order geometric mean of an entire set of CF regarding the contaminating elements as follows:

$$\mathrm{PLI} = \sqrt[n]{\prod_{i=1}^{n} C_{F,i}},$$

where *n* equals the total number of contaminating elements. $C_F$, $I_{geo}$, and PLI were graded into classes based on the extent of contamination (Salman et al. 2019).

Statistical analysis

Pearson correlation coefficients were calculated using SPSS (V16.0) statistical software to explore the relationships between areca nut or husk samples and the corresponding soils in terms of the amount of elements present by applying the confidence levels of $p < 0.05$ and $p < 0.01$.

## Results

The elemental profile measured in areca nut and areca palm husk and the corresponding soil samples are summarized in Table 1. The soil pH data (Supplementary Table 1) showed that soils are moderately acidic and contain macronutrient, micronutrient, trace elements, as well as rare earth elements (REEs). The mean concentrations of elements (ppm), viz., 60,000 (Al), 8.5 (As), 1300 (Ca), 98 (Cl), 14 (Co), 15,125 (K), 3800 (Mg), 604 (Mn), 3100 (Na), 100 (Rb), 0.7 (Sb), 154,000 (Si), and 81 (Zn) in soil; 560 (Al), 0.2 (As), 1200 (Ca), 5200 (Cl), 0.5 (Co), 15,900 (K), 1200 (Mg), 25 (Mn), 400 (Na), 20 (Rb), 0.2 (Sb), 8000 (Si), and 14 (Zn) in husk; 13 (Al), 0.06 (As), 700 (Ca), 2000 (Cl), 1.0 (Co), 7000 (K), 700 (Mg), 40 (Mn), 90 (Na), 10 (Rb), 0.2 (Sb), 13 (Zn) in areca nut, respectively. Besides, the mean concentration of other elements in soil (ppm), viz., 10 (Sc), 4700 (Ti), 100 (V), 30,000 (Fe), 15 (Ga), 50 (Sr), 140 (Zr), 5.0 (Cs), 30 (La), 70 (Ce), 6.6 (Sm), 0.6 (Tb), 5.5 (Dy), 7.2 (Hf), 1.3 (Ta), 2.02 (W), 13.6 (Th), and 2.8 (U), respectively.

Elemental concentrations measured in the areca nut samples can be arranged in the sequence K > Cl > Ca > Mg > Na > Mn > Zn > Al>Rb> Co >Sb > As. Areca nut (endosperm) contains lesser quantities of Al, As, Ca, Cl, K, Mg, Na, and Rb than areca palm husk (pericarp), whereas Mn, Zn, and Sb contents are comparable both in husk and nut. The concentrations of Mn and Sb are lower than areca nut in husk by factors 0.62 and 0.64, respectively. Zn, Ca, Mg, Rb, K, Cl, As, and Na contents were higher by factors, in the increasing order, ranging from 1.1 to 4.76; besides, concentration of Al is markedly higher by a factor of 43.6 in areca husk compared with betel nut. It is intriguing to note that even though the essential micronutrient element, Fe is present in substantial amounts in the tested acidic soils, probably in inorganic form; however it was not detected in the husk (except in one husk sample) and areca nut, whereas other micronutrient elements such as Mn and Zn (in areca nut and

**Table 1** Elements concentration[a] in areca nut, husk, and soil samples

| Elements | Technique applied | Areca nut | Husk | Soil |
|---|---|---|---|---|
| Na[b] | NAA | 90±40 | 450±300 | 3000 ± 1500 |
| Mg[b] | NAA | 700±150 | 1200±500 | 3800 ± 2000 |
| Al[b] | NAA | 13±4 | 600±700 | 60,000 ± 7000 |
| Si[b] | NAA | ND | 8000±2000 | 154,000 ± 44,000 |
| Cl[b] | NAA | 2000±650 | 5500±2000 | 100 ± 80 |
| K[b] | NAA | 7000±1500 | 15,000±4000 | 15,000 ± 5000 |
| Ca[b] | NAA | 670±100 | 120±300 | 1200 ± 600 |
| Sc[b] | NAA | ND | ND | 11 ± 2 |
| Ti[b] | NAA | ND | 229[d] | 5000 ± 600 |
| V[b] | NAA | ND | 2.3±1.5 | 100 ± 20 |
| Mn[b] | NAA | 40±20 | 30±10 | 600 ± 300 |
| Fe[b] | NAA | ND | 1160[d] | 30,000 ± 7000 |
| Co[b] | NAA | 1.0±0.3 | 0.5±0.1 | 13 ± 2 |
| Zn[b] | NAA | 13±3 | 15±5 | 80 ± 30 |
| Ga[b] | NAA | ND | ND | 15 ± 4 |
| As[b] | NAA | 0.06±0.02 | 0.2±0.1 | 8 ± 5 |
| Rb[b] | NAA | 11±5 | 25±5 | 100 ± 30 |
| Sr[b] | NAA | ND | 15±5 | 60 ± 20 |
| Zr[b] | NAA | ND | ND | 150 ± 60 |
| Sb[b] | NAA | 0.4±0.1 | 0.2±0.1 | 0.7 ± 0.2 |
| Cs[b] | NAA | ND | 0.32[d] | 5 ± 1.5 |
| La[b] | NAA | ND | 0.72[d] | 30 ± 5 |
| Ce[b] | NAA | ND | ND | 60 ± 5 |
| Sm[b] | NAA | ND | 0.13±0.07 | 6.5 ± 1.4 |
| Tb[b] | NAA | ND | ND | 0.6 ± 0.2 |
| Dy[b] | NAA | ND | ND | 5.5 ± 2.0 |
| Hf[b] | NAA | ND | ND | 7.0 ± 1.0 |
| Ta[b] | NAA | ND | ND | 1.3 ± 0.6 |
| W[b] | NAA | 0.34 | ND | 2.0 ± 0.6 |
| Th[b] | NAA | ND | 0.3[d] | 14 ± 3 |
| U[b] | NAA | ND | 0.033[d] | 3 ± 0.3 |
| Cu[c] | AAS | 14±3.4 | 15±4 | 20 ± 5 |
| Cd[c] | AAS | 0.06±0.03 | 0.05±0.02 | 0.07 ± 0.04 |
| Pb[c] | AAS | 0.7±0.7 | 0.8±1.1 | 20 ± 3 |

*ND* not etected

[a] Mean concentration ± Standard deviation

[b] Unit is in μg/g

[c] Unit is in μg/Kg

[d] Detected only in one sample

husk) along with trace element cobalt, Co, (areca nut) were detected. Besides, inessential element Si is only detected in the husk albeit it is not detected in the edible areca nut. However, the contents of the trace elements, viz., Ba, Cr, and Ni in the areca nut, areca palm husk or the soil samples, in our study were below the detection limits of the applied techniques.

The concentration levels of some trace elements, viz., Cd, Cu, and Pb in areca nut, husk, and soil were quantitatively determined using AAS methods. It should be mentioned that concentrations of Hg in all analysed samples were below the limits of detection of AAS. The mean concentration levels of Cd in nut, husk, and soil samples were 0.06, 0.05, and 0.07 μg/kg, respectively, while the mean concentration levels of Cu in nut, husk, and soil samples were 14.00, 14.53, and 22.68 μg/kg, respectively (Table 1). The concentration levels of Pb in nut, husk, and soil samples were 0.67, 0.87, and 20.06 μg/kg, respectively. Cd, Cu, and Pb contents are comparable both in husk and nut. The concentrations of Cd, C,u and Pb are modestly higher in nut by the factors 1.2, 1.03, and 1.29, respectively. The contents of selected trace elemental species in areca nut and husk determined by using AAS methods did not exhibit significant correlations with the corresponding trace elemental levels of soil samples.

On the basis of their grain size distribution, in this study, all soil samples may be classified as sandy soil (Siddiqui and Fatima 2017). In addition, the measured soil pH was in the range 4.7–5.92, with an average of 5.33 corresponding to moderately acidic, and the soils of Northeast India were also rich in organic matter (Supplementary Table 1). Importantly, physicochemical features of the soil can influence the translocation and distribution of elements in plants (Jin et al. 2005; Zeng et al. 2011). Hence, correlation analysis was performed to evaluate the relationships between the soil attributes and contents of all elements in various parts of the areca nuts and husks.

In areca nut, K concentration levels have a positive correlation trend with soil pH, while Al, Mg, and Cl contents have a negative correlation trend with soil pH albeit statistical significance was not observed (Table 3). Other elements (Rb, Ca, Na, As, Mn, and Sb) have displayed no correlation with soil pH. Besides, in areca husk, concentration levels of K showed significant and positive correlation with soil pH ($p < 0.05$), while Sb levels displayed significant and negative correlation ($p < 0.01$). Subsequently, the correlations between SOM and the amount of elements in areca palm were explored. Sb levels ($p < 0.05$) showed significantly positive correlation with SOM. However, statistically non-significant strong and positive correlation between Na and Al levels in areca nut and SOM were observed. Non-significant strong negative correlation between Mg, Cl, as well as Mn contents and SOM and non-significant weak negative correlation between K levels and SOM were observed (Table 3). In husk samples, weak negative correlations without statistical significance between Zn, Rb, As, Al, Mn, K, and Mg contents and SOM were observed, while Na levels showed a weak positive and non-significant correlation, whereas no correlations were observed for other elements (Cl, Ca, and Sb).

The BA index was calculated for areca nut and husk for each plantation site. The BA index provides the information on the extent of uptake and translocation of heavy elements by plant from the soil (Kabata-Pendias 2011). The order of BA index for heavy metals in areca nut was Cd > Sb > Cu > Zn ≥ Mn ≥ Co > Pb ≥ As. The calculated BA index values of areca nut samples were in the range of 0.27–2.65 for Cd; 0.2–1.25 for Sb; 0.42–0.74 for Cu; 0.05–0.3 for Zn; 0.03–0.2 for Mn; 0.043–0.15 (Co); 0.007–0.09 for Pb; 0.002–0.05 for As, respectively. The order of BA index for heavy metal in the husk was Cd > Cu > Sb > Zn > As ≥ Pb > Co. In addition, for husk samples, the calculated BA index values were in the range of 0.23–2.50 for Cd; 0.33–1.19 for Cu; 0.1–0.6 for Sb; 0.04–0.5 for Zn; 0.007–0.2 for As; 0.005–0.14 for Pb; 0.01–0.1 for Mn; and 0.043–0.052 (Co), respectively. Similarly, in order to assess the degree of anthropogenic contamination in soil due to heavy elements, $C_F$, $I_{geo}$, and PLI were calculated for the following elements normally considered as the environmental pollutants: V, Co, Zn, As, Sb, U, Cd, Cu, and Pb. The pollution indices are summarized in Table 2.

## Discussion

The advantage of NAA methods over other spectrometric methods is that the amenability of soil in its natural setting (soil in insoluble solid state) which may provide total elemental content as both the inorganic form (soluble and insoluble inorganic compounds) as well as the inorganic species bound to humic acids as coordination complexes (Spěváčková and Kučera 1989; Olafisoye et al. 2017). Because of their lower concentration levels, perhaps below the detection limits of the employed INAA method, the elements, viz.,Ba, Cr, Hg, and Ni species, were neither in soil nor in the areca nut else in husk, as detected in the present study. Besides, *Areca catechu* palm trees are raised in an 'organic' manner as small-scale households across North-eastern India grow their plantation crops without the application of any

**Table 2** Calculated pollution indices for soil samples

| | Lengpui | Lengte | Zampui | Zamuang | Vairengte | Kolasib | Sibsagar | Golaghat |
|---|---|---|---|---|---|---|---|---|
| $C_F$ | | | | | | | | |
| V | 1.0 | 1.4 | 1.1 | 1.1 | 0.9 | 0.8 | 1.3 | 1.1 |
| Co | 0.6 | 0.8 | 0.9 | 0.9 | 0.9 | 0.5 | 1.0 | 0.8 |
| Zn | 0.7 | 0.9 | 2.2 | 0.8 | 1.4 | 1.0 | 1.3 | 1.4 |
| As | 4.0 | 3.2 | 0.4 | 1.6 | 1.5 | 0.9 | 1.5 | 1.2 |
| Sb | 2.0 | 2.3 | 2.0 | 1.8 | 1.6 | 0.9 | 1.6 | 1.1 |
| U | 1.2 | 1.2 | 1.1 | 1.1 | 1.1 | 0.8 | 0.9 | 0.9 |
| Cu | 0.6 | 0.8 | 1.0 | 0.8 | 1.0 | 0.5 | 1.0 | 0.8 |
| Cd | 0.2 | 0.3 | 0.9 | 1.1 | 0.8 | 0.4 | 1.5 | 0.9 |
| Pb | 1.0 | 1.1 | 1.4 | 1.1 | 1.4 | 1.0 | 1.4 | 1.1 |
| $I_{geo}$ | | | | | | | | |
| V | 0.69 | 0.94 | 0.72 | 0.72 | 0.63 | 0.52 | 0.86 | 0.71 |
| Co | 0.41 | 0.53 | 0.62 | 0.58 | 0.63 | 0.32 | 0.64 | 0.55 |
| Zn | 0.45 | 0.60 | 1.49 | 0.54 | 0.94 | 0.64 | 0.86 | 0.97 |
| As | 2.64 | 2.11 | 0.26 | 1.07 | 0.97 | 0.58 | 0.97 | 0.81 |
| Sb | 1.32 | 1.55 | 1.33 | 1.17 | 1.03 | 0.61 | 1.08 | 0.72 |
| U | 0.79 | 0.81 | 0.73 | 0.74 | 0.70 | 0.53 | 0.58 | 0.62 |
| Cu | 0.40 | 0.52 | 0.64 | 0.55 | 0.69 | 0.32 | 0.64 | 0.56 |
| Cd | 0.14 | 0.20 | 0.60 | 0.76 | 0.50 | 0.24 | 1.01 | 0.61 |
| Pb | 0.65 | 0.74 | 0.92 | 0.72 | 0.94 | 0.66 | 0.94 | 0.73 |
| PLI | | | | | | | | |
| | 0.26 | 0.26 | 0.27 | 0.36 | 0.00 | 0.75 | 0.15 | 0.26 |

commercial fertilisers or insecticides/fungicides (Baruah et al. 2013) implying that anthropogenic inputs for these trace elemental species may be minimal or negligible.

The detected elemental profile of the soil is most likely a consequence of natural geochemical background features of the soil. Besides, in this study, the natural trace element background concentrations may be in moderate levels at the plantation sites in Mizoram and Assam. BA index provides the relationship between the relative bioavailability of various elements in soil and their concomitant distributions in different plant tissues. In most cases, BA index can also be related to the nature of the soil and approximate geographical background besides the ability of a specific plant species to translocate heavy elements from soil to the plant tissues (Kabata-Pendias 2011; Zhuang et al. 2009). When BA index > 1.0, then the plant species not only can absorb the heavy elements but also can accumulate heavy elements in the aerial parts of the plant including edible parts such as fruits, whereas for BA index < 1.0, the plant species can absorb the heavy elements albeit they may not accumulate them in the aerial parts (Sonu et al. 2019; Zhuang et al. 2009). In the present study, it is most likely that except for Cd (mean BA index = 1.09 ± 0.82), areca palm may not be able to accumulate other heavy elements in the edible areca nut.

In all soil samples, except for Vairengte and Kolasib sites, the $C_F$ values of vanadium were between 1 and 2 indicating moderately polluted soils. In case of Co, except Sibsagar site, all soil samples can be considered as uncontaminated. Soils collected in Zampui, Vairengte, Sibsagar, and Golaghat are moderately contaminated with zinc, whereas Lengpui and Lengte sites are considerably contaminated with arsenic. Based on $C_F$ values, Sb, U, Cu, Cd, and Pb contents in analysed soils can be classified as low or moderately contaminated (Table 2). $I_{geo}$ values for V, Co, Zn, U, Cu, Cd, and Pb were lower than 1.0 indicating uncontaminated to moderately contaminated soil. For all soil samples, essentially $I_{geo}$ values for As and Sb ranged from uncontaminated to moderately contaminated. The $I_{geo}$ value of As in soils has suggested

that Lengpui and Lengte sites are moderately to significantly contaminated soils (Table 2). PLI index values for all elements were less than 1.0 indicative of unpolluted soils (Salman et al. 2019).

Below pH 6, soil acidity entails highly ion exchangeable $Al^{3+}$ as well as lower ion exchangeable alkaline earth mineral elements such $Mg^{2+}$ and $Ca^{2+}$ (Natesan 1999; Di Meo et al. 2003). However, as a non-acidophilic plant species, *Areca catechu* tended to uptake and transport through roots to other parts of the plant, with less amount of inessential $Al^{3+}$ and more amounts of essential micronutrient elements $Ca^{2+}$ and $Mg^{2+}$ which is reflected in the mineral distributions of nut and husk samples (Table 1).

In the present study, the amount of As present in the areca nut samples were in the range of 0.043–0.107 $\mu gg^{-1}$, which is higher than the mean As concentration level of 0.013 $\mu gg^{-1}$ present in betel nuts of Bangladesh (Al-Rmalli et al. 2011), whereas it is considerably less than the As content present in betel nuts (0.34 $\mu gg^{-1}$) of Pakistan (Zaidi et al. 2002). This observation also suggests that the As content in areca nut samples of NEI are significantly less than the USFDA permissible levels of As in fruits and vegetables, viz., 1.4 $\mu gg^{-1}$ (Roshila et al. 2007).

Moreover, the Cd content with a mean concentration of 0.06 $\mu gg^{-1}$and the Pb species with a mean concentration of 0.67 $\mu gg^{-1}$ are present in the areca nut samples, which are higher than the amounts of Cd and Pb species present in the areca nut samples available in Bangladesh (Al-Rmalli et al. 2011). Furthermore, the observed Cd content in areca nut samples is comparatively lower than FAO/WHO's permissible levels of Cd, viz., 0.2 $\mu gg^{-1}$, whereas the Pb content is relatively higher than FAO/WHO's safe levels of Pb in fruits and vegetables, viz., 0.3 $\mu gg^{-1}$ (Elbagermi et al. 2012). However, it is pertinent to note that cadmium and lead exhibit strong bioaccumulation potential due to their long biological half-lives in mammals (Bernard 2008; Gairola and Wagner 1991; Mussalo-Rauhamaa et al. 1986). Chronic exposure to and the concomitant bioaccumulation of these heavy elements in various organs may entail adverse health effects.

Available experimental studies, all of them from South India, have indicated that due to the unendurable agricultural practices, Cu contents in areca nut and the soil were significantly higher (Alexander et al. 2019; Khan et al. 2015) (Table 3). Typically, in South India, copper is administrated, prior to the monsoon rains, as a fungicidal spray to the fruit-bearing areca palm trees known as Bordeaux mixture which

**Table 3** Pearson correlation between soil parameter and tested plant parts

| Soil parameters | Plant part | Na | Mg | Al | Cl | K | Ca | Mn | Zn | As | Rb | Sb | Cu | Cd | Pb |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Soil pH | Husk | 0.321 | −0.443 | 0.143 | 0.532 | 0.719* | 0.341 | −0.389 | −0.174 | 0.31 | 0.255 | −0.850** | 0.226 | 0.047 | −0.074 |
| | Areca Nut | −0.019 | −0.275 | −0.591 | −0.21 | 0.505 | 0.173 | 0.013 | 0.038 | −0.015 | −0.197 | 0.012 | −0.057 | −0.258 | −0.056 |
| SOM | Husk | 0.263 | −0.199 | −0.381 | 0.174 | −0.32 | 0.102 | −0.343 | −0.6 | −0.353 | −0.367 | 0.105 | 0.41 | 0.517 | 0.458 |
| | Areca Nut | 0.582 | −0.489 | 0.325 | −0.368 | −0.211 | 0.209 | −0.298 | 0.008 | 0.174 | 0.018 | 0.757n | 0.395 | 0.138 | −0.259 |

**Correlation is significant at $p < 0.01$ level (2-tailed)

*Correlation is significant at $p < 0.05$ level (2-tailed)

can be freshly prepared as an aqueous mixture of copper sulphate and slaked lime at an appropriate ratio (Mathew et al. 2015). Moreover, the copper content in Indian-made cigarettes was also found to be significantly higher possibly due to the application of a variety of Cu sprays employed in tobacco crop protection in India (Mishra et al. 1986). Nearly all heavy elemental species sequestered in the tobacco plant tissues were attributed to either atmospheric depositions or the use of fertilizers and sewage water/sewage sledge for irrigations (Mann et al. 2011). The observed contents of Cu, Cd, and Pb present in NEI soils (Table 1) were well below the range of permissible limits for these trace elements in soils as prescribed by Indian standards for agricultural soils, viz., 135–270 mg/kg (Cu); 3–6 mg/kg (Cd); and 250–500 mg/kg (Pb) (Awashthi 2000), as well as the soil guideline values of Canada, China, and Poland (Kumar et al. 2019). Besides, these observed heavy elements contents were also relatively below the range of heavy elements concentrations detected in uncontaminated soils, viz., 2–100 mg/kg (Cu); 0.01–0.7 mg/kg (Cd); and 2–200 mg/kg (Pb) (Bowen 1966). However, the Cu content is within the limits, whereas Pb content in NEI soils exceeded the standard Indian natural soil back ground level, viz., 56.5 mg/kg (Cu) and 13.1 mg/kg (Pb) (Kumar et al. 2019).

It is important to note that the observed Cu content in areca nut samples is significantly less than FAO/WHO's permissible levels for Cu in fruits and vegetables, viz., 40 $\mu g g^{-1}$ (Elbagermi et al. 2012). One reasonable possibility is that in northeast India, rural small landscape holders, in general, hardly apply intensive agricultural methods (Baruah et al. 2013) which are reflected by the elemental distributions in areca nut and husk samples. In this study, the concentration levels of heavy elements (As, Cd, and Cu) except Pb are well within the permissible limits in biomatrices that may be attributed to edaphic and biogeochemical features of the soil in addition to the rudimentary 'organic' agricultural practices that may be employed (Barbosa et al. 2014).

Uranium and thorium as well as few REEs were detected sporadically, only in some of the assayed areca palm husk samples (Table 1). The natural radioactive elements thorium and uranium are widely distributed in soils and rocks of the earth's crust; besides, under certain environmental conditions, these elements can be accumulated in plant matrices. In the tested soil samples, the amounts of uranium and thorium were present in the range of 2.13–3.3 $\mu g g^{-1}$ and 8.6–17.2 $\mu g g^{-1}$, respectively. The manifestations of their radioactivity normally observed as decay products, viz., radon or thoron, have been detected at multiple sites in India including the northeast India (Singh et al. 2007; Singh et al. 2016; Zoliana et al. 2016).

The results presented in this study provide the detailed information available to date on the distribution of macro-, micro-, and trace elements at different parts (endosperm and pericarp) of the 'mature and fresh' unprocessed areca palm fruit. More akin to other palm species, *Areca catechu* fruit also germinates in soil when both an endosperm (nut) that contains embryo and a fibrous pericarp (husk) are intact (Davis et al. 1978). Moreover, in this study, we have observed that fibrous husk contained a higher amount of various elements than present in the areca nut. It is likely that the pericarp of areca palm fruit tends to act as a reservoir of nutrient mineral elements and, in addition perhaps, sequesters trace amounts of heavy elements.

It should be noted that areca palm husks are commercially processed and consumed as a masticatory article in Hunan province of China, in Taiwan, Indonesia, Philippines, Papua New Guinea, and few Micronesian islands (Papke et al. 2020), and normally young tender unripe fresh areca palm fruit with husk is consumed (Lee et al. 2011). Hence, the population which consumes the areca palm husk as a masticatory article may be potentially exposed to higher contents of trace elements in addition to areca alkaloids. It has been suggested that higher amount of areca alkaloids (Srimany et al. 2016; Yuan et al. 2018) in addition to relatively higher heavy metal contents present in the pericarp (husk) of areca palm may potentiate the carcinogenic risks of areca husk chewing (Yuan 2018; Khan et al. 2015).

## Conclusion

In summary, concentration levels of 14 elements present in areca nut and husk of areca palm fruits besides concentrations of 34 elements present in the corresponding soil samples collected from NEI were determined, for the first time, using INAA and AAS methods. The range of elemental concentration levels were at ppm to ppb levels in all areca palm fruit samples. No REEs were detected in areca nut samples. There was a direct relationship between the potassium and chloride concentrations in areca palm and soil pH and SOM, whereas concentrations of As, Cd, Cu, and Pb were found to be not correlated with soil pH and SOM of the corresponding soil samples. However, the elemental contents in

areca nut and husk samples did not appear to be correlated with the corresponding total elemental concentrations in the soil. Pb content was relatively higher than FAO/WHO's permissible levels, while As, Cd, and Cu contents were within the permissible levels in edible areca nuts. Based on the Indian standards for agricultural soils (Awashthi 2000), NEI plantation soils may be classified as unpolluted soil which is consistent with the calculated pollution indices. More detailed studies need to be carried out using more areca samples to further assess the preliminary finding of the current study.

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# ABSTRACT

# PHYSICO-CHEMICAL CHARACTERIZATION AND THE EVALUATION OF OXIDATIVE POTENTIAL OF *ZOZIAL*, THE INDIGENOUS HAND-ROLLED CIGARETTE OF MIZORAM

**A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY**

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## ABSTRACT

Organic cultivation of tobacco plant (*Nicotiana tabacum*) in Mizoram is primarily for an indigenous tobacco product called 'Zozial' which is a smoking tobacco product in which, similar to 'bidis', cellulose acetate type filter is absent. It has a distinctive processing method where matured tobacco leaves, harvested by priming method, are first thoroughly squashed by using bare foot on a bamboo mat . Oriental curing is practiced only for a very short period (~10-15 days) and then made into coarse flakes using wooden mortar and pestle and is then rolled with cigarette paper using bare hands. The indigenous 'zozial' is manufactured on a cottage level industry, entirely in the absence of modern 'automated' machineries using 'organic' tobacco exclusively grown within Mizoram.

Instrumental neutron activation analysis (INAA) provides simultaneous multi-elemental determination with high accuracy; low detection limits, and is rather free from array of interferences and was employed for elemental analysis. INAA detects the presence of alkaline earth metals such as Na, Mg, K, Ca, Rb, Cs, Sr, Ba along with Transition elements such as V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Zr, and Rare earth elements such as La, Ce, Nd, Sm, Eu, Tb, Dy, Yb, Hf, Ta, W, Th, and U in the tobacco leaf samples at ppm (µg/g) levels. The most abundant elements analyzed from our tobacco samples were Mg, Al, Si, Cl, K, Ca and Fe. Elements such as Na, Ti, Mn, Cu, Zn, Br, Rb, Sr and Ba were found to be present at minor levels, and Sc, V, Cr, Co, Ni, As, Zr, Sb, Cs, La, Ce, Nd, Sm, Eu, Tb, Dy, Yb, Hf, Ta, W, Th and U at trace levels.

Inductively coupled plasma optical emission spectroscopy (ICP-OES) method was used to determine the trace element content since it is sensitive, selective, and reliable method for the simultaneous and accurate determination of various elements in trace levels. The ICP-OES data indicated the presence of high concentrations of trace elements such as As, Cd, Pb, Hg, Ni and Cr in at ppm (µg/g) levels. The comparison between of the heavy metal concentrations with their corresponding permissible limits indicated that the mean concentrations of Cd and Pb are above

their permissible limits while those of Hg, Ni and Cr were within the permissible limits. From calculation using heavy metal data, estimated daily intakes (EDI) of all the analyzed heavy metals for zozial users lies below the tolerable daily intake (TDI) and target hazard quotient (THQ) of all heavy metals also shows a value within the acceptable range, the hazard index (HI) and carcinogenic risk (CR) of zozial and its users is significantly high and above the permissible levels with the probability of occurrence of adverse effects due to exposure to zozial.

Liquid chromatography – mass spectrometry (LC-MS/MS) study has indicated the presence of TSNAs such as N'- nitrosonornicotine (NNN), 4-methylnitrosamino-1-(3-pyridyl)-1-butanone (NNK) and its metabolite 4-(methylnitrosamino)-4-(3-pyridyl)-butanal (NNAL) and tobacco alkaloids such as Nicotine, Cotinine and Nornicotine along with nitrogen oxide species in *zozial* samples, Tobacco leaf filler (TLF), Indian and Myanmar commercial cigarette samples in our present study. The nicotine and nornicotine concentrations were highest in commercial cigarettes followed by tobacco leaf filler and then zozial. The concentration of detected alkaloids, TSNAs and nitrogen oxide species were significantly higher in commercial cigarettes than those of tobacco samples collected from Mizoram (zozial and TLF).

The smoker population within Mizoram may be broadly classified into two groups; the commercial cigarette smokers and the *zozial* smokers with the availability of commercial cigarettes from Indian and Myanmar manufacturers along with *zozial*. The epidemiological data indicated that majority of regular *zozial* smokers belongs to farmer population within rural areas with lesser annual income and lower educational qualifications as compared to commercial cigarette users. Current findings indicate that there is an elevated urinary 8-hydroxy 2deoxyguanosine (8-OHdG) concentration and superoxide dismutase (SOD) activity among *zozial* consumers in comparison with non smokers (controls). The present study also exposed a significant difference in the frequency of micronucleus (MN) between smokers and non-smokers and that among smoking tobacco users, *zozial*

smokers shows elevated concentration of MN compared to commercial cigarette users.

The high concentrations of toxic heavy metals and the absence of cellulose acetate filter in *zozial* may be the underlying motive behind the elevated frequencies of DNA damage among the users. The higher rate of smoking (sticks per day) by *zozial* consumers, when compared with commercial cigarette users, also shows a strong association with elevated incidence of DNA damage. *Zozial* smoking is considered to putatively pose a much higher health risk due to the potential synergistic effects of various xenobiotic compounds that are potentially hazardous to ingest. It is pertinent to mention here that the present study is the first scientific study on the characterization and evaluation of oxidative potential of *zozial* that will help in understanding and a much needed awareness among the *Zozial* smokers within the community.