

**DETERMINATION OF NORNICOTINE, NITRATE AND
NITRITE IN SELECTED COMMERCIAL AND INDIGENOUS
TOBACCO PRODUCTS AVAILABLE IN MIZORAM**

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

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**DETERMINATION OF NORNICOTINE, NITRATE AND NITRITE IN
SELECTED COMMERCIAL AND INDIGENOUS TOBACCO PRODUCTS
AVAILABLE IN MIZORAM**

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

Thesis Certificate

This is hereby certified that the research work for the dissertation entitled *“Determination of Nornicotine, nitrate and nitrite in Selected Commercial and Indigenous Tobacco Products Available in Mizoram”* submitted by **Ms. Bomngam Kakki** to Mizoram University, Tanhril, Aizawl-04, for the award of Doctor of Philosophy (Ph.D.) Degree in Chemistry is a *bona fide* record of the research work carried by Ms. Bomngam Kakki 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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DECLARATION
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AUGUST, 2025

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

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To my Parents

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ABBREVIATIONS

Abbreviations	Full Form
μl	Microlitre
AA	Ammonium acetate
AAS	Atomic absorption spectroscopy
ABTS	2,2'-azinobis-(3-ethylbenzthiazolin-6-sulfonic acid)
ACN	Acetonitrile
AD	Alzheimer's disease
ADI	Acceptable daily intake
AFL	Pamplified fragment length polymorphism)
AFLP	Amplified Fragment Length Polymorphism
AMI	Acute myocardial infarction
As	Arsenic
BaCl_2	Barium chloride
BD	bulk density
BE	Binding energy
BHI	Brain Heart Infusion
BLAST	Basic Alignment Search Tool
BSI	Botanical Survey of India
C	Carbon
Ca	Calcium
CaCO_3	Calcium carbonate
CaClOH	Calcium hydroxide chloride
$\text{CaMg}(\text{CO}_3)_2$	Dolomite
CaMgSiO_4	Calcium magnesium silicate
CAN	Acetonitrile β -NADPH β -nicotinamide adenine dinucleotide 2'-phosphate
$\text{Ca}_5\text{Si}_6\text{O}_{16}(\text{OH})_{2 \cdot 4\text{H}_2\text{O}}$	Tobermorite
CE	Collision energy
CIMAP	Central Institute of Medicinal and Aromatic Plants
CLSI	The clinical and laboratory standards institute
Cl	Chlorine
cm	Centimeter
CMC	Carboxy-methyl-cellulose
COI	Cytochrome c oxidase
CO_3^{2-}	Carbonate ion
COOH	Carboxyl
Cr	Chromium
CRPs	CORESTA Reference Products
CSIR	Council of Scientific and Industrial Research
C–S–H	Calcium-silicate hydrate

CTAB	Cetyl Trimethyl Ammonium Bromide
CVR	Coronary vascular resistance
CXP	Collision cell exit potential
CYP1	Lanosterole 14-demethylase
DAN	2,3-diaminonaphthalene
DDA	Disc Diffusion Assay
DMSO	dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DP	Declustering potential
DPPH	2,2-diphenylpicrylhydrazyl
DW	Distilled Water
EC	Electrical conductivity
EDS	Energy Dispersive X-ray Spectroscopy
EDTA	Ethylene diaminetetra acetic acid
EO	Essential oil
EOs	Essential oils
EP	Entrance potential
FA	Formic acid
FAD	Flavin adenine dinucleotide disodium salt hydrate
FCT	Flue-cured tobacco
Fe	Iron
FE-SEM	Field Emission Scanning Electron Microscope
GC	Gas Chromatography
GC-MS	Gas chromatography-mass spectrometry
gm	Gram
HCl	Hydrochloric acid
HPLC	High-performance liquid chromatography
HPLC- MS	High performance liquid chromatography mass spectrometry
HPLC-UV	High-performance liquid chromatography ultra-violet
H ₂ SO ₄	Sulphuric acid
IARC	International Agency for Research on Cancer
IC ₉₀	90 percent Inhibition concentrations
ISSR	Inter-Simple Sequence Repeats
ITK	Indigenous Technical Knowledge
<i>ITS2</i>	Internal transcribed spacer gene2
K	Potassium
KCl	Potassium chloride/Sylvite
K ₆ Si ₃ O ₉	Potassium silicate
K ₂ SO ₄	Potassium sulfate
LC	Liquid chromatography
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
MAP	Mean arterial pressure

<i>matK</i>	MaturaseK
MBC	Minimum bacterial concentration
MDA	Malondialdehyde
MeOH	Methanol
Mg	Magnesium
MIC	Minimum inhibitory concentration
ml	Mililitre
ML	Maximum likelihood
MgO	Magnesium oxide
Mg (OH) ₂	Magnesium hydroxide
Mn	Manganese
MRM	Multiple Reaction Monitoring
MSWI	Municipal solid waste incineration
N	Nitrogen
Na	Sodium
NAB	N'-nitrosoanabasine
nAChRs	Nicotinic Acetylcholine Receptors
NaCl	Sodium Chloride
NaNO ₂	Sodium nitrite
NaOH	Sodium hydroxide
NAT	N'-nitrosoanatabine
NCCLS	The national committee for clinical laboratory standards
NEI	North East India
NER	North East Region
NIST	National Institute of Standard and Technology
NJ	Neighbor-Joining
NNAL	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NNN	N'-nitrosonornicotine
NO	Nitric oxide
NO ₃ ⁻	Nitrate
O	Oxygen
O.D	Optical density
OECD	Organization for Economic Co-operation and Development
P	Phosphorus
PAHs	Polycyclic Aromatic Hydrocarbons
PASS	Prediction of Activity Spectra for Substances
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDB	Protein structure database
PGN	Peptidoglycan network
PMT	putrescine methyltransferase
PVP	Polyvinyl pyrrolidone
QPT	Quinolinate phosphoribosyl transferase

QQQ	Triple Quadrapole Mass Spectrometer
QTP	Qinghai-Tibetan Plateau's
RAPD	Random amplified polymorphic DNA
RBC	Red blood corpuscles
<i>rbcL</i>	Ribulose-bisphosphatecarboxylase
RFLP	Restriction fragment length polymorphism
RNA	Ribo nucleic acid
ROS	Reactive Oxygen Species
SDA	Sabouraud's Dextrose Agar
S	Sulphur
Si	Silicon
SID	Sudden Infant Death
SIL-IS	Stable isotope-labeled internal standards
SiO ₂	Silicon dioxide/ Quartz
SLT	Smokeless Tobacco
SMC	Soil moisture content
SPE	Solid-phase extraction
SSR	Simple sequence repeats
ST	Smokeless Tobacco
STPs	Smokeless Tobacco Products
TDW	Triple Distilled Water
TGA	Thermo gravimetric analysis
TLC	Thin-layer chromatography
TN	Total nitrogen
TOC	Total Organic Carbon
TSNAs	Tobacco-Specific Nitrosamines
WBC	White blood corpuscles
WHC	Water holding capacity
WHO	World Health Organization
XRPD	X-ray powdered diffraction
XPS	X-ray Photoelectron Spectroscopy
Zn	Zinc
ZnO	Zinc Oxide
ZOI	Zone of Inhibition

Chapter-1

Introduction and Review of Literature

1.1 Introduction and Review of Literature

The narrative of tobacco is a complex interweaving of cultural acceptance, economic expansion, and public health crises. Tobacco has evolved from its ritualistic beginnings to its current status as a global commodity (Stanfill *et al.*, 2015; Stepanov *et al.*, 2005). Tobacco belongs to the genus *Nicotiana* of the Solanaceae family, which comprises a diverse range of species, including *Nicotiana tabacum* and *Nicotiana rustica*. Among the various *Nicotiana* species, *Nicotiana tabacum* is extensively cultivated for commercial purposes (DeptaandDoroszewska, 2023; Siva Raju *et al.*, 2008). Tobacco use is a significant global public health concern, with varying consumption preference among smoking and smokeless tobacco products (STPs). The key differences between smoking and smokeless tobacco (ST) lie in their mode of consumption and the specific health risks associated with them. Smoking involves the combustion of tobacco, a process that not only volatilizes several hazardous chemical species present in tobacco matter but also generates thousands of harmful compounds through pyrolysis and oxidation (Klus *et al.*, 2009). During combustion, tobacco generates thousands of toxic substances, including nicotine, tar (a complex mixture of polycyclic aromatic hydrocarbons, or PAHs, metal ions, phenols, quinones, and other non-polar organic compounds), carbon monoxide (which binds to hemoglobin and reduces oxygen delivery), volatile aldehydes (like formaldehyde and acrolein), and reactive oxygen species (ROS) that contribute to oxidative stress and cellular damage. Moreover, the elevated temperatures associated with smoking generate nitrogenous carcinogens such as nitrosamines and aromatic amines, which are potent carcinogens and mutagens. On the contrary, smokeless tobacco does not undergo combustion during its consumption; instead, it is consumed through chewing, oral retention, or nasal inhalation. STPs release toxic chemicals through dissolution in saliva. Although it may not possess any combustion-related toxins such as carbon monoxide, acrolein, however it possesses preformed carcinogens, notably tobacco-specific nitrosamines (TSNAs), heavy elements such as cadmium, arsenic, and lead, PAHs such as benzo[a]pyrene, as well as radioactive elements such as polonium, most of them

present considerable non-cancer hazards and/or cancer risks, especially to oral and pancreatic tissues (Janbaz *et al.*, 2014; Noor and Ramanarayana, 2024).

Smoking facilitates the delivery of nicotine and the consequent rapid absorption into the bloodstream through the lungs, enabling it to reach the brain within seconds, which contributes to its high addictive potential (Le-Houezec, 2003). However, the chemical delivery of nicotine in smokeless tobacco (STP) relies on a more gradual absorption through the oral mucosa and is primarily influenced by the pH levels of STPs, which determine the proportion of free-base (unprotonated) nicotine available for absorption through the oral mucosa (Wilhelm *et al.*, 2022). Although both are chemically addictive and carcinogenic, however smoking causes the exposure to a wider range of combustion-derived toxins, whereas smokeless tobacco primarily increases the exposure to alkaloids, nitrosamines, nitrate and heavy elements. Consequently, both kinds of tobacco products pose significant health hazards owing to their unique chemical compositions.

Tobacco use varies significantly across regions and countries, with some areas experiencing higher prevalence due to factors such as weak regulatory frameworks, aggressive marketing tactics by tobacco companies, and deeply entrenched social norms that normalize tobacco consumption (Wakefield *et al.*, 2010). India is the second largest consumer of different tobacco products in the world, reflecting the diverse tobacco use culture across the country (Thomas *et al.*, 2019). In India, tobacco use takes several forms, including both smoked and smokeless forms (Shaikh *et al.*, 2022). Tobacco consumption pattern in India is very different from many Western countries, where smoking is the most common type of tobacco use. Socioeconomic factors, regional norms, and cultural practices also affect the consumption pattern (Rai and Bramhankar, 2021). Attempts to reduce tobacco usage are made more difficult by the cultural integration of tobacco, especially in East and North India, where it is frequently served to visitors as a sign of respect and appreciation. Due to the introduction of ready-to-use STPs in the market, over the past few decades, the high rate of tobacco uses among people aged 15 and older in India has changed, but it has always been >25%, (Jena *et al.*, 2023; Nugroho and Atmanti, 2020). Approximately 60% of 275 million tobacco users in India use

smokeless tobacco products. Another 15% of population in the country uses both smoking and smokeless tobacco products (Stanfill *et al.*, 2018). The affordability of tobacco products, especially beedis and smokeless varieties, makes even the economically weaker populationsto spend ~10-20% of their income on tobacco products (NFHS-5, 2019–21). Smokeless tobacco products have gained significant popularity in India (Jena *et al.*, 2023). More importantly, India encounters challenges associated with the illicit tobacco trade, which compromises tobacco control initiatives by providing consumers with access to cheaper, unregulated products (Arda and Santiago, 2023).

Tobacco use in India varies greatly from region to region. Northeastern India (NEI) has especially unique and varied patterns of tobacco use that warrant special investigation. Nationwide, 28.6% of Indian adults consume tobacco in some form or other (GATS-2, 2016-17). However, these results disguise dramatic regional differences; states like Punjab and Goa report incidence below 15%, whereas NEI consistently reports the highest rates of tobacco consumption in the country. The unique geography, ethnic customs, and socioeconomic conditions of the region makes tobacco smoking a major public health issue (Phukan *et al.*, 2005). The tobacco consumption trend in NEI deviates greatly from the rest of the country. The humid subtropical climate with significant rainfall (> 200 cm annually) provides the ambience for tobacco cultivation at the local level, while its porous international borders allow cheap tobacco products from Bangladesh and Myanmar to be smuggled into the state, making tobacco both economically significant and deeply ingrained in the local culture (Ladusingh *et al.*, 2017; Mizoram Excise Department Report, 2022). With distinctive consumption forms like tuibur (tobacco smoke-infused water) and meizial (traditional roll-your own cigarettes) playing a major part in social and ceremonial life, tobacco is ethnically firmly ingrained in tribal rituals (NFHS-5, 2019–21). This cultural normalization contributes to remarkably high usage rates, especially among women (68%), a phenomenon rarely seen elsewhere in the country (DLHS-4). Mizoram, one of the Northeastern states has the highest rate of gastro-intestinal cancers in the country and a high prevalence of tobacco-related non-communicable diseases (PBCR Aizawl, 2018-22; Mudur, 2005). Current active

smokers in Mizoram are more prone to an increased risk of stomach cancer (Phukan *et al.*, 2005).

1.2 Smoking tobacco products

In India, Tobacco use encompasses a diverse and complex landscape, with a wide array of smoking products such as cigarette, beedi, hookah, gutkha, khaini, zarda, etc. Each variant carries distinct cultural, social, and health implications, contributing to the multifaceted challenges surrounding tobacco consumption. Cigarettes, the most prevalent form of tobacco smoking, consist of finely cut tobacco leaves tightly rolled in paper, often supplemented with additives to enhance flavor and accelerate nicotine absorption. Despite their global ubiquity, cigarette smoking is less common in some regions than beedi smoking or smokeless tobacco use (Shaikh *et al.*, 2022). Both commercially manufactured and hand-rolled cigarettes contain over 7,000 chemicals, including at least 70 known carcinogens and other toxic elements. These toxic compounds significantly elevate the risk of severe health consequences, including lung cancer, cardiovascular disease, gastritis, and chronic respiratory conditions (Benowitz, 2010).

Beedis are hand-rolled, non-filter ‘cigarettes’ that originated from South Asian countries, mainly India (Oladipupo *et al.*, 2019). Alarming levels of population in India smoke-beedi, especially amongst lower-income groups. People often consider beedi as a cheaper and more natural alternative to regular cigarettes, even though they have the same or even higher health risks (McAfee and Kruger, 2012). Beedis are made up of roughly 0.2 g of sun-dried and processed tobacco flakes wrapped in a tendu leaf (Oladipupo *et al.*, 2019). Beedi smokers may be exposed to higher nicotine content since they puff more intensely and often to keep it lit. Tendu leaves, on the other hand, are not porous, which resulted in absorption of higher levels of tar and carbon monoxide (Oladipupo *et al.*, 2019). Studies have shown that smoking beedi is associated with a range of negative health impact, including coronary heart diseases, glaucoma, lung cancer, and head and neck cancers (Oladipupo *et al.*, 2019). Apart from normal smoking forms, unlike cigarettes and beedis, hookah, also referred to as shisha, narghile, or waterpipe, is a way to smoke tobacco that is

different from traditional smoking forms such as cigarettes and beedis. Hookah use, which originated in India, has expanded to the Middle East and North Africa and is gradually spreading to other regions of the world (Patel *et al.*, 2019).

Hookah smoking is often regarded as a fun social activity, which boosts its increasing popularity (Kakaje *et al.*, 2021). Hookah smoking involves flavored tobacco (shisha) being passed through water, which makes it seem safer because of the water filtration. However, a single session can expose users to more smoke than a cigarette, which can lead to lung disease, heart problems, and infections from sharing mouthpieces (WHO, 2023; Maziak *et al.*, 2015). The popularity of hookah consumption is expanding, particularly due to the introduction of flavored and scented hookah tobacco (Kakaje *et al.*, 2021). In some countries, smoking hookah is part of their culture and is considered a sign of status and pride (Nimma *et al.*, 2022). Contrary to the belief that hookah smoking is less detrimental than cigarette smoking, research indicates that it poses considerable health risks. The identified risks include: Long-term exposure to high levels of toxins from hookah smoking, which involves longer sessions and bigger puffs, is linked to a higher risk of lung cancer, respiratory illnesses, and possibly heart disease and other chronic conditions. Furthermore, secondhand smoke exposure poses significant health risks, especially for at-risk populations like pregnant women and children (Sultana, 2021). The IARC has included tobacco smoke and smokeless tobacco products in Group 1 carcinogens, which means there is strong evidence that they can cause cancer (Xia *et al.*, 2013). Additionally, market dynamics that encourage tobacco use in low- and middle-income nations may elevate tobacco-related mortality to exceed eight million by 2030 (Yalçın and Monte, 2016). Secondhand smoke comprises carcinogenic substances, including benzene, 1, 3-butadiene, benzopyrene, and 4-1-1-butanone (Pierce and Leon, 2008).

Tobacco smoking remains a significant global health challenge, impacting a substantial portion of the world's population. Smoking prevalence varies considerably across regions and genders, with men consistently showing higher rates than women (Islami *et al.*, 2015). The World Health Organization's 2023 Global Report documented around 1.3 billion adult tobacco users (aged 15+), with

combustible cigarette smoking representing the dominant form of consumption (WHO 2023). This figure aligns with the 2021 Global Burden of Disease Study, which estimated 1.14 billion current smokers in 2019 and highlighted persistent gender disparities; men accounted for about 82% of smokers globally, with prevalence rates nearly five times higher than women.

Globally, smoking prevalence remains heavily concentrated in Asia, where China, India, and Indonesia collectively contribute over 600 million smokers according to Tobacco Atlasdata (Eriksen *et al.*, 2012). India ranked as the second-largest tobacco-consuming nation globally, with approximately 275 million users (Stanfill *et al.*, 2018). On the production front, India is the world's second-largest tobacco producer, accounting for 13.4% of total unmanufactured global production. Consequently, due to its high consumption patterns, India is positioned at the top of the Southeast Asia Region in terms of tobacco use prevalence.

While worldwide smoking rates have declined from 23% in 2007 to 19% in 2023, population growth has kept the total number of smokers persistently high, especially in low- and middle-income countries. According to GLOBOCAN reports, Cigarette smoking alone accounts for approximately two-thirds of global lung cancer deaths, contributing to 1.8 million fatalities in 2020 (Sung *et al.*, 2021). The highest smoking prevalence occurs in South and Southeast Asia, with dramatic variations between countries and genders. In South-East Asia, male smoking rates showed considerable variation, with the highest rates observed in Indonesia (72.3%), followed by Timor-Leste (69.5%), Bangladesh (60.0%), and Maldives (47.3%). Comparatively lower prevalence was found in India (34.1%), Nepal (33.6%), Cambodia (34.7%), and Pakistan (31.6%). Among women, smoking rates were significantly lower across all countries, with the highest prevalence in Nepal (9.8%), followed by the Philippines (5.2%), Maldives (4.6%), and Pakistan (4.02%) (Sreeramareddy *et al.*, 2014).

The prevalence of cigarette smoking in South and South-East Asian countries demonstrates clear socioeconomic and demographic gradients. Rural areas consistently have higher smoking rates compared to urban settings across nations,

with India showing 36.8% prevalence in rural regions compared to 29.5% in urban settings (Sreeramareddy *et al.*, 2014). Educational attainment strongly influences smoking patterns, with lower education associated with higher prevalence (Islam *et al.*, 2022). Wealth disparities are equally striking, as poorer individuals are more likely to smoke (Bandyopadhyay and Irfan, 2019), as evidenced in Bangladesh, where smoking prevalence drops from 70.1% in the poorest compared to 46.8% in the wealthiest. Age is also a factor, with older men generally smoking more than younger cohorts (Sinha *et al.*, 2016). It is also evident that the tobacco consumption patterns in Asia differ significantly from Western countries, with high smoking rates among men but low rates among women due to cultural factors (Tsai *et al.*, 2008). The larger expansion of transnational tobacco companies into Asia, facilitated by U.S. trade policies, has significantly undermined regional tobacco control efforts. These challenges are further compounded by deeply entrenched smoking cultures and the dominance of state-owned tobacco monopolies, creating formidable barriers to effective regulation (Mackay, 1995; Mackay *et al.*, 2022).

Tobacco consumption patterns indeed exhibit striking differences between Western and Asian markets due to cultural traditions and regulatory environments (Anwar and Sa, 2008). In Western nations, the market is dominated by commercial filtered cigarettes, increasingly accompanied by vaping products and limited smokeless tobacco options like Swedish snus, all produced under strict manufacturing standards and heavy taxation (Al-Hamdani and Hopkins, 2023; Kasza *et al.*, 2017). In contrast, Asian markets feature a diverse array of traditional products, including India's beedis (hand-rolled tobacco) (Oladipupo *et al.*, 2019), and various smokeless tobacco products (Siddiqi *et al.*, 2020), often with higher toxin levels and less regulation (Stepanov *et al.*, 2005). While tobacco in Western countries is mostly manufactured by multinational corporations and faces strict packaging and flavor regulations, several Asian nations continue to have state-managed tobacco industries that offer cheaper, locally tailored products. These fundamental differences—from the cultural significance of tobacco in Asian traditions to the West's focus on harm reduction innovations—underscore why effective tobacco control requires region-

specific strategies that account for varying product types, consumption habits, and regulatory frameworks across these diverse markets.

1.3 Smokeless tobacco products

Smokeless tobacco (ST) comprises a wide variety of non-combustible products consumed through chewing, oral retention, or nasal inhalation, with distinct regional preferences and diverse global compositions and production methods. This diversity leads to significant variations in carcinogenic potential, as documented by the US Centers for Disease Control and Prevention and the International Agency for Research on Cancer (Hecht and Hatsukami, 2022).

Although public health efforts often prioritize smoking cessation, smokeless tobacco remains a substantial global health burden. According to Sinha *et al.* (2015), more than 350 million individuals across 121 countries consume STPs, with men comprising 67% of users. The vast majority (95%) reside in developing nations, with an overwhelming 82.7% concentrated in the South-East Asia Region (Siddiqi and Mishu, 2019; WHO, 2023), where smokeless tobacco frequently exceeds cigarette smoking as the primary form of nicotine consumption (Sinha *et al.*, 2015). Smokeless tobacco products vary widely in composition and use patterns globally, with modern Western products generally considered less harmful than those used in Asia, Africa, and South America (Klus *et al.*, 2009).

Globally, smokeless tobacco use is most prevalent in South Asia, with 237.4 million users in India, 30.9 million in Bangladesh, 10.1 million in Pakistan, and 2.7 million in Nepal, accounting for the highest tobacco-related burden. Southeast Asia also demonstrates substantial consumption, particularly in Myanmar (12.6 million) and Indonesia (3.2 million), while China (4.1 million) represents the dominant East Asian market.

India shows the highest SLT use among men at 36.7% in rural areas versus 31.3% in urban centers. Unlike smoking, SLT use among women is particularly notable in India (9.0%), Cambodia (5.1%), and Nepal (4.8%). Globally, smokeless tobacco accounts for an estimated 1.5 to 2 million disability-adjusted life years (DALYs) lost

to upper aerodigestive tract cancers, with the South-East Asia region bearing the highest burden (Sinha *et al.*, 2018; Siddiqi *et al.*, 2020).

India is among the few countries globally with a high prevalence of both smoking and smokeless tobacco use, exhibiting a distinct pattern of dual tobacco use, the concurrent consumption of smoked and smokeless forms. This dual use contributes significantly to the overall burden of tobacco-related morbidity and mortality in the country. Smokeless tobacco is the most widely used form of tobacco in India, surpassing smoking and dual use among both males and females. Its prevalence varies substantially across states, with the highest usage among males recorded in Bihar. Among females, Mizoram shows the highest prevalence of smokeless tobacco use. The practice is especially common in the eastern and central regions of the country, and the northeastern states show increasing consumption trends. Among males, the peak age group for smokeless tobacco use is 35–39 years, while for females, use tends to rise steadily with age. Unlike smoking, the gender gap in smokeless tobacco use is narrower, though male's still show higher usage rates overall.

STPs exhibit remarkable global diversity in their composition and production methods, with significant variations in carcinogenic potential as documented by monographs from the US Centers for Disease Control and Prevention and the International Agency for Research on Cancer (Hecht and Hatsukami, 2022). In India, consumers predominantly use three main types: gutkha (a processed blend of areca nut, tobacco, and calcium hydroxide), khaini (a traditional tobacco and lime combination), and zarda (tobacco cured through smoking and often mixed with spices) (Singh, 2014; Mutti *et al.*, 2016). Smokeless tobacco (SLT) use is especially concerning, with Myanmar having the highest SLT use among ASEAN countries (Sein *et al.*, 2014) with widespread use of kun-ya, a betel quid variant containing tobacco, areca nut, and slaked lime, often chewed with additional flavorings (Sein *et al.*, 2014).

In India, smokeless tobacco products can be systematically categorized according to their primary routes of administration, which include chewing, sucking,

gargling, nasal insufflation (sniffing), and dental application (dentifrice). This categorization reflects fundamental differences in product formulation, delivery mechanisms, and user engagement patterns, with each method exhibiting distinct physicochemical properties that influence nicotine pharmacokinetics and bioavailability. Chewing products typically consist of coarser tobacco particles mixed with alkaline additives to enhance buccal absorption, while sucking preparations employ finer particulate matter designed for gradual dissolution and sustained release. Gargling formulations represent aqueous-based delivery systems, and nasal products feature finely milled tobacco optimized for mucosal absorption. Dentifrice applications incorporate tobacco extracts into oral hygiene products, presenting unique exposure pathways. Some popular STPs commonly consumed in India are listed as follows; Gutkha is a commercially manufactured smokeless product containing crushed betel nut and tobacco, often combined with slaked lime and sweet or savory flavorings, making it appealing and readily available, particularly among young people. Gutkha mixed with slaked lime is frequently consumed in eastern and central India (Stanfill et al., 2018; Dobe et al., 2006). Consumers typically place the mixture in the oral cavity, and are masticate to release its active compounds. During this process, most users expectorate the tobacco-laden saliva, though incidental ingestion may occur in some cases. It may result in oral submucous fibrosis and is linked to periodontal inflammation (Javed et al., 2010). Excessive consumption of gutkha can induce a cocaine-like state of intoxication, marked by amnesia, poor judgment, and psychosis. Prolonged usage may result in dependency (Dwivedi & Aggarwal, 2010). Gutkha has penetrated traditional society and among individuals of lower socioeconomic position as an alternate form of smoking (Nayak, 2011). The product's ready-to-use packaging and flavored composition contribute to its widespread availability and consumption patterns. Tobacco industries promote it as an alternate method of smoking, though studies confirm that it causes significant public health complications owing to its carcinogenic and genotoxic characteristics (Sankhla *et al.*, 2018).

Khaini, is a traditional smokeless tobacco preparation consisting of sun-dried unprocessed tobacco, slaked lime, and occasionally flavoring substances (Stanfill *et*

al., 2018). The preparation method typically involves manually processing the tobacco leaves by vigorously rubbing and slapping them between the palms to achieve the desired consistency before mixing with lime. Users typically take a small quantity of the mixture and place it in the oral cavity, where it is gradually sucked or held between the gum and cheek for extended periods facilitating nicotine absorption (Nasrin *et al.*, 2020). Khaini is predominantly utilized in India, especially in the states of Maharashtra and Gujarat. Khaini comprises elevated concentrations of tobacco-specific nitrosamines, which are strong carcinogens produced during the processing and storage of tobacco (Stepanov *et al.*, 2005). Khaini is a perilous smokeless tobacco product associated with detrimental health concerns, especially oral cancer.

Zarda is a form of smokeless tobacco product that is often used in South Asian countries. It is made out of processed tobacco flakes that are often boiled, baked, or roasted (Stanfill *et al.*, 2018). Additionally, it is frequently combined with slaked lime, spices, sweeteners, and flavorings such as menthol, camphor, herbs, saffron, and silver flakes to improve its sensory appeal. Consumers may use zarda either as a standalone chewing product or as a key component in betel quid preparations (Nasrin *et al.*, 2020). According to Stanfill *et al.* (2018), Zarda products frequently include significant concentrations of taste compounds, including camphor and coumarin. Zarda, like any other smokeless tobacco product, is associated with serious dangers to one's health.

Pan masala, a commercial mixture widely marketed as a mouth freshener worldwide, poses significant health risks (Garg *et al.*, 2015). This mixture, typically composed of areca nut, catechu, slaked lime, and flavorings, may or may not contain tobacco (Shah *et al.*, 2009). (Garg *et al.*, 2015) Describe it as a readily available product popular across various societal segments. Despite being promoted as harmless refreshment, pan masala is a known etiological factor in oral submucous fibrosis, a precancerous condition that frequently progresses to oral cancer (Garg *et al.*, 2015). Areca nut, a primary component, is itself neuroactive, toxic, and classified as a Group 1 carcinogen by the International Agency for Research on Cancer (Nigam & Bhatt, 2013). Some pan masala products also include tobacco; further

compounding health risks (Stepanov *et al.*, 2005). Even when tobacco-free, these products are often sold alongside tobacco sachets, encouraging combined use (Stepanov *et al.*, 2005). Chemical analysis has revealed the presence of harmful substances in both plain and blended pan masala (Nigam & Bhatt, 2013). Its wide availability and appealing flavor contribute to its popularity, especially among young people (Shah *et al.*, 2009). Its carcinogenic and genotoxic properties, coupled with widespread use, make pan masala a serious public health concern, especially given the elevated incidence of oral cancer among users (Mehrotra *et al.*, 2016).

Sadah is a type of air-cured, roasted, and hand-processed loose coarse tobacco that is marketed with or without slaked lime. Sadah tobacco is typically in a 'dry' form, either chewed or retained in the lower buccal cavity between the cheek and gum for approximately 15 to 20 minutes before expelling out. Sadah sold in the local market comprises specific grading depending on the type of tobacco used and levels of slaked lime added to the products. It is frequently utilized as an additive in betel quid, primarily among women and populations of lower socio-economic status in Mizoram. The International Agency for Research on Cancer (IARC) classifies chewing betel quid, with or without tobacco, as a group 1 carcinogen. This practice is prevalent among both genders in Mizoram and is similar to the consumption of smokeless tobacco (IARC, 2007). The high prevalence of tobacco product use in Mizoram may be a significant factor contributing to the observed rise in cancer incidence. Existing research in India and internationally has predominantly concentrated on commercial tobacco products, resulting in a notable gap in understanding the composition and potential hazards associated with indigenous tobacco varieties. Comprehending the chemical profiles of these products is crucial for precise risk assessment, particularly given their widespread use and cultural significance in areas such as Mizoram.

Snuff represents a finely processed tobacco product created by grinding or pulverizing cured tobacco leaves into a powder-like consistency (Dobe *et al.*, 2006). This preparation exists in both dry and moist varieties, often enhanced with aromatic additives or flavorings to modify its sensory characteristics. The product is typically administered nasally, with users either sniffing small quantities directly from their

fingers or employing specialized inhalation devices designed for this purpose. The manufacturing process involves careful control of particle size and moisture content to ensure proper delivery, while the addition of various scents and flavors serves to make the product more appealing to consumers. This form of tobacco consumption differs significantly from other smokeless products in its unique delivery method and physical properties. Recent trends indicate snuff has emerged as a preferred tobacco alternative in the United States, demonstrating significant sales growth that correlates with rising youth consumption rates (Alpert *et al.*, 2008).

The disruption of the oral microbiome caused by smokeless tobacco use can lead to dysbiosis, potentially promoting the growth of harmful microbes that produce carcinogenic metabolites and induce inflammation, thereby facilitating oral carcinogenesis (Oladipupo *et al.*, 2019). Furthermore, laboratory animal studies have consistently shown that pan masala exhibits carcinogenic properties and promotes tumor growth (Nigam *et al.*, 2013). Beyond its cancer-causing potential, scientific evidence indicates this product induces DNA damage, triggers oral submucous fibrosis, and impairs liver, kidney, and testicular function (Garg *et al.*, 2015).

1.4 The Role of pH and Pyrolysis in Nicotine Bioavailability and Addiction Potential

Although smokeless tobacco (ST) consumption does not involve pyrolysis, it delivers a complex mixture of chemical constituents with significant toxicological implications. Unprocessed tobacco leaf contains over 3,000 identified chemical species, with additional compounds generated during curing, fermentation, and product formulation (Roberts., 1988; IARC 2007; Stanfill *et al.*, 2015). Notably, several of these constituents exhibit well-characterized carcinogenic and addictive properties (IARC 2007; Stepanov *et al.*, 2016).

The primary addictive agent, nicotine, exists as a major alkaloid in the fresh tobacco plant, with concentration dependent on genetic factors and agricultural conditions. As a weak base ($pK_a = 8.02$), nicotine exists in equilibrium between three protonation states: the unprotonated (free base), monoprotonated, and diprotonated forms. The unprotonated species, being lipophilic, readily crosses biological

membranes, facilitating rapid systemic absorption and central nervous system delivery (Tomar *et al.*, 1997; Henningfield *et al.*, 2005). Natural tobacco (pH ~5-6) contains nicotine predominantly in the monoprotonated state, exhibiting poor buccal absorption (Richter *et al.*, 2003). However, deliberate pH modification through alkaline additives (e.g., Ca(OH)_2 , Na_2CO_3) shifts this equilibrium, dramatically increasing the concentration of unprotonated nicotine. This modification significantly increases nicotine bioavailability and enhances its addictive potential (Pickworth *et al.*, 2014; Tomar *et al.*, 1997). Moreover, pH manipulation is a key strategy used to regulate nicotine absorption rates across STPs, with more alkaline products delivering higher doses (Richter and Spierto, 2003). Elevated pH not only boosts nicotine absorption but also affects the uptake of other harmful constituents, including carcinogenic tobacco-specific nitrosamines (TSNAs), thereby compounding the health risks associated with STP use (Richter *et al.*, 2008). The inclusion of flavorings and stimulants in these products may further mask harshness, increase appeal, and expand their user base, particularly among new and younger users.

1.5 Chemical Composition of Tobacco: Focus on Alkaloids, Nitrate, Nitrite and TSNAs

Tobacco contains a diverse array of chemical compounds, many of which contribute to its pharmacological effects, addictiveness, and toxicity. This study focuses on the critical components that play a central role in tobacco chemistry and toxicology; Tobacco alkaloids, nitrate, nitrite, and tobacco-specific nitrosamines (TSNAs). These constituents are interconnected through biochemical pathways that significantly influence the ultimate health impacts of tobacco products. Below is a detailed breakdown of these key chemical groups. Several studies have been conducted periodically to assess the alkaloids, TSNA, and nitrate contents in different tobacco products (Djordjevic *et al.*, 1989; Pakhale and Maru, 1998; Wu *et al.*, 2004; Lisko *et al.*, 2013; Edwards *et al.*, 2017; Erythropel *et al.*, 2021).

1.5.1 Tobacco Alkaloid

In the history of tobacco research, tobacco alkaloids have always garnered attention across the world. Traditionally, tobacco alkaloids were considered as an indicator for assessing the quality of tobacco. Tobacco is known to contain a complex mixture of alkaloids, which tend to play significant roles in plant defense mechanisms. Tobacco alkaloids are a wide range of nitrogen-containing compounds primarily found in tobacco plants. The four principal alkaloids in tobacco plants (*Nicotiana* spp.) generally occur in the following increasing order of concentration: Anabasine < Nornicotine < Anatabine < Nicotine in fresh tobacco leaves. The tobacco alkaloids have been extensively investigated by researchers for their physiological and toxicological effects on humans (Rodgman and Perfetti, 2008).

Nicotine [3-(1-methyl-2-pyrrolidinyl)pyridine], a pyridine-pyrrolidine compound, is the predominant alkaloid found in tobacco (*Nicotiana tabacum*), which comprises 90–95% of the total alkaloid content (Benowitz, 2009; Saitoh *et al.*, 1985). The biosynthesis of nicotine primarily occurs in the roots of the tobacco plant (*Nicotiana tabacum*). A complex series of enzymatic reactions converts primary

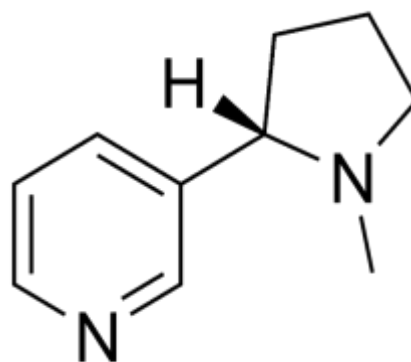


Fig. 1.1: Nicotine

metabolites into the characteristic pyridine and pyrrolidine rings of these compounds followed of accumulation in the leaves through translocation (Zenkner *et al.*, 2019). Nicotine is the main psychoactive component of tobacco, and it exerts its effects by binding to nicotinic acetylcholine receptors (nAChRs) in the brain (Rollema *et al.*, 2010). There are two enantiomeric forms of nicotine: (S)-nicotine also called (–)-nicotine and (R)-nicotine also called (+)-nicotine (Song *et al.*, 2021). This is because the pyrrolidine ring has a chiral carbon center at the 2' position. Compared to (R)-(+)-nicotine, (S)-(–)-nicotine binds to nicotinic acetylcholine receptors (nAChRs) significantly more strongly. This distinction makes it far better at getting the neurological system going and causing people to become addicted (Hukkanen *et al.*, 2005). Nicotine's interaction with the nicotinic acetylcholine receptors triggers a set

of neurochemical events, which leads to the release of dopamine in the brain's reward centers (Picciotto *et al.*, 2021). The surge of dopamine is the major cause of nicotine addiction, as it reinforces the behavior of tobacco use. Long-term exposure to nicotine can also promote a process called neuroadaptation, which indicates that the brain becomes less susceptible to the nicotinic effect over time. This can further lead to greater tolerance capacity and more dependency on tobacco use (Chiara, 2000). When tobacco use is ceased, this neuroadaptation makes withdrawal symptoms worse in users, which keeps the cycle of addiction going forward (Tiwari *et al.*, 2020). Nicotine also stimulates the release of catecholamines like epinephrine and norepinephrine into the bloodstream, leading to increased heart rate, blood pressure, and vasoconstriction, thereby contributing to the risks of cardiovascular diseases (D'Souza and Markou, 2011). The nicotine metabolism is controlled by the enzyme CYP2A6 (Benowitz, 2010). Person with faster metabolisms tend to consume more batches of tobacco per day than people with slower metabolisms to keep their nicotine levels intact. The addictive nature of nicotine is attributed to its neurological effects, with users experiencing greater stimulation, pleasure, and mood regulation (Benowitz, 2010). As a weak base ($pK_a = 8.02$), nicotine exists in equilibrium between three protonation states: the unprotonated (free base), monoprotonated, and diprotonated forms. The unprotonated species, being lipophilic, readily crosses biological membranes, facilitating rapid systemic absorption and central nervous system delivery (Tomar *et al.*, 1997; Henningfield *et al.*, 2005).

Tobacco (*Nicotiana tabacum*) synthesizes a diverse array of alkaloids beyond nicotine, which, although present in smaller quantities, play significant roles in the plant's biochemistry and human toxicology. Minor tobacco alkaloids including nornicotine, anabasine, anatabine, and myosmine, are structurally related to nicotine but exhibit distinct pharmacological and metabolic properties. These compounds are generated as secondary metabolites through the pyridine alkaloid biosynthetic pathway. The minor tobacco alkaloids nornicotine, anatabine, and anabasine occur in varying concentrations across *Nicotiana* species. Anatabine is typically the most abundant, followed by nornicotine and anabasine (Lisko *et al.*, 2013). Nornicotine and anatabine constitute only up to 2–3% of the total alkaloids present in tobacco,

whereas anabasine is far less abundant in comparison to other alkaloids, accounting for only about 0.3% of the alkaloid content (Jacob *et al.*, 1993). Nornicotine levels can increase during post-harvest processing due to enzymatic conversion from nicotine via CYP82E4-mediated demethylation (Siminszky *et al.*, 2005).

Despite their relatively minor concentrations in tobacco compared to nicotine, the secondary metabolites anabasine and anatabine have emerged as valuable analytical targets, serving both as exposure biomarkers and as compounds of pharmacological interest (Caine *et al.*, 2014). These alkaloids can be detected in urine, with traditional cutoff values of >2 ng/mL for both compounds (Jacob *et al.*, 2002). However, recent research suggests lower thresholds of >0.097 ng/mL for anatabine and >0.236 ng/mL for anabasine may improve sensitivity and specificity in differentiating smokers from non-smokers (Colsoul *et al.*, 2023). Toxicologically, they are precursors to carcinogenic tobacco-specific nitrosamines like N-nitrosoanabasine and N-nitrosoanatabine, formed via nitrosation during tobacco processing and metabolism, contributing to the carcinogenic burden of tobacco products (Hongzhi *et al.*, 2013). Pharmacologically, their structural similarity to nicotine allows binding to nicotinic acetylcholine receptors; anatabine may have anti-inflammatory potential via $\alpha 7$ nAChR activation, while anabasine may influence addiction pathways (Caine *et al.*, 2014). There are still a lot of contradicting findings in the literature about the stereochemical makeup of the minor tobacco alkaloids nornicotine, anatabine, and anabasine. Recent studies have challenged earlier assumptions about the enantiomeric composition of tobacco alkaloids. Armstrong *et al.* (1999) found that anabasine consistently had the lowest enantiomeric excess among common tobacco alkaloids, with 54-60% of the (S)-(-)-enantiomer. Liu *et al.* (2008) reported similar findings, showing S-(-)-anabasine proportions of 60-65% across different tobacco types. For anatabine, Liu *et al.* (2008) observed S-(-)-anatabine proportions ranging from 77.5% to 86.6%, depending on the tobacco type.

1.5.1.1 Nornicotine: Structural Analog and Metabolic Product

Nornicotine is a naturally occurring secondary metabolite of nicotine, formed through enzymatic N-demethylation of nicotine (Papke *et al.*, 2007). The metabolic conversion of nicotine to nornicotine primarily occurs during leaf senescence and curing process (Gavilano and Siminszky, 2007). Nicotiana species are classified into two distinct groups based on their metabolic profiles. The first group is referred to as

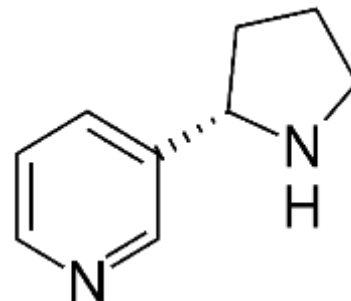


Fig. 1.2: Nornicotine

"converters," which extensively convert nicotine into nornicotine during senescence and the curing process, and the second group is referred to as "nonconverters," which prohibit the conversion process and retain nicotine in their leaves (Mannet *et al.*, 1974). This metabolic conversion not only modifies nicotine's pharmacological effects but also facilitates the formation of carcinogenic derivatives (Papke *et al.*, 2007). Cytochrome P450-dependent monooxygenases, especially CYP82E4, are the main N-demethylase enzymes that facilitate the metabolic conversion of nicotine into nornicotine (Siminszky *et al.*, 2005; Gavilano *et al.*, 2007). The amount of nornicotine in a product can vary based on the type of tobacco used, curing and fermentation processes, and storage conditions. These are the key factors that influence the overall toxicity profile of the product (Stanfill *et al.*, 2015).

Nornicotine is a well-known precursor to the formation of carcinogenic nitrosamines. Nornicotine's conversion to N'-nitrosornornicotine involve nitrosation reaction occurring during tobacco curing and processing (Hongzhi *et al.*, 2013). As a secondary alkaloid nornicotines readily interact with nitrosating agents, primarily oxides of nitrogen (nitrites), to form NNN (Nestor *et al.*, 2003). In the nitrosation of secondary amines, the reaction results in the replacement of N-H by N-N=O, yielding compounds like N'-nitrosornornicotine, N'-nitrosoanabasine and N'-nitrosoanatabine (Hecht *et al.*, 1978). The extent of nornicotine conversion is influenced by factors such as the initial nicotine concentration and the degree to which nicotine is converted to nornicotine within the tobacco plant (Richmond *et al.*, 2017). Nornicotine's impact on human health extends beyond its role as a precursor

to the production of carcinogenic NNN. Nornicotine may independently contribute to adverse health outcomes. It can induce aberrant protein glycation, alter the pharmacological properties of certain steroid drugs like prednisone, disrupt retinoid homeostasis (potentially leading to birth defects and age-related macular degeneration), and contribute to periodontal disease in smokers (Dickerson *et al.*, 2002; Brogan *et al.*, 2005; Katz *et al.*, 2005). It also has its pharmacological effects, which result from interacting with nicotinic acetylcholine receptors, potentially contributing to the addictive properties of tobacco (Drug and Therapeutics Bulletin, 2014). Furthermore, high nornicotine levels can negatively impact tobacco flavor and smoking characteristics. Tobacco alkaloids, particularly nornicotine, undergo nitrosation in the presence of nitrosating agents found in tobacco leaves to produce N-nitroso nornicotine (Brunnemann *et al.*, 2017; Castonguay and Vunakis, 1979; Schuller *et al.*, 2007). This conversion process is influenced by numerous environmental factors, including temperature, humidity, and the presence of microorganisms capable of reducing nitrate to nitrite (Brunnemann *et al.*, 2017; Stanfill *et al.*, 2018). Despite their lower concentrations, minor alkaloids are increasingly recognized for their toxicological significance, particularly in the context of smokeless tobacco and heated tobacco products, where alkaline conditions may enhance their bioavailability.

1.5.1.2 Cotinine: A Nicotine Metabolite

Cotinine, a principal alkaloid in tobacco leaves (*Nicotiana tabacum*), represents the major hepatic metabolite of nicotine, with approximately 70–80% of absorbed nicotine undergoing biotransformation into this compound (Benowitz, 2009). Cotinine is formed through the oxidative metabolism of nicotine, primarily catalyzed by the cytochrome P450 enzyme

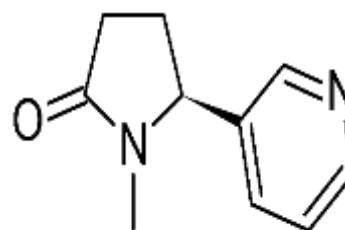


Fig.1.3: Cotinine

CYP2A6, which oxidizes nicotine to form a nicotine- $\Delta 1'(5')$ -iminium ion intermediate, subsequently converted into cotinine through the action of aldehyde oxidase (Tutka *et al.*, 2005). Compared to nicotine, cotinine is formed more slowly and is eliminated from the body at a reduced rate, leading to its accumulation and

sustained presence in biological fluids such as blood, urine, and saliva for longer durations, making it an ideal marker of recent tobacco use. Notably, the S(–)-enantiomer of cotinine, the biologically active form demonstrates preferential systemic retention due to stereoselective metabolic processes. This enantiomer also has a markedly prolonged elimination half-life of approximately 15 to 22 hours, in contrast to nicotine's much shorter half-life, thereby reinforcing its role as a robust and stable biomarker for assessing tobacco exposure (Narkowicz *et al.*, 2012; Hukkanen *et al.*, 2005).

Nicotine exhibits high affinity for neuronal nicotinic acetylcholine receptors (nAChRs), particularly $\alpha 4\beta 2$ and $\alpha 6\beta 2$ subtypes, while its primary metabolite cotinine shows significantly lower affinity (Grady *et al.*, 2010; Vainio and Tuominen, 2001). Cotinine, despite its lower affinity, can alter the assembly and trafficking of certain nAChR subtypes, increasing $\alpha 4\beta 2$ expression on the plasma membrane while down-regulating $\alpha 6\beta 2\beta 3$ receptors (Fox *et al.*, 2015).

Recent scientific investigations have revealed promising therapeutic potential for cotinine in addressing neurodegenerative conditions, particularly Alzheimer's disease, owing to its capacity to inhibit amyloid-beta plaque formation and exhibit neuroprotective anti-inflammatory characteristics (Echeverria *et al.*, 2017). The metabolic processing of cotinine through the CYP2A6 enzyme system, which displays significant genetic variability, introduces substantial individual differences in nicotine breakdown that influence smoking patterns and treatment efficacy, highlighting its importance in tailored smoking cessation strategies (Benowitz *et al.*, 2007). From a toxicological perspective, cotinine may transform into N-nitrosocotinine (NNC) through nitrosation reactions, a compound with suspected mutagenic properties, and prolonged exposure has been associated with increased oxidative damage and vascular impairment (Stepanov *et al.*, 2009). These diverse biological impacts justify expanded research into cotinine's applications beyond mere exposure detection, encompassing its pharmacological effects, health consequences, and possible medical uses.

1.5.2 Nitrate and Nitrite: Reactive Nitrogen Precursors

Nitrate and nitrite are ubiquitous inorganic anions that are naturally present in tobacco leaves as a result of nitrogen assimilation by the plant. Nitrate is present in the predominant form (Rodgman and Perfetti, 2008). Plants, including tobacco, absorb nitrate from the soil, and it plays a crucial role in plant growth and development. These compounds are introduced into the crop system through a series of processes, primarily through root uptake, which are then deposited into the foliar system.

Post-harvest processes may further lead to an increasing level of these compounds (Alori and Babalola, 2018). The levels of nitrate in tobacco are influenced by variety of factors, including soil type and composition, type of tobacco species, fertilization practices, the curing and fermentation processes. The global demand for tobacco production has increased drastically, which in turn has resulted in intensive tobacco production. This has led to heavy reliance on agrochemicals, such as inorganic fertilizers and pesticides. The heavy dependence on such fertilizers and pesticides may harm the environment (Aziz *et al.*, 2017). The prolonged and excessive use of agrochemicals can induce significant residues in the crop system, further raising concerns about potential risks to consumer health (Kahramanoğlu and Usanmaz, 2019). Particularly, excessive use of nitrogen-containing fertilizers elevates the level of nitrate available in the soil, influencing greater nitrate uptake by tobacco plants and in harvested leaves (Peterson, 1968; Bartucca and Buono, 2020; Hongzhi *et al.*, 2013).

Nitrate and nitrite serve as nitrosating agents in the formation of carcinogenic tobacco-specific nitrosamines during the process of curing and storage of tobacco products (Siminszky *et al.*, 2005). Elevated nitrate concentration in the tobacco products can highly aggravate the nitrosation process, leading to a concomitant

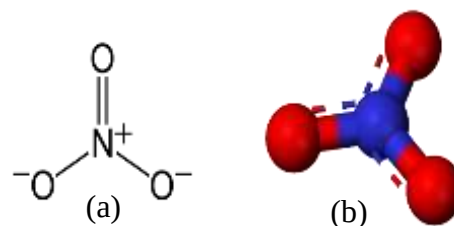


Fig.1.4: Nitrate 2D (a) and 3D (b)

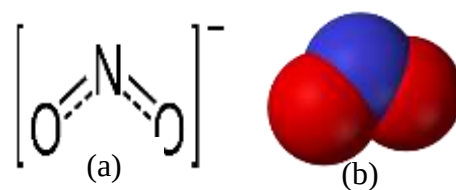


Fig.1.5: Nitrite 2D (a) and 3D (b)

increase in the levels of these detrimental compounds (Hongzhi *et al.*, 2013). During the curing and fermentation processes, nitrate can be reduced to nitrite by microbial activity. Additionally, nitrite acts as a reactive nitrosating species that facilitate the endogenous formation of tobacco-specific nitrosamines (TSNAs) from tobacco alkaloids (Stepanov *et al.*, 2008; Hecht, 2008). Nitrite can produce a large amount of NO_x in comparison with nitrate, thus promoting the formation of carcinogenic TSNAs (Wang *et al.*, 2017).

Dietary habits significantly influence nitrate and nitrite levels in the body. Especially leafy greens are associated with higher intakes of inorganic nitrate and nitrite; consequently, higher concentrations of nitrite are found in saliva and plasma (Ashworth *et al.*, 2019). In the northeastern regions of India, the indigenous people practice distinct dietary practices owing to their unique cultural heritage, such as smoked meat, fermented soybeans, fermented pork fats, and traditionally produced food additives. They traditionally consumed fermented food, which is an integral part of the regular diet that could potentially contribute to nitrite levels due to microbial activity during fermentation. It is believed that the microbial fermentation process not only acts as preservative but also enhances the texture, flavor, and boosts the nutritional value of the foods. There is sufficient experimental evidence that suggests that the traditional plant-based food additives like ashes, frequently consumed in the region, could be a contributing factor to the overall nitrate exposure to consumers (Ngoualem *et al.*, 2020). These could possibly interact with the chemicals obtained from other dietary and lifestyle products, such as tobacco. Exposure to both dietary nitrates and/or nitrites and tobacco products leads to a range of adverse health effects that are more severe than those associated with either exposure alone. The combined effect arises from the presence of carcinogens in tobacco products and the nitrate/nitrite from dietary products, which may potentially increase nitrosation of amines and amides from both sources.

Tobacco-specific nitrosamines are a class of compounds known to have carcinogenic properties found in tobacco and tobacco-related products (Hecht and Hoffmann, 1988; Shifflett *et al.*, 2018). The formation of TSNAs is significantly influenced by the concentration of nitrate present in the tobacco, which signifies that

the nitrosamines are positively correlated to the concentration of nitrate present in the tobacco (Ding *et al.*, 2008).

1.5.3 Tobacco-Specific Nitrosamines (TSNAs)

TSNAs are a class of potent carcinogens and are present in cured tobacco and tobacco smoke. TSNAs are generally undetectable or at very low levels in fresh tobacco leaves, but their levels increase during curing and storage (Hongzhi *et al.*, 2013). The presence of TSNAs in smokeless and smoking tobacco products is well documented (Hecht, 1998; Hecht and Hoffmann, 1988; Magee, 1996; Preston-Martin and Correa, 1989; Wu *et al.*, 2004; Edwards *et al.*, 2017, 2021; Hecht and Hatsukami, 2022). N'-nitrosornicotine (NNN), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), N'-nitrosoanabasine (NAB), N'-nitrosoanatabine (NAT), and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), along with other related compounds, are collectively known as TSNAs (Hecht, 2008; Shifflett *et al.*, 2018).

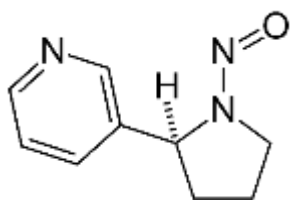


Fig.1.6: N'-nitrosornicotine

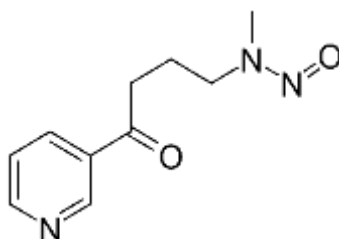


Fig. 1.7: 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone

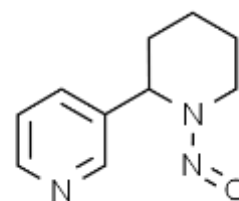


Fig. 1.8: N'-nitrosoanabasine

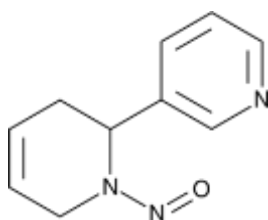


Fig. 1.9: N'- nitrosoanatabine

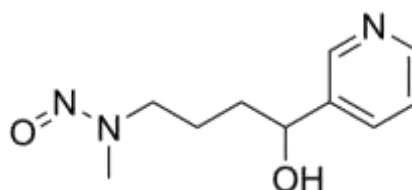


Fig. 1.10: 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol

NNN and NNK are the most prevalent and well-documented Group 1 carcinogens in both tobacco and tobacco smoke (Edwards *et al.*, 2017, 2021; Hecht and Hatsukami, 2022). Another potent Group 1 carcinogen, NNAL, is frequently detected in smokeless tobacco products, albeit at lower concentrations (Brunnemann and Hoffmann, 1994; Richter *et al.*, 2008; Stepanov *et al.*, 2016). However, NNAL is

rarely reported in combustible tobacco products or mainstream smoke, likely due to differences in formation pathways or analytical detection limits (Wu *et al.*, 2002; Stanfill *et al.*, 2018)

In 1974, the first analysis of tobacco smoke identifying NNN, a tobacco-specific nitrosamine, was published (Hoffmann *et al.*, 1974). That same year, NNN's presence in unburned tobacco was also reported (Hoffmann *et al.*, 1974). Then, in 1978, came the first report of NNAL in tobacco (Hecht *et al.*, 1978). These discoveries marked the beginning of research into TSNA, a class of potent carcinogens found in tobacco products (Edwards *et al.*, 2021). Initial studies indicated that NNN forms during tobacco curing (Burton *et al.*, 1989). Subsequent research has demonstrated that post-harvest processing significantly impacts TSNA levels (Nestor *et al.*, 2003). However, TSNA levels are also influenced by other factors, including tobacco genotype, field conditions, and storage duration and conditions (Djordjevic *et al.*, 1989; Cui, 1998; Burton *et al.*, 1992; Wei *et al.*, 2014; Richmond *et al.*, 2017).

During the post-harvest processing of tobacco leaves, microbial activity plays a critical role in the formation of tobacco-specific nitrosamines (TSNAs). When the tobacco leaves are subjected to curing, the leaves start losing their moisture content, and cellular exudate increases, which in turn provides substrates for microbial activity. Nitrate-reducing bacteria metabolize excess nitrate present in tobacco, converting it to nitrite, which accumulates in the extracellular environment (Shi *et al.*, 2013). Under favourable conditions, this nitrite undergoes further reactions, generating reactive nitrogen oxides (NO_x). Concurrently, the mechanical damage to leaf tissues releases endogenous alkaloids into the surrounding matrix. The interaction between these liberated alkaloids and NO_x facilitates the nitrosation of secondary amines, leading to the formation of carcinogenic TSNAs (Wang *et al.*, 2017).

Environmental factors such as temperature, humidity, and microbial composition significantly influence the kinetics of TSNA formation, with higher levels typically observed in air-cured and fermented tobaccos compared to flue-cured

varieties (DeRoton *et al.*, 2005). There are many reported studies on the formation of TSNA during the process of storage as well (Wang *et al.*, 2017; Hongzhi *et al.*, 2013). Temperature plays a vital role in increasing the level of nitrite and TSNA during the storage conditions (Hongzhi *et al.*, 2013). It was further confirmed that during the post cured storage under warm temperature condition, the formation of TSNA is attributed to the production of nitrogen oxides from nitrate and nitrate (Wang *et al.*, 2017).

1.5.3.1 Carcinogenicity of TSNA

The carcinogenic effects attributed to TSNA are mediated through the metabolic activation by cytochrome P₄₅₀ isozymes. The ensuing electrophilic diazo-intermediates can readily react with the nucleophilic sites in DNA, RNA, and proteins, forming the corresponding adducts which are considered as the putative biomarkers of tobacco exposure carcinogenesis (Hecht and Hatsukami, 2022). Indeed, NNN and NNAL are some of the potent DNA-alkylating agents (Shih *et al.* 2021). Moreover, in vitro studies also indicated that directly DNA-alkylating agent(s) are present predominantly in tobacco matter and to a lesser extent in the tobacco smoke which can inflict sporadic DNA lesions (Hu *et al.* 2018). Thus, polar and non-polar chemical constituents of tobacco smoke can interact with several biological factors that may influence the development and progression of cancers at different sites including lung, oropharyngeal, esophagus, stomach, pancreas, urinary bladder and prostate (Wooten *et al.* 2006; Shih *et al.* 2021; Hecht and Hatsukami, 2022).

The preformed TSNA levels in tobacco filler is a major determinant of the yields in tobacco smoke as some fraction of preformed TSNA are directly transferred as the predominant source of TSNA into mainstream smoke, whereas modest amounts of TSNA are produced as a secondary fraction during pyrosynthesis (Fischer *et al.* 1989; Wu *et al.* 2012; Stepanov *et al.* 2016; Edwards *et al.* 2017). Moreover, higher concentrations of reactive nitrogen oxides in tobacco smoke derived from high nitrate content more likely leads to a high rate of TSNA pyrosynthesis (Jin *et al.* 2022). The accumulated scientific evidence thus shown that the TSNA content in tobacco filler play a significant role in carcinogen exposure

levels and commensurate with the mainstream smoke TSNA data (Wu *et al.* 2005; Stepanov 2010; Edwards *et al.* 2017).

NNK and NNN metabolism have been extensively studied in experimental animals, as well as in animal and human cells and tissues. Bioassays in mice, rats, and hamsters have identified NNK and NNN as the most potent carcinogenic tobacco-specific nitrosamines (Hecht and Tricker, 1999). NNK induces tumors in the lung, nasal cavity, and liver of F344 rats, as well as lung, trachea, and nasal cavity tumors in Syrian golden hamsters, and lung tumors in mice (Hecht *et al.*, 1980). NNN causes tumors in the esophagus and nasal cavity of rats, the trachea and nasal cavity of Syrian golden hamsters, and the lungs of mice. While NAB is a weak esophageal carcinogen in rats and inactive in hamsters, NNAL induces lung tumors in A/J mice (Furukawa *et al.*, 1997). The striking organospecificity of NNK for the lung suggests its significant role in tobacco carcinogenesis (Brown *et al.*, 2009). The organospecificity of nitrosamines depends on the structure of the nitrosamine and the animal species (Tennant *et al.*, 2023). For nitrosamines to interact with DNA and cause cellular damage, they require metabolic activation (Preussmann and Stewart 1984). Understanding their metabolic pathways is crucial for determining the types of DNA modifications they induce and their role in cancer initiation. NNN's major metabolic pathways involve α -hydroxylation at the 2'- and 5'-carbons and oxidation of the pyridine nitrogen. Cytochrome P-450 enzymes catalyze the α -hydroxylation pathways. The specific cytochrome P-450 isozymes involved in these transformations and their tissue distribution could provide insights into the organospecificity of NNK and NNN carcinogenesis (Hecht and Hoffmann 1988). The α -hydroxylation of nitrosamines is important for their metabolic activation, leading to intermediates that bind to DNA (Zarth *et al.*, 2016).

1.6 Factors Influencing the Chemical Composition of Tobacco

The chemical composition of tobacco is shaped by a complex interplay of genetic, environmental, agronomic, and post-harvest factors. These determinants affect key constituents such as alkaloids, sugars, polyphenols, and TSNA, influencing both product quality and health risk.

1.6.1 Genetic and Cultivar Differences

Tobacco chemistry is fundamentally regulated by genotype. Differences across *Nicotiana* species, particularly *N. tabacum* and *N. rustica*, lead to significant variations in alkaloid levels due to differential expression of enzymes like PMT and QPT involved in nicotine biosynthesis (Saunders & Bush, 1979; Sierro *et al.*, 2018). While *N. rustica* accumulates 5–10% nicotine, *N. tabacum* typically ranges between 1–3%. Intraspecies variation is also substantial, with nicotine content differing up to 30-fold among *N. tabacum* cultivars (Djordjevic & Doran, 2009). Oriental and Burley tobaccos differ in their accumulation of polyphenols and nitrates, respectively, due to underlying genetic polymorphisms (Tsaballa *et al.*, 2020; Li *et al.*, 2017). Genetic factors also influence heavy metal accumulation patterns and stress-responsive metabolite profiles, such as cadmium localization in leaves versus roots (Huang *et al.*, 2021; Jassbi *et al.*, 2017).

1.6.2 Agricultural and Environmental Factors

1.6.2.1 Soil and Fertilization

Soil properties and fertilization significantly impact tobacco's chemical makeup. Nitrogen application, particularly in ammonium or split doses, has been shown to increase nicotine levels, especially in upper leaves (Dawson, 1938; Crockford, 1977; Xi *et al.*, 2005). As a key component in nicotine biosynthesis, nitrogen supports the formation of pyrrolidine and pyridine rings. Optimal nicotine yields are achieved at 90–100 kg N ha⁻¹ (Huan *et al.*, 2024). The positive correlation between nitrogen supply and nicotine concentration is well-established (Henry *et al.*, 2019). Soil pH also affects nutrient and heavy metal uptake, with acidic soils (pH 5.8–6.2) promoting optimal growth, while alkaline conditions impair biomass accumulation and alter metal transport (Zaprjanova *et al.*, 2010; Zeng *et al.*, 2014).

1.6.2.2 Climate and Environmental Stress

Climatic factors such as soil temperature and moisture significantly influence tobacco's chemical profile. Optimal growth occurs at 22°C and 75% soil moisture, with nicotine levels decreasing under cooler or wetter conditions (Parups *et al.*,

1960). During germination, high temperatures accelerate alkaloid transformation (Weeks & Bush, 1974).

1.6.2.3 Cultivation Practices

Agronomic practices from transplanting to topping play a critical role in determining chemical outcomes. Topping at the button stage increases nicotine accumulation (Fazli *et al.*, 1999; Mesbah *et al.*, 2014) and rapidly activates biosynthetic pathways within 24 hours (Qin *et al.*, 2020). Mechanical topping can trigger wound-induced alkaloid increases, while chemical suckercides such as maleic hydrazide and butralin offer effective alternatives (Richmond *et al.*, 2020). These practices influence both yield and chemical uniformity (Henry *et al.*, 2019).

1.6.3.4 Post-Harvest Processing (Curing Methods)

Tobacco curing is a meticulously controlled process that induces major physical and biochemical changes in the leaves, shaping their quality, flavor, aroma, nicotine content, and usability. During the initial stages of curing, moisture content drops from ~75% to 10%, triggering starch degradation, chlorophyll breakdown, and the buildup of reducing sugars and amino acids (Abubakar *et al.*, 2000; Zhang *et al.*, 2021). Tobacco is mainly classified by curing method: flue-cured, air-cured, and sun-cured. Each method produces distinct chemical profiles, influencing tobacco's quality and smoking properties (Leffingwell, 2001).

Flue-cured tobacco (FCT)—also known as Virginia or Brightleaf tobacco—is cured in enclosed barns using indirect heat at 70–80°C for 7–10 days, with carefully regulated ventilation to control humidity (Nestor *et al.*, 2003). This process promotes starch hydrolysis, leading to high sugar content, moderate nicotine levels (1.5–3.5%), and reduced nitrogen compounds due to Maillard reactions and enzymatic degradation (Wang, 2017). Carotenoids are preserved, producing a distinctive light yellow to orange color. Sugar-derived pyrazines and carbonyls enhance aroma and combustion, making FCT a key component in premium cigarette blends (e.g., Marlboro, Camel) and select pipe tobaccos (Darkis *et al.*, 1937). FCT thrives in warm, moderately rainy climates and well-drained sandy loam soils, with major production in the USA, Brazil, Zimbabwe, China, and India (particularly Andhra

Pradesh and Karnataka) (Ramesh *et al.*, 2020; Chikkala, 2014; Shava *et al.*, 2020). Flue-curing ensures uniform drying, suppresses microbial growth, and preserves volatile compounds, contributing to its smooth sensory profile and reduced harshness. Nitrogen-rich fertilizers are commonly applied, as nitrogen is vital for optimizing leaf yield and quality (Mason *et al.*, 2018)

Air-cured tobacco undergoes natural dehydration over 4–8 weeks in well-ventilated barns, relying on ambient temperature and humidity rather than applied heat (Christian, 2018; Tobacco Curing Methods, 2020). Humidity control is critical to prevent fungal growth, often managed using smokeless combustion sources like charcoal. This slow curing enables enzymatic and oxidative transformations distinct from thermal curing, producing two main types: light air-cured (e.g., *Burley*) and dark air-cured tobaccos. *Burley tobacco*, a light air-cured type, develops a light brown color and is chemically defined by low reducing sugars, high nicotine (2–4%), and elevated nitrogenous compounds—results of suppressed carbohydrate metabolism and enhanced protein retention. Its porous structure and alkaline pH (~6.0–7.5) promote flavorant absorption, making it ideal for cigarette blends, smokeless products, and cigar binders. In India, *Burley* is mainly grown in the eastern and northeastern regions, particularly Bihar (Patna, Nalanda, Gaya), due to favorable agroclimatic conditions (Curing and Grading, 2024). Air-curing facilitates slow chlorophyll degradation and polyphenol oxidation, creating mild flavor profiles and dark pigmentation from melanoidin formation. West Bengal's humid microclimate enhances terpenoid aromas and leaf pliability, while Uttar Pradesh and Gujarat produce varieties with higher lignin and cellulose for beedis and chewing tobacco. In the northeast (Assam, Manipur), *Nicotiana rustica* is cultivated, rich in nitrates and alkaloids for local use. Unlike flue-curing, which promotes Maillard reactions and sugar accumulation, air-curing retains nitrogenous compounds and results in reduced sugar levels. Fertilization practices, especially balanced NPK and organic inputs, are crucial for optimizing nitrogen uptake, leaf thickness, and nicotine synthesis (Gediya *et al.*, 2020), directly influencing curing efficiency and product quality. Maryland tobacco, an air-cured *Nicotiana tabacum* variety historically from the U.S., is now under trial in Bihar and West Bengal. It cures similarly to *Burley*,

producing golden-brown leaves with low nicotine (1.0–2.5%), minimal sugars (<0.5%), and moderate nitrogen content. Its high cellulose-to-lignin ratio supports clean combustion and low tar formation. Though primarily used in cigarette blends as a combustion modifier and flavor neutralizer, its low alkaloid content limits use in pipe tobaccos. Commercial expansion in India remains experimental due to agronomic constraints, though interest in low-nicotine filler tobaccos may drive further development.

Sun-cured tobacco undergoes a photochemically driven process where harvested leaves are exposed to direct sunlight and natural air circulation for 2–4 weeks. This light-mediated curing induces oxidative and hydrolytic reactions, degrading chlorophyll and carotenoids under UV exposure to produce thin, delicate leaves with light yellow to golden-brown pigmentation. The resulting tobacco has reduced sugar content, moderate nicotine levels, and elevated terpenoid volatiles (e.g., solanone, megastigmatrienone), contributing to a mild, sweet-aromatic flavor profile. The most notable sun-cured tobaccos are Oriental varieties (e.g., Turkish types), valued for their small leaves and intense spicy-sweet aroma, commonly used in premium cigarettes and hookah blends. Oriental tobacco is not commercially grown in India. Instead, India's sun-cured production centers around Andhra Pradesh and Telangana, where the coarse *Natu* variety is cultivated in districts such as Prakasam, Guntur, Nellore, Khammam, and Warangal. These leaves, cured in open fields over several weeks, develop an earthy flavor and are primarily used in chewing tobacco, beedis, and snuff. Smaller-scale sun-cured production also occurs in Gujarat (Lal Chop variety), Karnataka, and Tamil Nadu, supplying local traditional markets. Unlike the refined Oriental tobaccos of the Mediterranean, Indian sun-cured types are hardier and tailored for traditional rather than premium products. While Bihar and Karnataka lead in air- and flue-cured varieties respectively, sun-curing remains a niche yet culturally important practice in India's tobacco sector, particularly for supplying the beedi and chewing tobacco industries. Sun-curing is cost-effective and preserves natural flavors but is labor-intensive and highly dependent on consistent sunlight, with risks of mold or uneven curing under unfavorable conditions. Despite

these challenges, sun-cured tobacco retains global relevance in specialty and artisanal blends due to its unique sensory characteristics.

1.7 Analytical methods

Tobacco products comprise a complex mixture of chemicals, therefore employment of advanced analytical methodologies is necessary for the accurate quantification of their constituents (such as the minor alkaloid nornicotine and the inorganic anions nitrate and nitrite) and for advancement of tobacco research. Various methods have been developed and published in the literatures for the determination of minor tobacco alkaloids since the advancement of science and technology (Brunnemann *et al.*, 1983; Yang *et al.*, 1998; Nyiredy *et al.*, 1986; Cai *et al.*, 2003; Troje *et al.*, 1997; Peyton *et al.*, 1993; Wu *et al.*, 2002; Zhang *et al.*, 1990).

In the past, steam distillation process has been considered as a foundational method for the extraction of volatile alkaloids from complex tobacco mixtures. Employing this method further, facilitated subsequent qualitative and quantitative analyses (Cheng & Barthel, 1943; Larson & Haag, 1944). This technique exploits the volatile nature of alkaloids in steam, enabling their separation from non-volatile components. Steam distillation is particularly advantageous when dealing with alkaloids prone to degradation at higher temperatures if subjected to direct distillation (Cheng & Barthel, 1943). The process involves heating a mixture of the tobacco sample and water, generating steam that carries the volatile alkaloids into a condenser, subjected to cooling and collected as a distillate (Larson & Haag, 1944). The collected distillate, comprising of alkaloids can further be analyzed by employing different analytical methods ‘Gas Chromatography’, ‘High-Performance Liquid Chromatography’, ‘Gas Chromatography-Mass Spectrometry’ (Lisko *et al.*, 2013).

Various analytical techniques have been developed to measure nornicotine levels in tobacco products, each offering its own set of advantages and limitations. The accurate quantification of nornicotine is challenging due to its structural similarity to nicotine and its relatively low abundance in many tobacco varieties. Chromatography is a popular choice, with gas chromatography and liquid

chromatography standing out due to their high sensitivity and selectivity. Gas chromatography coupled with mass spectrometry (GC-MS) is a particularly powerful technique, providing both qualitative and quantitative data for identifying and measuring nornicotine in complex tobacco matrices (Stanfill *et al.*, 2009). This sophisticated instrument also allows for the simultaneous determination of tobacco alkaloids and their related nitrosamines in complex tobacco matrices. GC-MS technique is also reported to be great assets for the enantiomer separation minor tobacco alkaloids involving original derivatization process. The chiral separation of enantiomers is important due to its varied physiological properties, which tends to reflects its activity in their metabolized TSNA's particularly in N'-nitrosonornicotine. These methods often require extensive sample preparation steps, including extraction, cleanup, and derivatization steps to remove interfering compounds and enhance analyte detection, given the complexity of the tobacco matrix.

Modern techniques, such as liquid chromatography-tandem mass spectrometry (LC-MS/MS), have emerged as more rapid alternatives offering improved accuracy in measuring this minor tobacco alkaloids. In fact, CORESTA research from 1999-2005 indicated that LC-MS/MS demonstrates better reproducibility compared to GC-TEA for determining tobacco alkaloids. Furthermore, LC-MS/MS methods have been successfully used for the simultaneous determination of tobacco alkaloids and their related nitrosamines (Li *et al.*, 2015; Yong *et al.*, 2015). The principle of LCMS/MS involves separating compounds using liquid chromatography, followed by mass spectrometric detection and fragmentation (Stanfill *et al.*, 2015). The liquid chromatography stage separates analytes based on their physical and chemical properties, using a stationary phase and a mobile phase (Stepanov *et al.*, 2005). Reversed-phase chromatography, utilizing a non-polar stationary phase and a polar mobile phase, is commonly employed for separating alkaloids. The apparatus consists of a column packed with a porous granular material (stationary phase), such as silica or polymer beads, through which the mobile phase (solvent) flows, carrying the injected sample. Upon injection, the sample components are adsorbed onto the stationary phase, and as the solvent moves through the column, separation occurs based on each compound's differential affinity for the stationary

and mobile phases. Compounds with stronger adsorption to the stationary phase migrate more slowly, eluting last, while those with higher solubility in the mobile phase exit the column earlier. The mass spectrometry stage measures the mass-to-charge ratio (m/z) of the separated analytes, providing qualitative and quantitative information (Steinmann & Ganzera, 2010). The mass spectrometer consists of an ionization source, mass analyzer, and detector (Mukherjee, 2019).

Accurate quantification in LC-MS/MS relies on robust calibration strategies to ensure precision, sensitivity, and reproducibility. Calibration establishes the relationship between analyte concentration and instrument response, correcting for variations in sample preparation, matrix effects, and instrumental performance. Among the various calibration approaches, isotopic dilution (using stable isotope-labeled internal standards, SIL-IS) is considered the most accurate method. The isotopic dilution method using SIL-IS is widely recognized as the gold standard for quantitative LC-MS/MS analysis. SIL-IS, typically containing ^2H , ^{13}C , or ^{15}N isotopes, are added to samples before processing to correct for matrix effects and improve measurement accuracy (Arrivault *et al.*, 2015). While deuterium-labeled compounds are commonly used, they may exhibit unexpected behavior, such as different retention times or recoveries compared to the analyte (Stokvis *et al.*, 2005). C-labeled internal standards have shown superior performance, co-eluting with their analytes under various chromatographic conditions and better compensating for ion suppression effects (Berg & Strand, 2011; Berg *et al.*, 2014). These isotopically enriched analogs are chemically identical to the target analytes but are distinguished by their predictable mass differences, allowing for clear differentiation by the mass spectrometer. During sample preparation, the SIL-IS and analyte exhibit matched extraction efficiencies and recovery rates due to their structural equivalence. Chromatographic separation demonstrates virtually identical retention times as both compounds interact similarly with the stationary phase. In mass spectrometric detection, they show nearly equivalent ionization efficiencies while maintaining distinct mass differences that enable clear differentiation (Ulvik *et al.*, 2021). This parallel behavior allows the analyte-to-SIL-IS peak area ratio to effectively

normalize multiple sources of variability, including matrix effects (ion suppression/enhancement), extraction inconsistencies, and instrumental fluctuations.

LC-MS/MS relies critically on efficient ion sources to convert analytes from the liquid phase into gas-phase ions for mass analysis. Electrospray ionization (ESI) and atmospheric pressure ionization (API) techniques serve as critical interfaces between liquid chromatography and mass spectrometry, enabling the analysis of diverse chemical compounds. In ESI, the sample solution passes through a charged capillary needle, where it's transformed into a fine mist of charged droplets (Tarr *et al.*, 2006). As these droplets move toward the mass spectrometer inlet, a countercurrent drying gas (usually nitrogen) facilitates solvent evaporation, causing the droplets to progressively shrink until Coulombic repulsion forces overcome surface tension, ultimately producing gas-phase analyte ions (Nguyen & Fenn, 2007). The resulting ions typically form protonated $[M+H]^+$ or deprotonated $[M-H]^-$ ions in positive or negative mode, and are then guided into the mass analyzer through a series of focusing elements. Related API techniques like APCI and APPI employ different mechanisms. APCI uses a heated nebulizer and corona discharge to ionize analytes through gas-phase reactions, while APPI utilizes UV light for direct or dopant-assisted ionization (Cai *et al.*, 2005; K. Hanold *et al.*, 2004). These complementary techniques expand the range of analyzable compounds, with APCI better suited for small, semi-polar molecules and APPI excelling with non-polar compounds. Together, these ionization methods provide researchers with versatile tools for detecting and quantifying compounds across numerous applications, from pharmaceutical development.

Mass analyzers, such as triple quadrupole mass spectrometers, are used to select specific ions, fragment them, and then analyze the resulting fragment ions. The triple quadrupole mass spectrometer (QQQ) consisting of two mass analyzers (Q1 and Q3) separated by a collision cell (Q2) (James & Arnott, 2001). The Q-Q-Q design allows for selective transmission of precursor ions, controlled fragmentation, and analysis of resulting product ions (Hager & Yves Le Blanc, 2003). The ionized samples enter the first quadrupole (Q1), which functions as a mass filter to selectively transmit precursor ions of a specific mass-to-charge ratio (m/z) while

excluding others. The selected ions subsequently pass into the second quadrupole (Q2), known as the collision cell, where they undergo controlled fragmentation through collisions with inert gas molecules under optimized collision energy conditions. Finally, the resulting product ions are analyzed by the third quadrupole (Q3), which can either scan across a range of m/z values or selectively monitor specific fragment ions. This sophisticated arrangement enables multiple operational modes, including highly sensitive multiple reaction monitoring (MRM) for quantitative analysis near the limit of quantitation (Hager & Yves Le Blanc, 2003).

The data obtained from LCMS/MS analysis include chromatograms and mass spectra. Chromatograms display the separation of analytes as a function of retention time(s), while mass spectra depict the abundance of ions at different mass-to-charge (m/z) ratios (Rodgman & Perfetti, 2008). Hybrid quadrupole time-of-flight mass spectrometers can be used, but have limited scanning functions when compared to triple quadrupole instruments (Pitt, 2009). This tandem mass spectrometry (MS/MS) approach enhances selectivity and sensitivity by monitoring specific precursor-to-product ion transitions, minimizing background interference.

In LC-MS/MS analysis, the Limit of Detection (LOD) and Limit of Quantification (LOQ) are fundamental parameters that define a method's sensitivity and reliability. The LOD represents the lowest analyte concentration that can be reliably detected, while the LOQ indicates the lowest concentration that can be quantitatively measured with acceptable precision and accuracy (Taleuzzaman, 2018). Various approaches exist for estimating LOD and LOQ, including signal-to-noise ratio, statistical analysis of blank samples, and calibration curve methods, with the most accurate results obtained using isotope-labeled internal standards to compensate for matrix effects (Shrivastava & Gupta, 2011). The signal-to-noise ratio criterion is commonly used, with LOD typically defined as 3:1 and LOQ as 10:1 (Taleuzzaman, 2018). The achievable LOD/LOQ values depend on multiple factors, including instrument sensitivity, ionization efficiency, chromatographic separation quality, and sample preparation techniques, typically ranging from pg/mL to ng/mL levels in pharmaceutical, environmental, and clinical applications. Method optimization strategies to improve LOD/LOQ include enhancing ionization

conditions, reducing matrix interference through improved sample cleanup, and employing high-sensitivity mass analyzers, with the ultimate goal of achieving reliable detection and quantification at the required concentration levels for each specific application (Li *et al.*, 2012).

Accurate measurement of nitrate and nitrite levels in tobacco is crucial for several reasons: it helps us understand tobacco product quality, assess safety, and evaluate the potential impact on human health. The determination of nitrate and nitrite in tobacco products presents its own set of analytical challenges. Spectrophotometric methods, ion chromatography, and electrochemical techniques are commonly employed for quantifying these inorganic anions. Spectrophotometric methods, based on the Griess reaction, involve the reaction of nitrite with a chromogenic reagent to form a colored product that can be measured spectrophotometrically. Ion chromatography provides a powerful means of separating and quantifying nitrate and nitrite based on their ionic properties. Electrochemical techniques, such as voltammetry, offer an alternative approach for determining nitrate and nitrite based on their electrochemical activity. LCMS/MS has emerged as a prominent analytical technique over traditional methods for the determination of nitrate and nitrite in diverse matrices, including tobacco, owing to its exceptional sensitivity, selectivity, and capability for simultaneous quantification of multiple analytes (Lisko *et al.*, 2013; Hongzhi *et al.*, 2013). Determining the levels of nornicotine, nitrate, and nitrite in tobacco products is essential for evaluating the potential health risks associated with their consumption. The role(s) played by the above-mentioned compounds as precursors to pro-carcinogens and/or carcinogens necessitate their accurate quantification for assessing the potential health risks and concomitantly develop effective mitigation strategies. Additionally, quantitative analysis of these compounds enables a more comprehensive understanding of adverse consequences associated with different tobacco products use.

Chapter-2

Methods and Material

2 Materials and Methods

2.1 Chemicals and reagents

All solvents and reagents used in the present study were of analytical grade or higher. Methanol (MeOH) and acetonitrile (ACN) were purchased from J. T. Baker (Center Valley, PA, USA). Ammonium acetate (AA), formic acid (FA), 2,3-diaminonaphthalene (DAN), nitrate reductase (from *Aspergillus niger*), β -nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt hydrate (β -NADPH), flavin adenine dinucleotide disodium salt hydrate (FAD), hydrochloride (HCl), sodium hydroxide (NaOH), nicotine, cotinine, sodium nitrate (NaNO_3), and sodium nitrite (NaNO_2) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Nornicotine, 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), d_4 -nornicotine, d_3 -4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (d_3 -NNK), and d_3 -NNAL were obtained from Toronto Research Chemicals (Ontario, Canada). d_4 -nicotine and d_3 -cotinine were obtained from Cerilliant (Round Rock, TX, USA). $\text{Na}^{15}\text{NO}_2$ and $\text{Na}^{15}\text{NO}_3$ were obtained from Cambridge Isotope Laboratories (Tewksbury, MA, USA). NNK and *N*-Nitrosornicotine (NNN) were kindly provided by Taiwan Food and Drug Administration (Taipei, Taiwan). $^{13}\text{C}_6$ -NNN was kindly provided by Dr. Hui-Lin Lee (Fu Jen Catholic University, Taiwan).

The stock solution of each standard and its corresponding stable isotope-labeled internal standard (SIL-IS) were prepared in 10% (v/v) MeOH at a final concentration of either 1000 $\mu\text{g/mL}$ or 100 $\mu\text{g/mL}$, and stored at -20°C . For the purpose of analyte quantification, a serial dilution of the stock solution was prepared using deionized water to prepare working solutions at different concentrations for establishing the calibration curves. The linear range for each analyte was as follows: 1.8 to 230 ng/mL for NO_2^- ; 0.24 to 62 $\mu\text{g/mL}$ for NO_3^- ; 2.44 to 1250 ng/mL for nicotine; 0.49 to 250 ng/mL for nornicotine; 0.049 to 25 ng/mL for cotinine; 0.0061 to 25 ng/mL for NNK, NNN, and NNAL. Each calibrator, depending on the purpose of NO_2^- , NO_3^- , TSAs or TSNAs quantification, contained the corresponding SIL-IS, including $^{15}\text{NO}_2^-$: 23 ng, $^{15}\text{NO}_3^-$: 155 ng, d_4 -nicotine: 1.5 ng, d_4 -nornicotine: 1.5 ng, d_3 -cotinine: 7.5 ng, d_3 -NNK: 0.8 ng, $^{13}\text{C}_6$ -NNN: 0.2 ng and d_3 -NNAL: 0.8 ng.

2.2 Sample Collection for Smoking Tobacco Products

Twenty-seven samples of smoking tobacco products were collected from various regions between April and December 2019. The collected samples were classified into four main categories: zozial (n = 9), beedi (n = 7), commercial cigarettes (n = 8), and water pipe tobacco (n = 3). **The photographs (Figure 2.1 a-e) and collection details of the collected samples are presented below (Table 2.1).** Among the commercial cigarettes, two brands from India (Wills SC and Capstan), two from Myanmar (Farstar and Valiant), and two from the USA (Marlboro Red Label and Camel Filters) were included, along with popular multinational brands (Marlboro) and the UK (Dunhill) available in India. The 3R4F Kentucky reference cigarettes were obtained from the Center for Tobacco Reference Products (University of Kentucky) and served as standard material for verifying the analytical method by comparing the data acquired from the current study with previous literature (Edwards *et al.*, 2017). All tobacco samples were carefully stored at -20°C until the sample pretreatment.

Tobacco filler from individual cigarettes was meticulously separated from the wrapper, except for beedis, which were processed with the entire filler and leaf wrapper. All tobacco samples were initially cut with scissors and then ground to achieve a fine, powder-like consistency to ensure homogeneity. 0.1 g of tobacco samples was weighed for the subsequent sample extraction of NO_2^- , NO_3^- , TSAs and TSNAs. The aqueous extraction of tobacco samples for each analyte was carried out using either deionized water or 100 mM AA buffer solution, which is described in detail below.



(a)



(b)



(c)



(d)



(e)



(f)

Fig. 2.1: Photographs of various smoking tobacco products

(a) Hookah, (b) Zozial, (c) Bidi, (d) Commercial Cigarette, (e) Eastern brand cigarette, (f) Western brand cigarette, (g) 3R4F (Reference cigarette)

Table 2.1: Details of Sample Collection for Smoking Tobacco Products

Sample category	Description	Brand name	Place of manufacture
Hookah	<u>Tobacco used for waterpipe</u> Tobacco type: Blend of Virginia and burley tobacco Fertilizer used: Yes Processing after harvesting: Flue-cured and sun-cured; hydrothermal treatment – washing at 85 to 90 °C hourly for 8 h; unflavoured and/or flavour additives; burned using charcoal pellets [Qasim <i>et al.</i> , 1995].	1. B freezer 2. Strawberry Island 3. Double Apple	Lucknow, North India
Zozial	<u>Hand-rolled local cigarette</u> Tobacco type: Locally grown oriental tobacco (<i>Nicotiana tabacum</i>) Fertilizer used: No Processing after harvesting: Pre-drying of mature tobacco leaves for 1-2 days under the sun; wilted tobacco leaves are crushed using feet; sun-cured for 10 -15 days; no cellulose acetate-type filter used (Northeast India) [Lalrammawia 2021]	1. Zozial (dilkawn) 2. Zozial (lianpui-1) 3. Zozial (lianpui-2) 4. Zozial (ZZ-3) 5. Zozial (ZZ-4) 6. Zozial (TLF-1) 7. Zozial (TLF-2) 8. Zozial (TLF-3) 9. STP precursor	Mizoram, Northeast India
Beedi	<u>Thin cigarette with tobacco wrapped in leaf</u> Tobacco type: Locally grown Beedi tobacco (<i>Nicotiana tabacum</i>) Fertilizer used: Yes Processing after harvesting: Whole plant is sun-cured for 2 - 3 weeks; coarsely shredded tobacco; wrapped in Tendu/Temburni leaf (<i>Diospyros melanoxylon</i>); Unfiltered and hand-rolled (India) [Mahimkar <i>et al.</i> , 1995; Gupta and	1. Beedi 501 2. Beedi Kajahseya du 3. Beedi Seyadu 4. Beedi 207 5. Beedi Pataka 502 6. Beedi Dum Dum 7. Beedi 82	1. Karnataka, Southwest India 2. Tamil Nadu, Southeast India 3. Tamil Nadu, Southeast India 4. Delhi, North Central India 5. Uttar Pradesh, North India 6. West Bengal, Eastern India 7. West Bengal,

	Asma, 2008]		Eastern India
Commercial cigagrette	<u>Eastern brand</u> (1) Capstan Tobacco type: Virginia tobacco Fertilizer used: Yes Processing after harvesting: Flue-cured (2) Wills Silk Cut Tobacco type: Virginia tobacco Fertilizer used: Yes Processing after harvesting: Flue-cured (3) Farstar Tobacco type: Virginia tobacco Fertilizer used: Yes Processing after harvesting: Flue-cured (4) Valiant Tobacco type: Burley tobacco (could be) Fertilizer used: Yes Processing after harvesting: Air-cured (could be)	Eastern brand 1. Capstan 2. Wills Silk cut 3. Farstar 4. Valiant	
	Western brand (5) Marlboro Red Label Tobacco type: Blend of Bright (Virginia) and Burley tobacco Fertilizer used: Yes Processing after harvesting: flue-cured (6) Marlboro International Label Tobacco type: Blend of Bright (Virginia) and Burley tobacco Fertilizer used: Yes Processing after harvesting: flue-cured (7) Camel Filters Tobacco type: Blend of Turkish/oriental tobacco and Virginia tobacco Fertilizer used: Yes Processing after harvesting: Sun-cured and Flue-cured (8) Dunhill international	Western brand 1. Marlboro Red label 2. Camel filters 3. Marlboro Premium 4. UK Dunhill	Western brand 1. Richmond, USA 2. Winston, USA 3. Multinational brand (India) 4. Multinational brand (India)

	Tobacco type: Virginia tobacco Fertilizer used: Yes Processing after harvesting: Flue-cured		
3R4F	(1) 3R4F, Kentucky Reference cigarette, Research cigarette Tobacco type: Flue Cured 35.41%, Burley 21.62%, Oriental 12.07%, Maryland 1.35%	Kentucky Tobacco Research & Development Center	Kentucky Tobacco Research & Development Center

2.3 Sample collection for smokeless tobacco products

Twenty-nine samples of smoking tobacco products were collected from various regions between April 2019 and December 2019. The collected samples were classified into six main categories: sahdah (n = 11), khaini (n = 6), zarda (n = 8), snus (n = 3), guthka (n=1), supari (n=4). A photograph of the samples is presented below (Fig. 2.2).

All tobacco samples were manufactured in India, except for three snus samples sourced from the USA. Additionally, the CORESTA Reference Products (CRPs) were procured from the Tobacco Analytical Science Lab at North Carolina State University (USA). These CRPs served as standardized reference materials for smokeless tobacco, enabling direct comparison between experimental data and the reference values in this study. To maintain sample integrity, all tobacco specimens, including the CRPs, were stored under controlled conditions at -20°C until sample pretreatment.

All tobacco samples were ground to achieve a fine, powder-like consistency to ensure homogeneity. 0.1 g of tobacco sample was weighed for the subsequent extraction of NO_2^- , NO_3^- , TSAs, and TSNAs. The aqueous extraction of tobacco samples for each analyte was performed using either deionized water or a 100 mM AA buffer solution, as described in detail below.



(a)



(b)



(c)



(d)



(e)



(f)



(g)

Fig. 2.2: (a-g) Photograph of various smokeless tobacco products

(a) Sadah, (b) Khaini, (c) Zarda, (d) Snus, (e) Guthka, (f) Supari

2.4 Simultaneous determination of nicotine, nornicotine and cotinine in tobacco

A volume of 5 mL–deionized water was used to extract TSAs from 0.1 g powdered tobacco samples. The resulting mixture was shaken using orbital shaker S101 (FIRSTTEK) at 200 rpm for 1 h at room temperature and sonicated for an additional 1 h using ultrasonic cleaner ELMA LC-130/H (Sumber Aneka Karya Abadi) to achieve complete extraction. After centrifugation at $2790 \times g$ for 10 minutes, the supernatant was filtered using a 13 mm, $0.22 \mu\text{m}$ Nylon syringe filter. The filtrate was subsequently diluted 500-fold with deionized water. A $50 \mu\text{L}$ aliquot of 500-fold diluted filtrate was collected and combined with $50 \mu\text{L}$ of mixture solution of SIL-IS (d_4 -nicotine: 30 ng/mL , d_4 -nornicotine: 30 ng/mL , and d_3 -cotinine: 15 ng/mL). The prepared sample was ready for injection $10 \mu\text{L}$ into the LC-MS/MS system.

2.5 Determination of nitrite and nitrate in tobacco

To extract NO_2^- and NO_3^- from tobacco samples, the same procedures of water extraction and subsequent filtration as those described in TSAs extraction were followed. The resulting filtrate was then diluted using deionized water to make 2-fold and 10-fold dilution filtrates for the analysis of NO_2^- and NO_3^- , respectively.

2.5.1 Nitrite Analysis

For NO_2^- analysis, a 250 μL aliquot of 2-fold diluted filtrates was collected and combined with 250 μL of SIL-IS ($^{15}\text{NO}_2^-$: 92 ng/mL). The resulting 500 μL sample mixture was then subjected to derivatization with DAN (6.25 mM) in 0.5 M HCl at 37 °C for 30 minutes to yield nitrite-DAN derivatives (i.e., 2,3-naphthotriazole, NAT) and ^{15}NAT . The reaction was terminated by adding 0.58 M NaOH. The resulting reaction mixture was then diluted 10-fold with 10% (v/v) methanol containing 1 mM AA solution, and the mixture was ready for injection of 10 μL into the LC-MS/MS system.

2.5.2 Nitrate Analysis

For NO_3^- analysis, it was required to convert NO_3^- to NO_2^- by nitrate reductase (from *Aspergillus niger*), as previously described (Chao et al. 2016) with minor modifications. A 10 μL aliquot of 10-folds diluted filtrates was combined with 10 μL of SIL-IS ($^{15}\text{NO}_3^-$: 15.5 $\mu\text{g/mL}$). The resulting mixture was then incubated at room temperature for 2 h with 95 μL of nitrate reductase solution containing 10 mU of nitrate reductase, 12 mM NADPH, 10 mM phosphate buffered saline (pH 7.5) and 0.1 mM FAD. This allowed for the reduction of NO_3^- to NO_2^- . To continue the derivatization reaction, the mixture containing the total NO_2^- (initial NO_2^- plus NO_2^- reduced from NO_3^-) was diluted 20-fold with deionized water and processed following the same procedure described for NO_2^- . The concentration of NO_3^- was calculated by subtracting the initial NO_2^- concentration from the total NO_2^- concentration.

2.6 Simultaneous determination of NNK, NNN and NNAL in tobacco

The extraction of TSNA from tobacco was conducted following the CORESTA recommended method⁴⁰ [CORESTA, 2021], with minor modifications. A volume of 3 mL of 100 mM AA solution was added into 0.1 g of tobacco. The resulting mixture

was then shaken at 200 rpm at room temperature for 1 h and sonicated for additional 1 h to achieve complete extraction. The extracts were subjected to centrifugation and filtration, following the same procedures described for TSAs. The resulting filtrate was subsequently diluted 10-fold with deionized water. A 100 μ L aliquot of 10-folds diluted filtrate was added with a 100 μ L of mixture solution of SIL-IS (d_3 -NNK: 8 ng/mL, $^{13}C_6$ -NNN: 2 ng/mL or d_3 -NNAL: 8 ng/mL). The prepared sample is ready for injection of 50 μ L into the LC-MS/MS system.

2.6.1 LC-MS/MS analysis

All calibrators and prepared samples were injected into an LC-MS/MS instrument coupled with an online solid phase extraction (SPE) system for the measurement of TSAs, NO_2^- , NO_3^- (Chao et al. 2016) and TSNA^s⁴¹ (Hu et al. 2018), as described previously. Detailed description of LC-MS/MS analysis is discussed below.

The online solid-phase extraction (SPE) coupled with liquid chromatography (LC) method for the determination of NAT (DAN derivatives of nitrite and nitrate) was carried out using a well-defined gradient program involving two synchronized pumps and a switching valve system. Initially, from 0.0 to 2.0 minutes, the system was set to valve position A, with Pump I delivering a mixture of 40% methanol [10% (v/v)], containing 1 mM ammonium acetate aqueous solution ($S1^a$) and 60% methanol [75% (v/v)], containing 1 mM ammonium acetate aqueous solution ($S2^b$) at 200 μ L/min to load and clean up the sample, while Pump II maintained the same solvent ratio and flow rate to equilibrate the LC-MS/MS system. At 2.0 minutes, the valve was switched to position B to allow analyte elution from the SPE column to the analytical column for separation, continuing at the same conditions. Shortly after, at 2.1 minutes, Pump I was switched to 100% $S2^b$ to flush the SPE column, while Pump II continued the elution at 200 μ L/min. From 4.5 to 6.5 minutes, Pump II shifted to 100% solvent $A2^b$, and the valve returned to position A to clean the SPE column. Re-equilibration began at 6.6 minutes with both pumps returning to the initial solvent ratios (40% $S1^a/A1^a$ and 60% $A1^a/A2^b$), maintaining this composition through 11.5 minutes to prepare for the next injection cycle. This automated online-SPE-LC setup ensured efficient sample loading, cleanup, analyte elution, column washing, and re-equilibration in a single, continuous run. The detailed program of online-SPE

coupled with LC separation for the determination of NAT (DAN derivatives of nitrite and nitrate is given in Table 2.2.

Table 2.2: Detailed program of online-SPE coupled with LC separation for the determination of NAT (DAN derivatives of nitrite and nitrate).

Time (min)	Pump I (online SPE)			Pump II (LC separation)			Valve position ^c	Remarks
	S1 ^a (%)	S2 ^b (%)	Flow rate (μL/min)	A1 ^a (%)	A2 ^b (%)	Flow rate (μL/min)		
0.0	40	60	200	40	60	200	A	Sample loading and cleanup; equilibration of LC-MS/MS system
2.0	40	60	200	40	60	200	B	Analyte elution from SPE to LC main column; analyte separation
2.1	0	100	200			200		
4.5			200	0	100	200	A	Cleaning of SPE column
6.5	0	100	200	0	100	200		
6.6	40	60	200	40	60	200		Re-equilibration of column
11.5	40	60	200	40	60	200		

^a 10% (v/v) methanol containing 1 mM ammonium acetate aqueous solution.

^b 75% (v/v) methanol containing 1 mM ammonium acetate aqueous solution.

^c The valve position was located at A to lead the fluid to waste, while the position at B led the fluid into LC-MS/MS.

The SPE for the determination of nicotine, nor nicotine, and cotinine was carried out based on the same time-programmed gradient described for the determination of NAT. At the start of the run (0.0 min), Pump I delivered 100% solvent S1^a (2% (v/v) methanol containing 0.1% trifluoroacetic acid) at a flow rate of 200 μL/min for sample loading and cleanup, while Pump II operated with 95% solvent A1^c (2% (v/v) methanol containing 0.1% formic acid) at 200 μL/min to equilibrate the LC-MS/MS system, with the valve set to position A. At 1.0 minute, the valve was

switched to position B to direct the analytes from the SPE column to the analytical LC column for separation. By 3.0 minutes, the valve returned to position A to allow continued separation and initiate SPE column cleaning. At 3.1 minutes, Pump I was switched to 100% solvent S2^b (75% (v/v) methanol containing 0.1% trifluoroacetic acid) to flush the SPE column, while Pump II maintained its flow. Between 4.6 and 6.6 minutes, Pump II shifted to a composition of 65% A1^c and 35% A2^d (50% (v/v) methanol containing 0.1% formic acid) for optimal separation. At 6.7 minutes, the mobile phase was changed to 100% A2^d to clean the LC column, and SPE cleaning continued with 100% S2 until 7.0 minutes. Re-equilibration of the SPE column began at 7.1 minutes by reverting Pump I to 100% S1. At 8.7 minutes, Pump II was maintained at 100% S2^b before returning to the initial condition (95% A1^c and 5% A2^d) at 8.8 minutes. The full cycle concluded at 15.0 minutes, ensuring full re-equilibration of both columns. This automated and synchronized SPE-LC setup enabled efficient online sample preparation, analyte transfer, separation, and system reconditioning for reliable quantification of nicotine, nornicotine, and cotinine. Detailed program of online-SPE coupled with LC separation for determination of nicotine, nornicotine and cotinine is given in Table 2.3.

Table 2.3: Detailed program of online-SPE coupled with LC separation for determination of nicotine, nornicotine and cotinine

Time (min)	Pump I (online SPE)			Pump II (LC separation)			Valve position ^e	Remarks
	S1 ^a (%)	S2 ^b (%)	Flowrate (μL/min)	A1 ^c (%)	A2 ^d (%)	Flow rate (μL/min)		
0.0	100	0	200	95	5	200	A	Sample loading and cleanup; equilibration of LC-MS/MS system
1.0			200			200	B	Analyte elution from SPE to LC main column
3.0	100	0	200	95	5	200	A	SPE column cleaning; analyte separation
3.1	0	100	200			200		Cleaning of SPE column
4.6			200	65	35	200		
6.6			200	65	35	200		
6.7			200	0	100	200		Cleaning of LC main column
7.0	0	100	200			200		Cleaning of SPE column
7.1	100	0	200			200		Re-equilibration of SPE column
8.7			200	0	100	200		
8.8			200	95	5	200		Re-equilibration of column
15.0	100	0	200	95	5	200		

^a 2% (v/v) methanol containing 0.1% trifluoroacetic acid.

^b 75% (v/v) methanol containing 0.1% trifluoroacetic acid.

^c 2% (v/v) methanol containing 0.1% formic acid.

^d 50% (v/v) methanol containing 0.1% formic acid.

^e The valve position was located at A to lead the fluid to waste, while the position at B led the fluid into LC-MS/MS.

The SPE for the determination of NNK, NNN, and NNAL was performed based on the same time-programmed gradient used for NAT determination. At the beginning of the run (0.0 min), Pump I delivered 90% solvent S1^a (5% (v/v) methanol containing 1 mM ammonium acetate aqueous solution.) and 10% S2^b (75% (v/v) methanol containing 1 mM ammonium acetate aqueous solution) at a flow rate of 200 µL/min to facilitate sample loading and cleanup, while Pump II operated with 80% solvent A1^c (5% (v/v) methanol containing 0.1% formic acid) and 20% A2^d (75% (v/v) methanol containing 0.1% formic acid) at 200 µL/min to equilibrate the LC-MS/MS system, with the valve in position A. The system was maintained under these conditions until 8.0 minutes, when the valve was switched to position B to allow the elution of analytes from the SPE column to the main LC column for separation. At 9.0 minutes, the LC mobile phase composition was adjusted to 70% A1^c and 30% A2^d to enhance analyte resolution. At 10.5 minutes, Pump I returned to the initial condition of 90% S1^a and 10% S2^b, followed by a switch to 100% S2^b at 10.6 minutes for SPE column cleaning. Analyte separation continued until 12.5 minutes. At 13.0 minutes, the LC column was cleaned using 100% A2^d, and then re-equilibrated back to 80% A1^c and 20% A2^d at 14.1 minutes. Meanwhile, SPE column re-equilibration began at 14.5 minutes by switching back to 100% S1^a, and by 15.0 minutes, the initial condition (90% S1^a/ 10% S2^b) was restored. The system returned to full re-equilibration for both SPE and LC channels by 19.0 minutes, ensuring readiness for the next injection. This fully automated and integrated online-SPE-LC allowed for efficient sample cleanup, analyte elution, and reliable separation of NNK, NNN, and NNAL. Detailed program of online-SPE coupled with LC separation for determination of NNK, NNN and NNAL is given in Table 2.4.

Table 2.4: Detailed program of online-SPE coupled with LC separation for determination of NNK, NNN and NNAL

Time (min)	Pump I (online SPE)			Pump II (LC separation)			Valve position ^e	Remarks
	S1 ^a (%)	S2 ^b (%)	Flow rate (μL/min)	A1 ^c (%)	A2 ^d (%)	Flow rate (μL/min)		
0.0	90	10	200	80	20	200	A	Sample loading and clean up; equilibration of LC-MS/MS system
7.0			200	80	20	200		
8.0			200			200	B	Analyte elution from SPE to LC main Column; analyte separation
9.0			200	70	30	200		
10.5	90	10	200			200		
10.6	0	100	200			200		Cleaning of SPE column
11.0			200	70	30	200		
12.5			200			200	A	
13.0			200	0	100	200		Cleaning of LC main column
14.0			200	0	100	200		
14.1			200	80	20	200		Re-equilibration of LC main column
14.5	0	100	200			200		Re-equilibration of SPE column
15.0	90	10	200			200		
19.0	90	10	200	80	20	200		

^a 5% (v/v) methanol containing 1 mM ammonium acetate aqueous solution.

^b 75% (v/v) methanol containing 1 mM ammonium acetate aqueous solution.

^c 5% (v/v) methanol containing 0.1% formic acid.

^d 75% (v/v) methanol containing 0.1% formic acid.

^e The valve position was located at A to lead the fluid to waste, while the position at B to lead the fluid into LC-MS/MS.

The simultaneous quantification of NAT (nitrite-DAN derivatives) and its isotopically labeled internal standard, ^{15}NAT , was performed using Multiple Reaction Monitoring (MRM) mode with specific mass spectrometric parameters. The precursor-to-product ion transitions were set at m/z 170 $\rightarrow$ 115 for NAT and m/z 171 $\rightarrow$ 115 for ^{15}NAT , respectively, both producing a common fragment at m/z 115. A dwell time of 100 ms was applied to each transition to ensure adequate signal collection. The declustering potential (DP) and entrance potential (EP) were both optimized at 20 V and 10 V, respectively, to enhance ion stability and efficient transmission into the mass analyzer. The collision energy (CE) was set at 30 eV to induce effective fragmentation of the precursor ions, while a collision cell exit potential (CXP) of 15 V was used to facilitate the transfer of product ions into the third quadrupole. These optimized parameters ensured high sensitivity and selectivity for accurate quantification of NAT in the presence of its labeled analog. MRM parameters used for the simultaneous quantification of NAT are given in Table 2.5.

Table 2.5: MRM parameters used for the simultaneous quantification of the NAT (nitrite-DAN derivatives)

Analytes	Q1 ion (m/z)	Q3 ion (m/z)	Dwell time (ms)	DP (V)	EP (V)	CXP (V)	CE (eV)
NAT	170	115	100	20	10	15	30
^{15}NAT	171	115	100	20	10	15	30

DP, declustering potential; EP, entrance potential; CXP, collision exit potential; CE, collision energy.

The simultaneous quantification of nicotine, nornicotine, and cotinine, along with their respective isotopically labeled internal standards, was carried out using MRM mode with optimized mass spectrometric parameters. For nicotine, two MRM transitions were monitored: m/z 163 $\rightarrow$ 132 with a collision energy (CE) of 22 eV and m/z 163 $\rightarrow$ 130 with a CE of 29 eV, while its internal standard, d_4 -nicotine, was detected using the transition m/z 167 $\rightarrow$ 136 at 22 eV. Nornicotine was quantified using transitions m/z 149 $\rightarrow$ 132 and 149 $\rightarrow$ 130 with collision energies of 19 eV and 25 eV, respectively, and its internal standard, d_4 -nornicotine, was monitored via the transition m/z 153 $\rightarrow$ 136 with a CE of 19 eV. For cotinine, the transitions m/z

177 → 80 and 177 → 98 were used with CE of 35 eV and 29 eV, respectively, and its internal standard, d₃-cotinine, was detected using the transition m/z 180 → 80 at 35 eV. A consistent dwell time of 100 ms was applied to all transitions, ensuring sufficient ion monitoring. The DP ranged from 31 V to 50 V, optimized for each compound, while the EP was set at 10 V across all analytes. CXP varied slightly to ensure efficient ion transmission, ranging from 4 V to 12 V depending on the compound and transition. These finely tuned MRM parameters enabled sensitive and selective quantification of nicotine, nornicotine, and cotinine in complex sample matrices.

Table 2.6: MRM parameters used for the simultaneous quantification of the nicotine, nornicotine and cotinine

Analytes	Q1 ion (m/z)	Q3 ion (m/z)	Dwell time (ms)	DP (V)	EP (V)	CXP (V)	CE (eV)
Nicotine	163	132	100	40	10	6	22
	163	130	100	40	10	6	29
d ₄ -Nicotine	167	136	100	40	10	6	22
Nornicotine	149	132	100	31	10	8	19
	149	130	100	31	10	8	25
d ₄ -Nornicotine	153	136	100	31	10	8	19
Cotinine	177	80	100	50	10	12	35
	177	98	100	50	10	4	29
d ₃ -Cotinine	180	80	100	50	10	12	35

The quantifier ion of each alkaloid was labeled in bold; DP, declustering potential; EP, entrance potential; CXP, collision exit potential; CE, collision energy.

The simultaneous quantification of NNK, NNN, and NNAL, along with their respective isotopically labeled internal standards, was performed using MRM mode with carefully optimized mass spectrometric parameters. For NNK, two transitions were monitored: m/z 208 → 122 with a CE of 15 eV and m/z 208 → 79 with a higher CE of 52 eV, enabling sensitive detection of both a stable and a high-energy fragment. Its internal standard, d₃-NNK, was monitored using the transition m/z 211 → 122 with the same CE of 15 eV. NNN was quantified using the transitions m/z

178 → 148 and 178 → 120, with respective collision energies of 15 eV and 25 eV. The internal standard for NNN, ¹³C₆-NNN, was detected using the transition m/z 184 → 154 at a CE of 15 eV. For NNAL, the transitions m/z 210 → 180 and 210 → 93 were used with collision energies of 15 eV and 30 eV, respectively, while its internal standard, d₃-NNAL, was monitored at m/z 213 → 183 with a CE of 15 eV. All analytes were monitored with a dwell time of 100 ms to ensure adequate signal acquisition. The DP ranged from 15 V to 40 V depending on the compound and transition, and the EP was between 5 V and 10 V. CXP varied from 4 V to 15 V, optimized to ensure effective transmission of product ions. These parameters enabled highly sensitive and specific quantification of the tobacco-specific nitrosamines NNK, NNN, and NNAL in samples.

Table 2.7: MRM parameters used for the simultaneous quantification of the NNK, NNN and NNAL

Analytes	Q1 ion (m/z)	Q3 ion (m/z)	Dwell time (ms)	DP (V)	EP (V)	CXP (V)	CE (eV)
NNK	208	122	100	25	5	8	15
	208	79	100	25	5	4	52
d ₃ -NNK	211	122	100	25	5	8	15
NNN	178	148	100	15	6	9	15
	178	120	100	40	10	5	25
¹³ C ₆ -NNN	184	154	100	15	6	9	15
NNAL	210	180	100	30	5	15	15
	210	93	100	30	5	15	30
d ₃ -NNAL	213	183	100	30	5	15	15

The quantifier ion of each alkaloid was labeled in bold; DP, declustering potential; EP, entrance potential; CXP, collision exit potential; CE, collision energy.

2.7 Measurement of pH

A portion of the water extracts from each tobacco sample, obtained from the extraction process described earlier, was collected separately for pH measurement. The pH of the aqueous extract was determined using a pH meter (6177M, JENCO).

Prior to measuring the pH of the tobacco samples, the pH meter was calibrated using pH buffer solution at pH 4, 7, and 10 to ensure accuracy.

2.8 Statistical analysis

The geometric mean (GM) was utilized to summarize the quantified results of all analytes detected in tobacco samples due to their non-normal distributions. Spearman's rank correlation coefficient was employed to assess the correlation between variables. Multivariate regression analysis was performed to examine the combined influence of multiple independent variables, such as pH and nitrate, on the conversion efficiency of TSAs to TSNAs. Furthermore, it was employed to evaluate the regression of the contents of sum of TSNAs against two nitrosation-associated factors, including the nitrate contents and the sum of TSAs, and a regression equation was developed for predicting the outcome (i.e., formation of TSNAs) based on both nitrate content and TSA levels. To ensure the accuracy and reliability of the multivariate regression model, the datasets were normalized using Box-Cox transformation (Marimuthu et al. 2022) to mitigate the effect of non-normality and outliers. All data analysis was conducted using either SPSS software (version 25) or SigmaPlot version 15.

Chapter-3

Result and Discussion

3.1 Smoking Tobacco Products

3.1.1 The pH of smoking tobacco products

The pH values of suspended tobacco filler powder of different tobacco products in water (0.02 g/ml) were measured and are summarized in Table 3.1. Hookah (Water pipe tobacco) exhibited a geometric mean (GM) pH of 5.3, with values ranging from 4.1 to 6.2. Zozial tobacco showed a GM pH of 6.7 with a range of 5.9–7.2, while beedi tobacco had a slightly higher GM pH of 6.9, ranging from 6.2 to 7.6. The pH of commercial cigarette tobacco suspensions demonstrated greater variability, with a GM value of 6.4 ranged between pH 4.5–7.3. The distribution of pH values of different smoking tobacco products is illustrated in Fig. 3.1. Interestingly, the water pipe tobacco exhibited a significantly lower pH compared to the other three types of smoking tobacco products, and this difference was found to be statistically significant ($P < 0.05$) according to one-way ANOVA analysis. In contrast, the variations in pH values of zozial, commercial cigarettes, and beedi tobacco were not statistically significant ($P > 0.05$).

Table 3.1: The pH values in various smoking tobacco products.

Category	pH values
Beedi	6.9 ^a (6.2-7.6) ^b
Zozial	6.7 (5.9-7.2)
Hookah	5.3 (4.1-6.2)
Commercial cigarettes	6.4 (4.5-7.3)

^aGeometric mean

^bRange

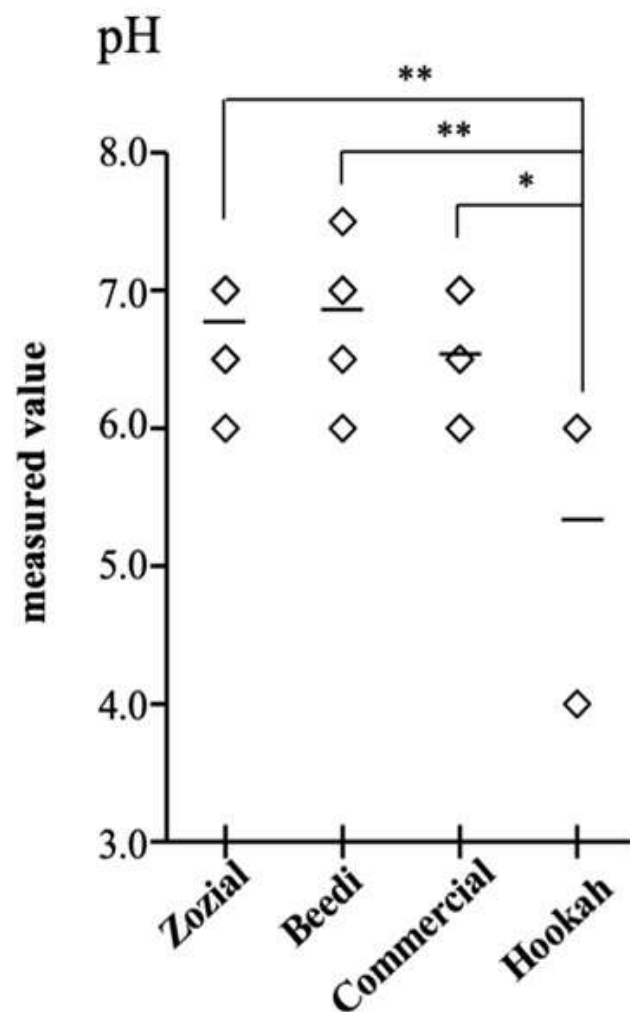


Fig. 3.1; Distribution of pH values in different smoking tobacco products including Zozial, Bidi, Commercial cigarette, and Hookah, is presented, with horizontal lines indicating GM. Group differences were assessed using one-way ANOVA, and results with significant differences are denoted by asterisks: * for $p < 0.05$ and ** for $p < 0.01$.

**highly significant; *significant; — GM of pH

3.1.2 Nitrite and nitrate levels in smoking tobacco products

The nitrite and nitrate concentrations in various tobacco products exhibited considerable variation. The quantitative results are summarized in Table 3.2. Beedi samples showed a geometric mean nitrite level of 0.81 µg/g, ranging from 0.75 to 0.95 µg/g, and a nitrate concentration of 7,362.27 µg/g (range: 5,663.95–17,140 µg/g). In zozial, a slightly higher nitrite levels with a GM of 1.19 µg/g (range: 0.78–1.63 µg/g), but markedly lower nitrate levels at 704.56 µg/g (535.55–920.64 µg/g) were observed. Hookah tobacco contained the lowest nitrite levels among all tobacco products analyzed, with a GM of 0.55 µg/g (0.46–0.78 µg/g), and nitrate levels at 742.97 µg/g (627.87–858.03 µg/g). Among commercial cigarettes, Myanmar cigarettes presented nitrite levels of 1.58 µg/g (1.24–2.00 µg/g) and nitrate concentrations of 7,985.74 µg/g (7,970.69–8,000.81 µg/g). USA cigarettes exhibited the highest nitrite content, with a GM of 1.94 µg/g (1.92–1.96 µg/g), and nitrate at 7,772.65 µg/g (7,394.57–8,170.04 µg/g). Indian cigarettes had nitrite levels of 1.23 µg/g (1.00–1.50 µg/g) and nitrate at 6,593.69 µg/g (4,368.41–9,952.51 µg/g). Among multinational brands available in NEI, Marlboro cigarettes contained 1.55 µg/g nitrite (range: 0–1.55 µg/g) and the highest nitrate content across all samples at 12,248.77 µg/g (0–12,248.77 µg/g). Dunhill cigarettes exhibited nitrite levels of 1.57 µg/g (0–1.57 µg/g) and lower nitrate content at 3,125.76 µg/g (0–3,126.76 µg/g). These results indicate that while nitrate levels varied substantially across product categories, nitrite concentrations were generally elevated in most commercial and multinational cigarette products compared to traditional forms like Hookah or Beedi.

The distribution of nitrite and nitrate in four different categories of smoking tobacco products is depicted in Fig. 3.2 and 3.3, respectively. Commercial cigarettes includes Myanmar cigarettes, USA cigarettes, Indian cigarettes and multinational cigarettes available in Northeast India (NEI). Furthermore, the nitrite-to-nitrate ratio was calculated using the measured concentrations to assess the conversion from nitrate to nitrite in various tobacco products. Pairwise comparisons following one-way ANOVA revealed statistically significant differences in nitrite concentrations among the tobacco product groups. Specifically, nitrite levels were significantly

higher in zozial compared to Beedi ($p < 0.05$), commercial cigarettes ($p < 0.05$), and hookah tobacco ($p < 0.05$). Commercial cigarettes exhibited significantly higher nitrite concentrations than both Beedi ($p < 0.01$) and hookah tobacco ($p < 0.01$). Similarly, **nitrate concentrations** varied significantly across the tobacco products. Pairwise comparisons showed statistically significant differences between Zozial and Beedi ($p < 0.01$), Zozial and commercial cigarettes ($p < 0.01$), Beedi and hookah tobacco ($p < 0.01$), and commercial cigarettes and hookah tobacco ($p < 0.01$).

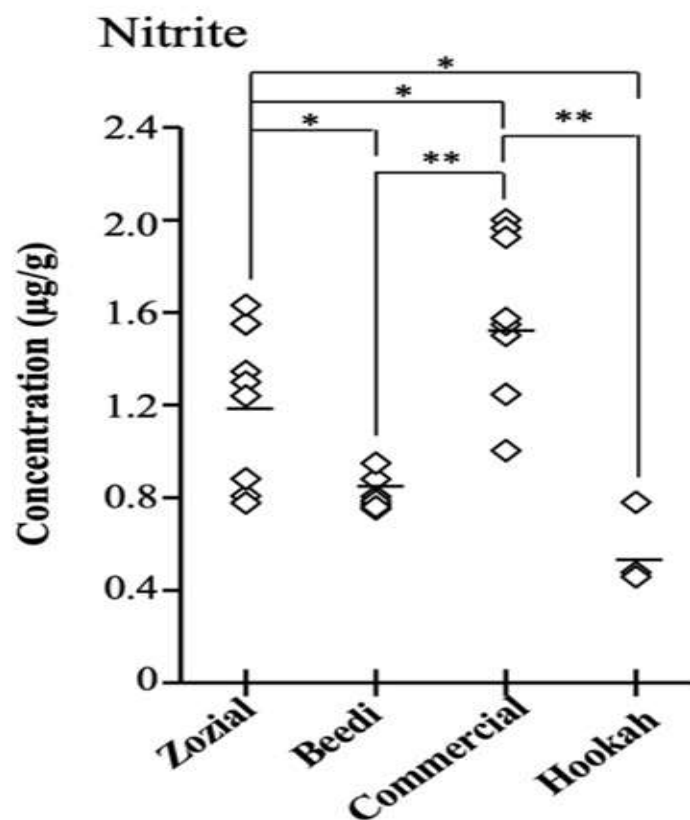


Fig. 3.2: The distribution of nitrite and nitrate in different smoking tobacco products including Zozial, Beedi, Commercial cigarette, and Hookah, is presented, with horizontal lines indicating GM. Group differences were assessed using one-way ANOVA, and results with significant differences are denoted by asterisks: * for $p < 0.05$ and ** for $p < 0.01$.

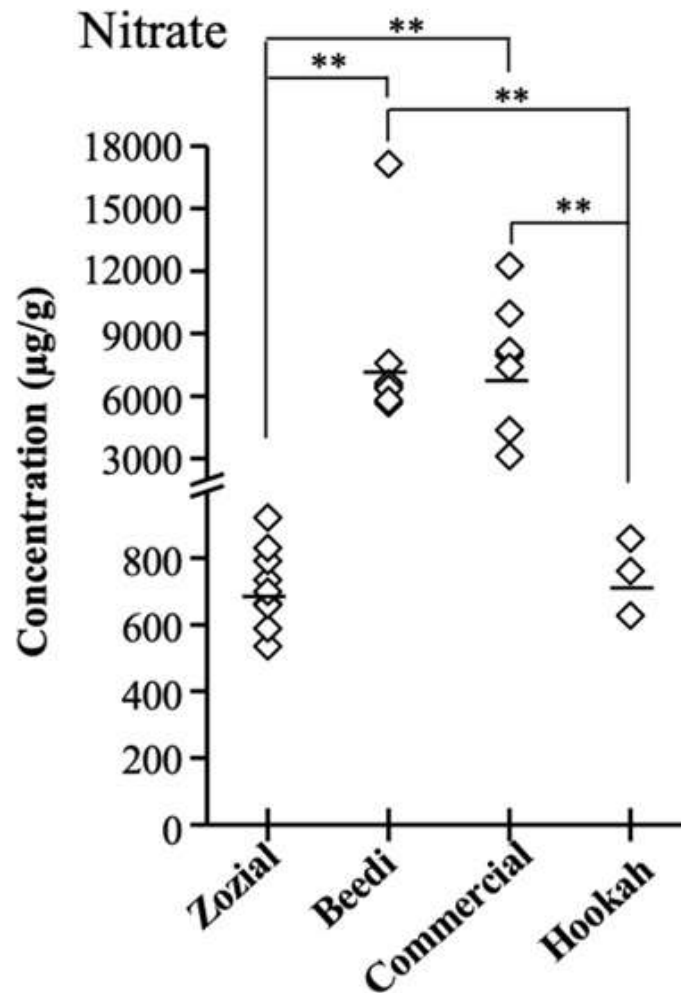


Fig. 3.3: The distribution of nitrate in different smoking tobacco products including Zozial, Beedi, Commercial cigarette and Hookah, are presented, with horizontal lines indicating GM. Group differences were assessed using one-way ANOVA, and results with significant differences are denoted by asterisks: ** for $p < 0.01$.

Table 3.2: GM value of nitrite and nitrate in different smoking tobacco products

Category		Nitrite (µg/g)	Nitrate(µg/g)
Beedi		0.81 ^a (0.75-0.95) ^b	7362.27 (5663.95-17140)
Zozial		1.19 (0.78-1.63)	704.56 (535.55-920.64)
Hookah		0.55 (0.46-0.78)	742.97 (627.87-858.03)
Commercial cigarettes	Myanmar cig	1.58 (1.24-2.00)	7985.74 (7970.69-8000.81)
	Usa cig	1.94 (1.92-1.96)	7772.65 (7394.57-8170.04)
	Indian cig	1.23 (1.00-1.50)	6593.69 (4368.41-9952.51)
	Multinational cigarettes (Marlboro)	1.55 (0-1.55)	12248.77 (0-12248.77)
	Multinational cigarettes (Dunhill)	1.57 (0-1.57)	3125.76 (0-3126.76)

^aGeometricmean^bRange

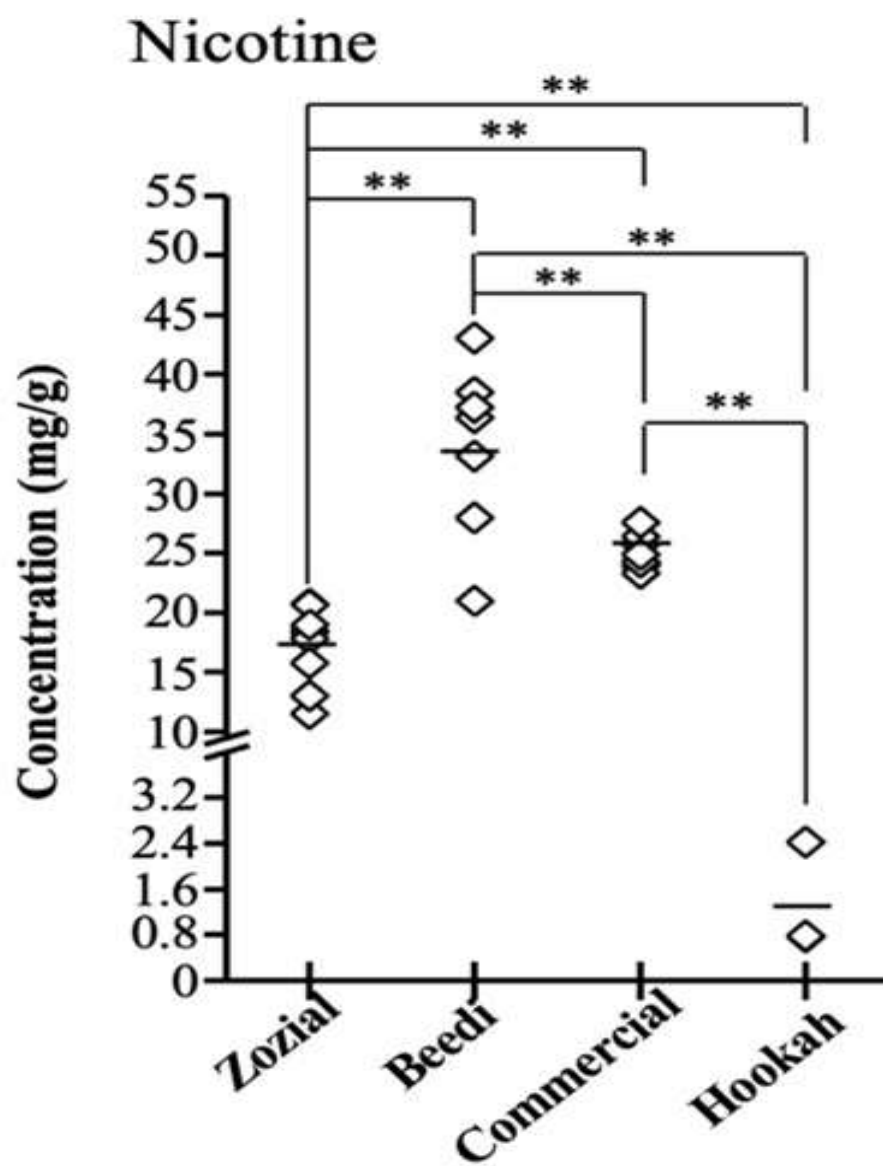
3.1.3 Alkaloids contents

The alkaloid profile—comprised of nicotine, cotinine, and nornicotine, in various smoking tobacco products were quantified. The measured values are summarized in Table 3.3. Beedi tobacco exhibited the highest total alkaloid content (34,606.87 µg/g), with nicotine as the predominant component (33,161.91 µg/g), followed by significant levels of cotinine (353.10 µg/g) and nornicotine (1,091.86 µg/g). Zozial tobacco showed substantially lower alkaloid levels, with a total of 17295.6 µg/g, consisting of 16995 µg/g of nicotine, 39.60 µg/g of cotinine, and 261 µg/g of nornicotine. Indian commercial cigarettes had a total alkaloid content of 25,871.15 µg/g, which included 25,307.13 µg/g of nicotine, 83.83 µg/g of cotinine, and 480.19 µg/g of nornicotine. USA commercial cigarettes displayed a comparable alkaloid profile with a total of 26,212.64 µg/g, comprising 25,427.57 µg/g of nicotine, 150.59 µg/g of cotinine, and 634.48 µg/g of nornicotine. Among the multinational brands, Marlboro cigarettes contained 24294.77 µg/g of nicotine,

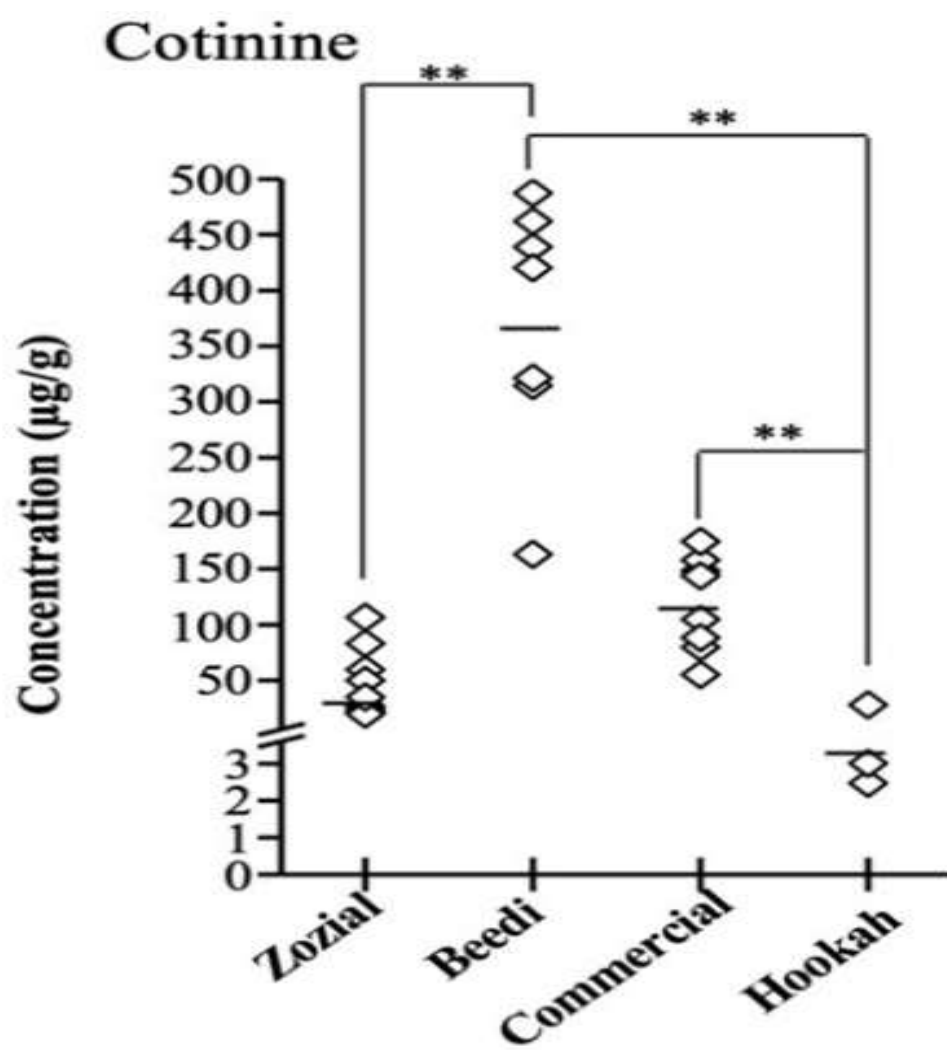
174.80 µg/g of cotinine, and 889.19 µg/g of nornicotine, giving a total alkaloid content of 25358.76 µg/g while multinational Dunhill cigarettes had a total of 25444.37 µg/g, with 24938.02 µg/g of nicotine, 55.01 µg/g of cotinine, and 451.34 µg/g of nornicotine. Myanmar cigarettes exhibited relatively high alkaloid concentrations as well, with a total of 25,977.13 µg/g, including 24,876.47 µg/g of nicotine, 124.54 µg/g of cotinine, and 976.12 µg/g of nornicotine. In contrast, hookah tobacco showed the lowest alkaloid content among all products, with a total of only 1167.73 µg/g, composed of 1144.66 µg/g of nicotine, 5.94 µg/g of cotinine, and 17.13 µg/g of nornicotine. These findings indicate a wide range of alkaloid concentrations among different tobacco products, reflecting differences in tobacco type, processing methods, and possibly user exposure. Beedi and commercial cigarette products generally exhibited the highest levels of all alkaloids, while Zozial and hookah tobacco had considerably lower levels.

Additionally, the distribution of nicotine (mg/g), nornicotine (µg/g), and cotinine (µg/g) in the four different tobacco categories is illustrated in Fig. 3.4 a,b and c, respectively. Pairwise comparisons revealed statistically significant differences in alkaloid concentrations among various tobacco products. For nicotine, significantly higher levels were observed in Beedi compared to Zozial ($p < 0.01$), commercial cigarettes compared to Zozial ($p < 0.01$), Zozial compared to Hookah ($p < 0.01$), Beedi compared to commercial cigarettes ($p < 0.01$), Beedi compared to Hookah ($p < 0.01$), and commercial cigarettes compared to Hookah ($p < 0.01$). For cotinine, Beedi exhibited significantly higher concentrations than Zozial ($p < 0.01$) and commercial cigarettes ($p < 0.01$), while commercial cigarettes had significantly higher levels than Hookah ($p < 0.01$).

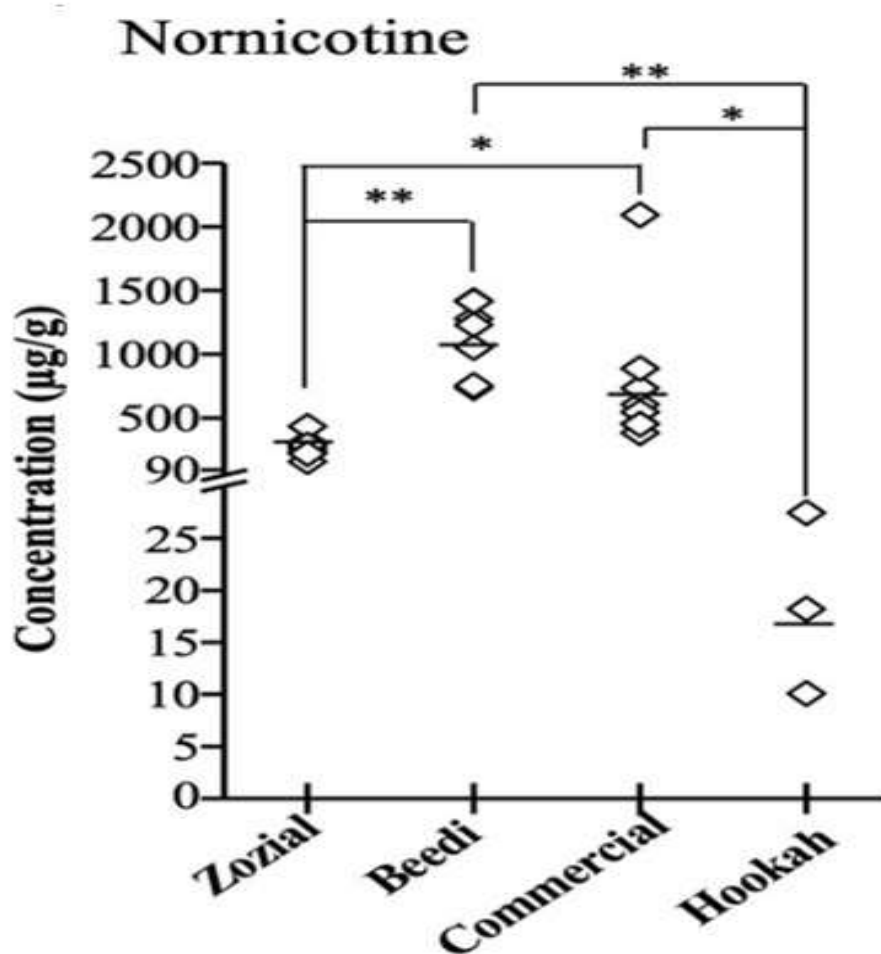
For nornicotine, significantly elevated concentrations were detected in Beedi compared to both Zozial ($p < 0.01$) and Hookah ($p < 0.01$). Similarly, commercial cigarettes exhibited significantly higher nornicotine levels than Zozial ($p < 0.05$) and Hookah ($p < 0.05$). It is important to note that during smoking, a part of the nornicotine in the tobacco matter is volatilized and it undergoes nitrosation and converted to group 1 carcinogen, NNN.



(a)



(b)



(c)

Fig. 3.4: The distribution of (a) Nicotine, (b) Cotinine, (c) Nornicotine in different smoking tobacco products including Zozial, Beedi, Commercial cigarette, and Hookah, are presented, with horizontal lines indicating GM. Group differences were assessed using one-way ANOVA, and results with significant differences are denoted by asterisks: * for $p < 0.05$ and ** for $p < 0.01$.

Table 3.3: GM value of nicotine, cotinine, and nornicotine in different smoking tobacco products

Category	*Nicotine (µg/g)	*Cotinine (µg/g)	Nornicotine (µg/g)	Total alkaloids (µg/g)
Beedi	33161.91 (21068.83- 43063.97)	353.10 (162.97- 487.32)	1091.86 (737.73- 1420.02)	34606.87
Zozial	16995 (11547-20814)	39.60 (20-106)	261 (158-432)	17295.6
Hookah	1144.66 (778.06- 2421.19)	5.94 (2.47-28.10)	17.13 (10.07-27.44)	1167.73
Indian cigarette	25307.13 (24249.65- 26410.11)	83.83 (79.75-88.12)	480.19 (381.34-604.66)	25871.15
USA cigarette	25427.57 (23380.66- 27653.68)	150.59 (143.78- 157.72)	634.48 (547.63-735.09)	26212.64
Multinational cigarettes (Marlboro)	24294.77 (0-24294.77)	174.80 (0-174.80)	889.19 (0-889.19)	25358.76
Multinational cigarettes (Dunhill)	24938.02 (0-24938.02)	55.01 (0.55.01)	451.34 (0-451.31)	25444.37
Myanmar cigarettes	24876.47 (23976.61- 25810.11)	124.54 (104.65- 148.19)	976.12 (455.60- 2091.33)	25977.13

*Data taken from Lalrinnawma's thesis for comparison.

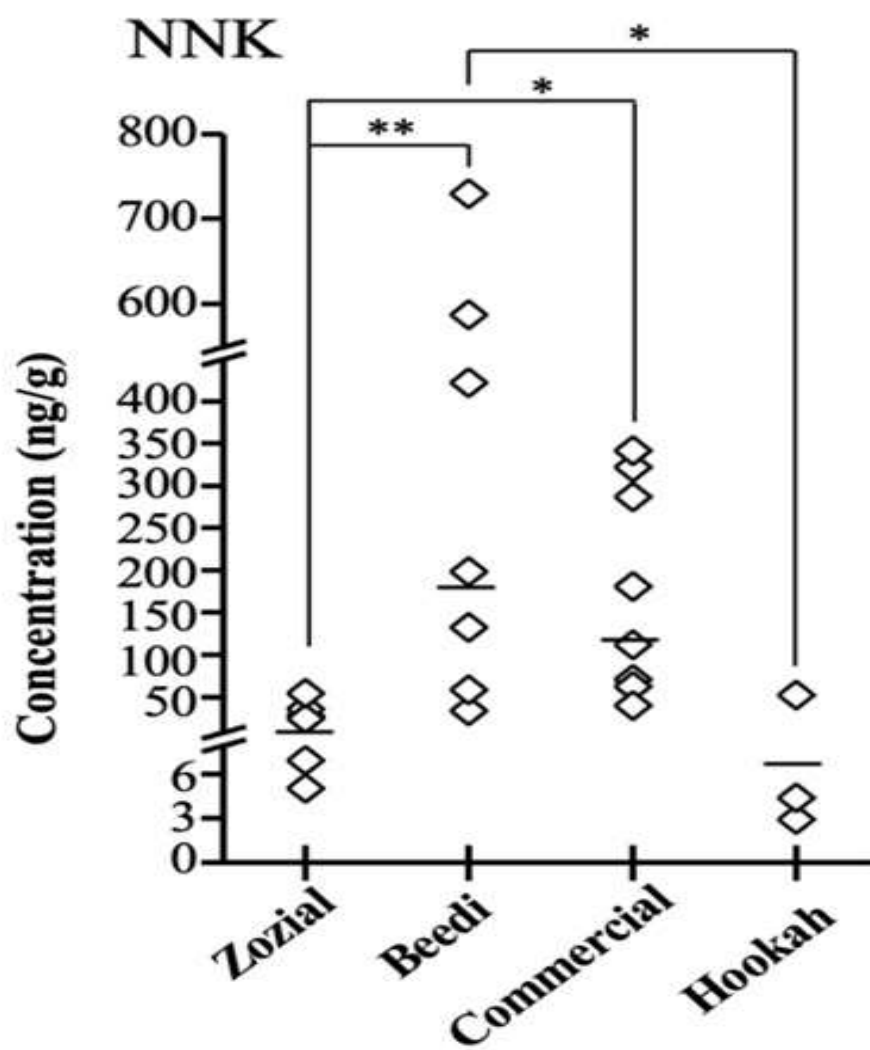
3.1.4 Preformed TSNA content

The concentrations of three major TSNAs; NNN, NNK, and NNAL, were quantified across different tobacco product types. Constituent TSNA levels in smoking tobacco products expressed as GM concentrations along with observed ranges are summarized in Table 3.4. Beedi showed the highest NNN level among the traditional products, with a geometric mean (GM) of 1114.06 ng/g (range: 479.55–1621.55 ng/g), followed by NNK at 192.59 ng/g (34.73–729.33 ng/g), and NNAL at 84.65 ng/g (18.02–470.01 ng/g), resulting in a total TSNA concentration of 1391.30 ng/g. Zozial tobacco exhibited considerably lower TSNA levels, with NNN at 34.70 ng/g (18.41–149.20 ng/g), NNK at 5.01 ng/g (0.35–55.20 ng/g), and NNAL at 1.57 ng/g (0.49–12.45 ng/g), yielding a combined TSNA content of 41.28 ng/g. Hookah tobacco displayed a similarly low TSNA profile, with NNN at 12.40 ng/g (2.33–86.64 ng/g), NNK at 10.80 ng/g (2.93–52.42 ng/g), and NNAL at 5.03 ng/g (0.49–60.80 ng/g), resulting in a total of 28.23 ng/g for all three TSNAs. Among commercial cigarettes, Indian brands recorded NNN levels of 301.38 ng/g (301.38–368.85 ng/g), NNK at 66.81 ng/g (62.84–71.04 ng/g), and NNAL at 10.84 ng/g (10.37–11.32 ng/g), amounting to a cumulative TSNA value of 379.03 ng/g. USA cigarettes demonstrated higher TSNA content with NNN at 376.83 ng/g (308.65–460.06 ng/g), NNK at 332.38 ng/g (322.59–342.48 ng/g), and NNAL at 32.11 ng/g (29.69–34.72 ng/g), summing to a total TSNA concentration of 741.32 ng/g. Multinational cigarette brands showed varying TSNA profiles. Marlboro had NNN at 511.67 ng/g (0–511.67 ng/g), NNK at 287.26 ng/g (0–287.26 ng/g), and NNAL at 24.39 ng/g (0–24.39 ng/g), with an overall TSNA burden of 823.32 ng/g. In contrast, Dunhill cigarettes recorded NNN at 33.74 ng/g (0–33.74 ng/g), NNK at 40.06 ng/g (0–40.06 ng/g), and NNAL at 2.35 ng/g (0–2.35 ng/g), reflecting a total TSNA level of 76.15 ng/g. Notably, Myanmar cigarettes exhibited the highest overall TSNA burden, with NNN at 1522.34 ng/g (523.53–4426.76 ng/g), NNK at 142.60 ng/g (112.23–181.17 ng/g), and NNAL at 34.44 ng/g (18.49–64.13 ng/g), giving a combined concentration of 1699.38 ng/g.

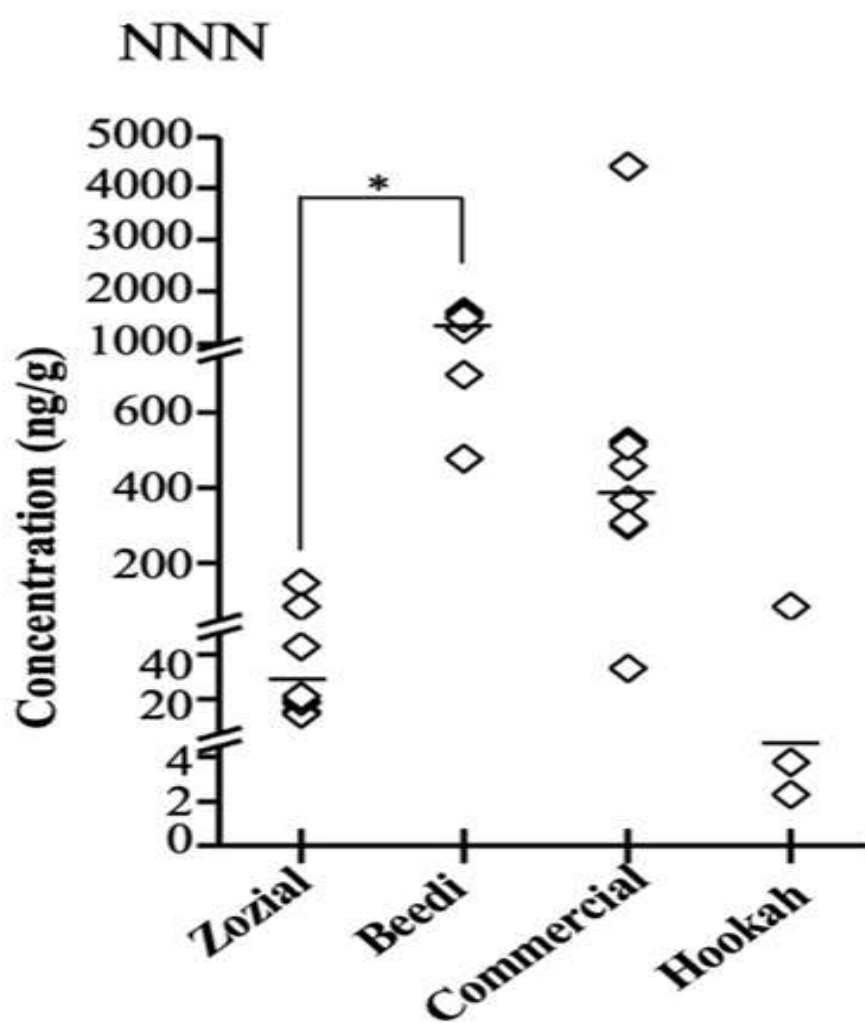
The distribution of NNK, NNN, and NNAL in different categories of tobacco products is displayed in Fig. 3.4 (A), (B), and (C). The pair wise comparisons of

TSNA concentrations among different tobacco product categories, Zozial, Beedi, commercial cigarettes, and Hookah, revealed several statistically significant differences. For NNK, significantly higher concentrations were found in Beedi compared to Zozial ($p < 0.01$), Beedi compared to Hookah ($p < 0.05$), and commercial cigarettes compared to Zozial ($p < 0.05$). For NNN, a significant difference was observed between Beedi and Zozial, with Beedi exhibiting higher concentrations ($p < 0.05$). For NNAL, significantly higher levels were found in Beedi compared to Zozial ($p < 0.05$), Beedi compared to Hookah ($p < 0.05$), and commercial cigarettes compared to Beedi ($p < 0.05$).

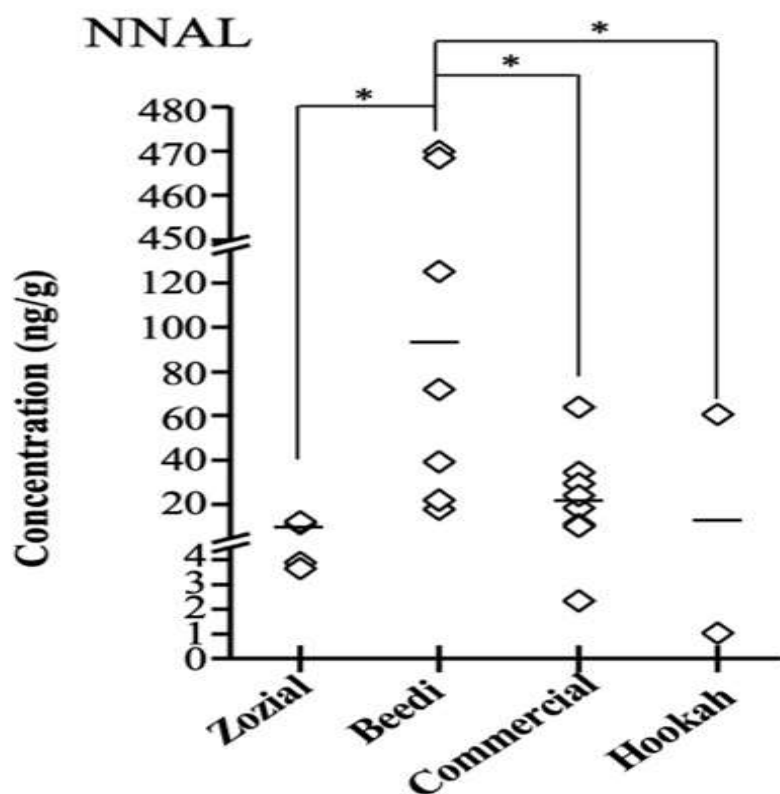
In addition, the ratio of NNAL to NNK was calculated and the results were expressed in the range: zozial, 0.14-0.317; commercial cigarettes, 0.0571-0.571 (with USA cigarettes and multinational cigarettes in the range 0.0571-0.108, and Indian and Myanmarrese cigarettes in the range, (0.102-0.571); water pipe, 0.35-1.16; beedi, 0.091-13.53.



(A)



(B)



(C)

Fig. 3.5: The distribution of (A) NNK, (B) NNN, (C)NNALin different smoking tobacco products including Zozial, Beedi, Commercial cigarette, and Hookah, are presented, with horizontal lines indicating GM. Group differences were assessed using one-way ANOVA, and results with significant differences are denoted by asterisks: * for $p < 0.05$ and ** for $p < 0.01$.

Table 3.4: GM value of NNN, NNK, and NNAL in different smoking tobacco products

Category		NNN (ng/g)	NNK (ng/g)	NNAL (ng/g)	Total TSNA (ng/g)
*Beedi		1114.06 (479.55-1621.55)	192.59 (34.73-729.33)	84.65 (18.02-470.01),	1391.3
*Zozial		34.70 (18.41-149.20)	5.01 (0.35-55.20)	1.57 (0.49-12.45)	41.28
Hookah		12.4 (2.33-86.64)	10.8 (2.93-52.42)	5.03 (0.49–60.8)	28.23
Commercial Cigarettes	Indian cigarette	301.38 (301.38-368.85)	66.81 (62.84-71.04)	10.84 (10.37-11.32)	379.03
	USA cigarette	376.83 (308.65-460.06)	332.38 (322.59-342.48)	32.11 (29.69-34.72)	741.32
	Multinational cigarettes (Marlboro)	511.67 (0-511.67)	287.26 (0-287.26)	24.39 (0-24.39)	823.32
	Multinational cigarettes (Dunhill)	33.74 (0-33.74)	40.06 (0-40.06)	2.35 (0-2.35)	76.15
	Myanmar cig	1522.34 (523.53-4426.76)	142.60 (112.23-181.17)	34.44 (18.49-64.13)	1699.38

*Data taken from Lalrinnawma's thesis for comparison

3.1.5 3R4F Reference Product

The measured chemical characteristics of 3R4F reference cigarettes in this study were generally consistent with previously published results, though some variations were observed (Table 3.5). The pH of tobacco matter was 4.5 ± 0.1 , slightly lower than the reported reference values 5.5 ± 0.1 . Nitrate content was measured at 10,114 $\mu\text{g/g}$, consistent with the published range of 10,000–10,600 $\mu\text{g/g}$. Nitrite concentration was low (1.1 $\mu\text{g/g}$), yet within the reported range of <1.0–4.7 $\mu\text{g/g}$. Among TSAs, nicotine was found at 18.2 ± 2.1 mg/g, which lies within the reported range (14.6–22.5 mg/g). Cotinine and nornicotine levels were 114 ± 21 $\mu\text{g/g}$ and 281 ± 87 $\mu\text{g/g}$, respectively. While cotinine lacked a direct reference value, the

nornicotine concentration was notably lower than the reported range of 645–910 µg/g. For TSNA, NNK was found at 654 ± 112 ng/g, consistent with the reference range (673–880 ng/g). However, NNN was slightly lower (2092 ± 77 ng/g) compared to reported values (2400–2650 ng/g). NNAL was measured at 38 ± 4 ng/g, though no reference range was available for comparison.

The pH values of tobacco products varied significantly across categories when compared with the 3R4F reference cigarette (pH: 4.5 ± 0.1). Beedi tobacco samples exhibited the highest pH, with a geometric mean (GM) of 6.9 ranged between 6.2–7.6, indicating a considerably more alkaline environment than the reference. Similarly, Zozial showed a moderately elevated pH (GM: 6.7; range: 5.9–7.2), while commercial cigarettes had a mean pH of 6.4, ranged between 4.5–7.3—spanning both acidic and basic ranges and thus overlapping with the 3R4F baseline. In contrast, Hookah tobacco had the lowest pH among the studied products (GM: 5.3; range: 4.1–6.2), which in some cases fell below or near the 3R4F value. Overall, traditional tobacco products like Beedi and Zozial demonstrated a more alkaline profile, while Hookah remained relatively acidic, aligning more closely with the pH characteristics of the reference cigarette.

The analysis of nitrate and nitrite levels in various tobacco products, in comparison to the 3R4F reference cigarette (10,114 µg/g nitrate and 1.1 µg/g nitrite), revealed notable variations across categories. Beedi samples exhibited lower concentrations of both nitrate (7,362 µg/g) and nitrite (0.81 µg/g) relative to the reference. In contrast, Zozial and Hookah tobacco products demonstrated substantially lower nitrate levels (705 and 744 µg/g, respectively); however, nitrite content in Zozial (1.19 µg/g) marginally exceeded that of 3R4F, whereas Hookah remained lower (0.56 µg/g). Among commercial cigarette products, Indian and USA cigarettes had moderately reduced nitrate levels (6,593.69 and 7,772.65 µg/g, respectively), though both exhibited elevated nitrite concentrations (1.23 and 1.94 µg/g). Myanmar cigarettes showed comparable trends, with nitrate levels at 7,985.74 µg/g and nitrite at 1.58 µg/g. Notably, Marlboro cigarettes recorded the highest nitrate concentration across all samples (12,249 µg/g), surpassing the reference standard, along with an increased nitrite level (1.55 µg/g). Dunhill cigarettes, although characterized by

lower nitrate content (3,126 µg/g), also showed elevated nitrite levels (1.57 µg/g). These findings indicate a general pattern of variable nitrate content and consistently elevated nitrite concentrations in most commercial tobacco products when compared with the 3R4F reference.

Compared to the 3R4F reference, Beedi had significantly higher nicotine (33,161.91 µg/g) and nornicotine (1,091.86 µg/g) concentrations, and a slightly elevated cotinine level (353.10 µg/g). Zozial showed slightly lower nicotine (16,995 µg/g), markedly lower cotinine (39.60 µg/g), and comparable nornicotine (261 µg/g). Hookah tobacco had substantially lower levels of all three alkaloids (nicotine: 1,144.66 µg/g; cotinine: 5.94 µg/g; nornicotine: 17.13 µg/g). Commercial cigarettes (Indian, USA, multinational, and Myanmar) generally had nicotine levels close to or slightly higher than 3R4F, with USA cigarettes (25,427.57 µg/g) and Myanmar cigarettes (24,876.47 µg/g) showing elevated nornicotine (634.48 µg/g and 976.12 µg/g respectively), and Marlboro cigarettes presenting the highest cotinine concentration (174.80 µg/g).

Compared to the 3R4F reference cigarette (NNN: 2092 ng/g, NNK: 654 ng/g, NNAL: 38 ng/g), Beedi exhibited lower NNN (1114.06 ng/g) and NNK (192.59 ng/g) levels but a notably higher NNAL concentration (84.65 ng/g). In contrast, Zozial (NNN: 34.70 ng/g; NNK: 5.01 ng/g; NNAL: 1.57 ng/g) and Hookah tobacco (NNN: 12.4 ng/g; NNK: 10.8 ng/g; NNAL: 5.03 ng/g) showed substantially lower TSNA concentrations across all three analytes. Commercial cigarettes (Indian, USA, and multinational brands) also demonstrated lower TSNA levels compared to 3R4F, with USA cigarettes having the closest NNK (332.38 ng/g) and NNAL (32.11 ng/g) values to the reference. Overall, all tobacco products tested exhibited lower NNN and NNK levels than 3R4F, while NNAL was highest in Beedi, suggesting possible differences in nitrosamine metabolism or tobacco processing.

Table 3.5: pH values and concentrations of nitrate, nitrite, TSAs, and TSNAs measured in 3R4F reference cigarettes. Some data were not available and is denoted by “–”

	3R4F		
	Our study	References	
pH	4.5 ± 0.1	5.5 ± 0.1	(Lawler <i>et al.</i> , 2017)
Nitrate (µg/g)	10114 ^a	10000-10600	(Eldridge <i>et al.</i> , 2015; Jain <i>et al.</i> , 2019)
Nitrite (µg/g)	1.1 ^a	<1.0– 4.7	(Eldridge <i>et al.</i> , 2015; Jain <i>et al.</i> , 2019)
Nicotine (mg/g)	18.2 ± 2.1	14.6 – 22.5	(Eldridge <i>et al.</i> , 2015; Jain <i>et al.</i> , 2019; Lawler <i>et al.</i> , 2017)
Cotinine (µg/g)	114 ± 21	–	
Nornicotine (µg/g)	281 ± 87	645 – 910	(Lisko <i>et al.</i> , 2013; Eldridge <i>et al.</i> , 2015; Jain <i>et al.</i> , 2019)
NNK (ng/g)	654 ± 112	673 – 880	(Eldridge <i>et al.</i> , 2015; Jain <i>et al.</i> , 2019; Edwards <i>et al.</i> , 2017)
NNN (ng/g)	2092 ± 77	2400–2650	(Eldridge <i>et al.</i> , 2015; Jain <i>et al.</i> , 2019; Edwards <i>et al.</i> , 2017)
NNAL (ng/g)	38 ± 4	–	

^a Single measurement

3.1.6 Correlation analysis

The quantitative results of *N*-nitrosation-associated chemicals, including alkaloids, nitrate, nitrite, and TSNAs, were further examined for their correlations by applying Spearman's Rank Correlation analysis. A significant positive correlation was observed between nitrate content and the levels of nicotine ($r = 0.642$, $p < 0.001$), cotinine ($r = 0.752$, $p < 0.0001$), nornicotine ($r = 0.727$, $p < 0.0001$), NNK ($r = 0.851$, $p < 0.0001$), NNN ($r = 0.807$, $p < 0.0001$), and NNAL ($r = 0.723$, $p < 0.0001$),

indicating that higher nitrate concentrations in tobacco are associated with elevated levels of both alkaloids TSNA's Fig. 3.6 (A-F).

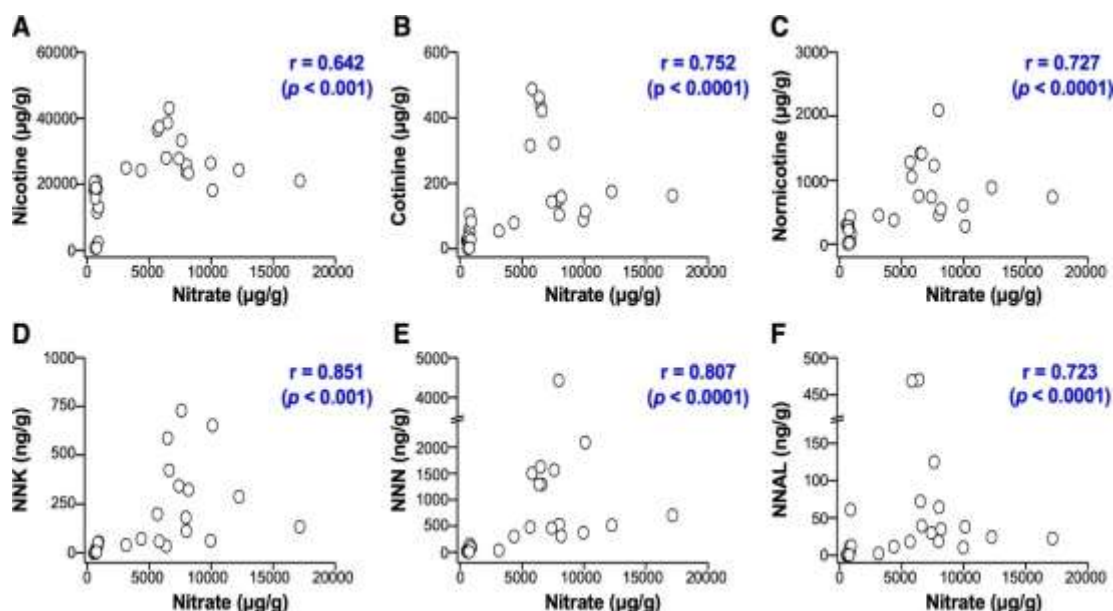


Fig. 3.6 Spearman's correlation between nitrate concentration (µg/g) and (A) nicotine, (B) cotinine, (C) nornicotine, (D) NNK, (E) NNN, and (F) NNAL.

Correlation analyses between the levels of tobacco alkaloids, particularly nicotine and nornicotine, and the concentrations of major TSNA's demonstrated consistently strong and statistically significant positive correlation, indicating that higher alkaloid concentrations are associated with increased TSNA formation (Fig. 3.7). Specifically, **nicotine** levels showed a strong correlation with **NNK** ($r = 0.719$, $p < 0.0001$), **NNN** ($r = 0.792$, $p < 0.0001$), and **NNAL** ($r = 0.655$, $p < 0.001$), suggesting that nicotine is a major precursor contributing to nitrosamine formation during tobacco curing and processing (Fig. 3.7 A–C). Notably, **nornicotine** showed an even stronger correlation with **NNN** ($r = 0.885$, $p < 0.0001$) (Fig. 3.7 D), reflecting its direct role as the primary precursor for NNN formation through nitrosation reactions.

A statistically significant positive correlation was observed between nitrate levels and the molar ratios of TSNA's to their alkaloid precursors, as illustrated in

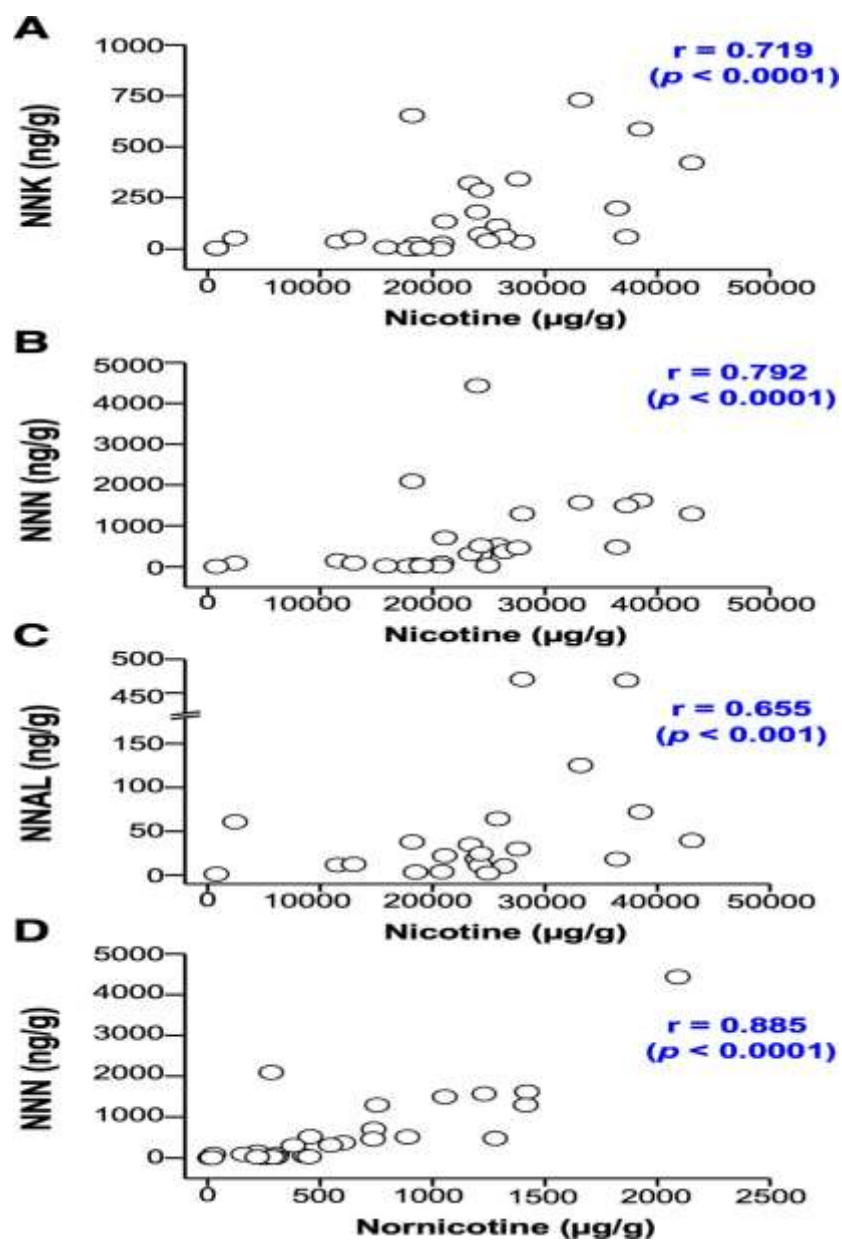


Fig. 3.7: Correlation between tobacco alkaloids (nicotine and nornicotine) and tobacco-specific nitrosamines (TSNAs). (A) NNK and nicotine, (B) NNN and nicotine, (C) NNAL and Nicotine, and (D) NNN and nornicotine

Fig. 3.8 (A–C). Specifically, NNK/nicotine (Fig. 3.8 A) showed a correlation coefficient of $r = 0.692$ ($p < 0.001$), NNN/nicotine (Fig. 3.8 B) had $r = 0.780$ ($p < 0.0001$), NNAL/nicotine (Fig. 3.8 C) exhibited $r = 0.586$ ($p = 0.001$) and NNN/nornicotine (Figure D) showed a correlation coefficient of $r = 0.695$ ($p < 0.001$).

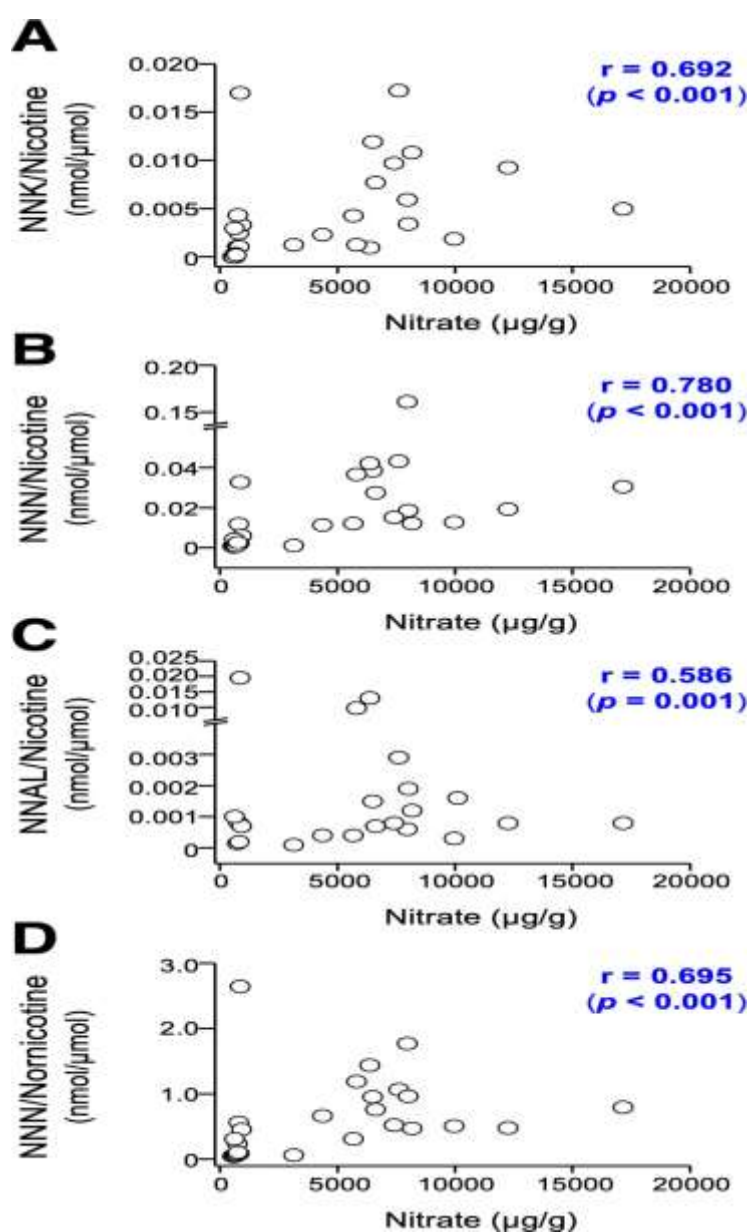


Fig.3.8:Correlation between nitrate concentration and the mole ratio of (A) NNK/nicotine, (B) NNN/nicotine or (C) NNAL/nicotine and (D) NNN/nirnicotine as determined by the Spearman's rank correlation coefficient (r).

A heatmap illustrating Spearman's rank correlation among key chemical constituents including pH, nitrate, nitrite, nicotine, cotinine, nornicotine, NNK, NNN, and NNAL is presented in the figures (Fig. 3.9). A strong positive correlation was observed between alkaloids and TSNA (s) ($r = 0.65$, $p < 0.001$ – 0.93 , $p < 0.0001$). Additionally, nitrate concentrations exhibited a significant association with the alkaloid's contents ($r = 0.64$, $p < 0.001$ – 0.75 , $p < 0.0001$) and TSNA concentrations ($r = 0.72$ – 0.85 , $p < 0.0001$). In contrast, pH and nitrite were not significantly correlated with other variables, except for cotinine, which exhibited a moderate correlation with pH ($r = 0.42$, $p = 0.029$). Furthermore, nitrate was also significantly correlated with the conversion efficiency of alkaloids to TSNA (s), such as the conversion of nicotine to NNK ($r = 0.72$, $p < 0.0001$), NNN ($r = 0.79$, $p < 0.0001$), NNAL ($r = 0.66$, $p < 0.001$), as well as nornicotine to NNN ($r = 0.89$, $p < 0.0001$). Since the nitrosation of alkaloids preferably occurs in an acidic condition, we further examined the combined influence of pH and nitrate on the conversion efficiency of alkaloids to TSNA (s). The multivariate regression analysis, reveals a strong linear correlation between nitrate, TSAs, TSNA (s) Fig. 3.10. A moderate positive correlation was found between nitrate and total TSAs ($r = 0.566$, $p < 0.002$), indicating that higher nitrate levels are generally associated with increased concentrations of alkaloids such as nicotine, cotinine, and nornicotine. More notably, a strong positive correlation was observed between nitrate and total TSNA (s) ($r = 0.824$, $p < 0.0001$), suggesting that nitrate may play a key role in the formation of carcinogenic nitrosamines like NNK, NNAL, and NNN (Fig 3.10). Additionally, total TSAs were also strongly correlated with total TSNA (s) ($r = 0.706$, $p < 0.0001$), reinforcing the idea that tobacco alkaloids serve as precursors for nitrosamine formation. These findings collectively imply that the nitrate content in tobacco, possibly through nitrosation reactions, significantly contributes to TSNA formation are shown in Fig. 3.11. Therefore, reducing nitrate and alkaloid levels during tobacco processing could be an effective strategy for minimizing harmful nitrosamine content in tobacco products.

The 3D mesh plot (Fig. 3.12) presents the outcome of a multiple linear regression analysis using Box-cox method assessing how nitrate and total tobacco-specific alkaloids (TSAs) together influence the formation of tobacco-specific nitrosamines

(TSNAs). The regression model shows a strong linear relationship with a high correlation coefficient ($R = 0.852$, $p < 0.001$), indicating that the combined effect of nitrate and TSAs can robustly predict TSNA levels. The derived regression equation $(\text{TSNAs}) = 2.651 + (0.0102 \times \text{nitrate}) + (0.0632 \times \text{TSAs})$ — implies that both nitrate and alkaloid content significantly contribute to TSNA formation, with TSAs exerting a stronger effect per unit increase compared to nitrate. The plane in the plot clearly demonstrates that higher values of both predictors are associated with elevated TSNA levels. These findings reinforce the hypothesis that nitrosation of alkaloids, facilitated by the presence of nitrate, is a key pathway in TSNA formation. The strong predictive power of this model highlights the importance of monitoring and controlling both nitrate and alkaloid content in tobacco products to reduce the levels of harmful nitrosamines.

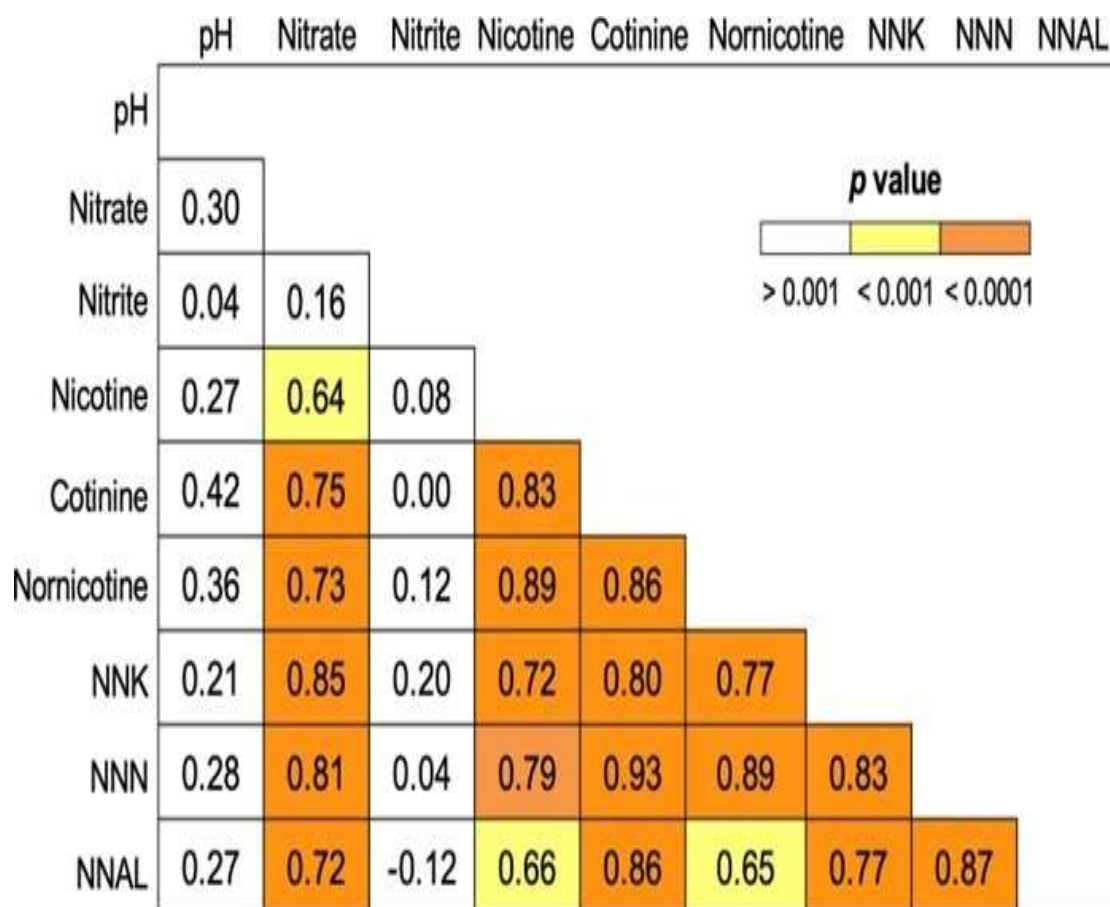


Fig. 3.9: Heatmap of Spearman's correlation coefficients for key chemical variables. Shown are correlations among pH, nitrate, nitrite, three tobacco-specific alkaloids (nicotine, cotinine, nor nicotine), and three tobacco-specific nitrosamines (NNK, NNN, NNAL). Color intensity reflects statistical significance: white ($p > 0.001$), yellow ($p < 0.001$), orange ($p < 0.0001$)

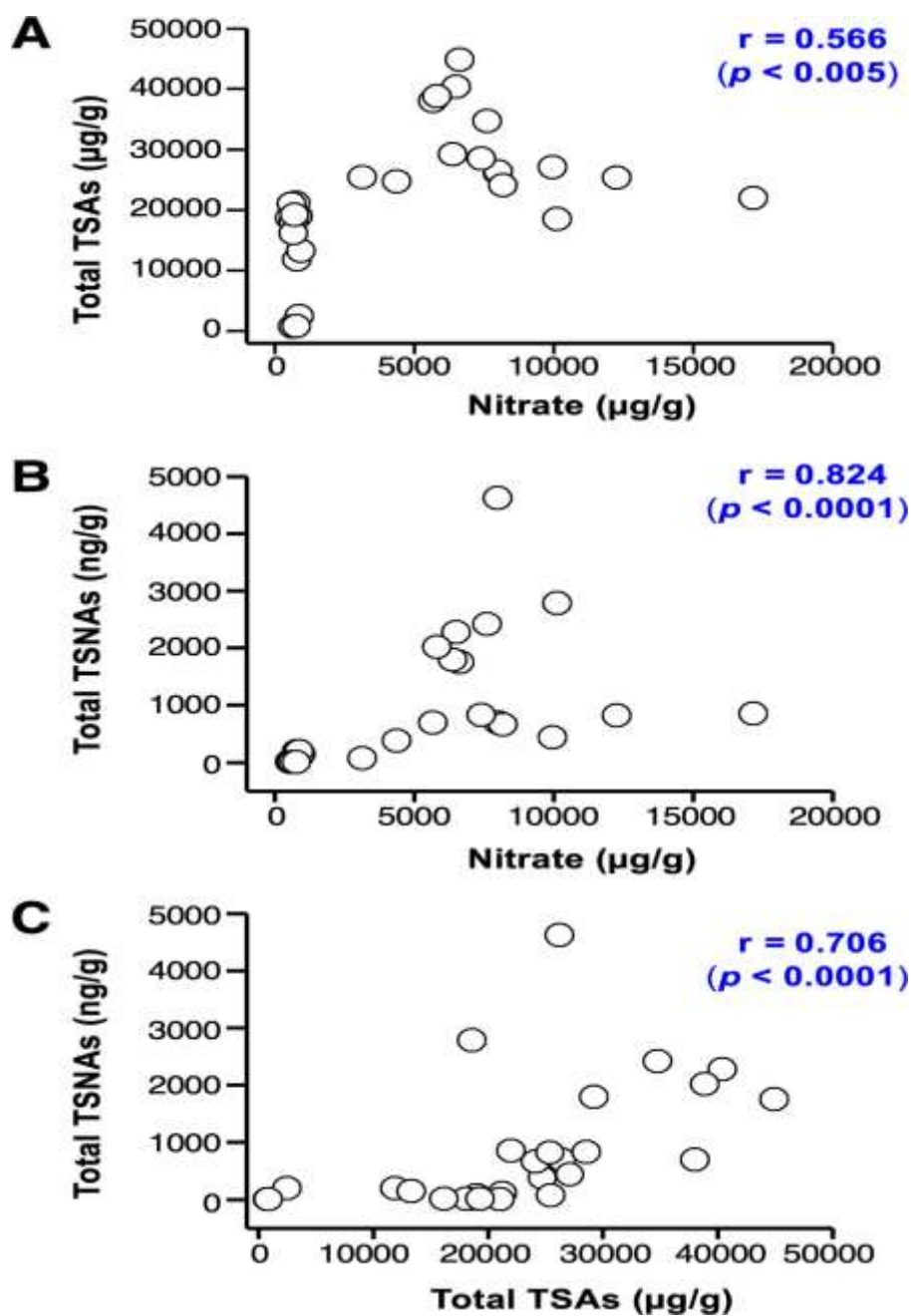


Fig. 3.10: Correlations between nitrate concentration and (A) total TSAs (ie., sum of nicotine, cotinine and nornicotine) or (B) total TSNAs (i.e., sum of NNK, NNAL and NNN) and (C) total TSNAs and total TSAs

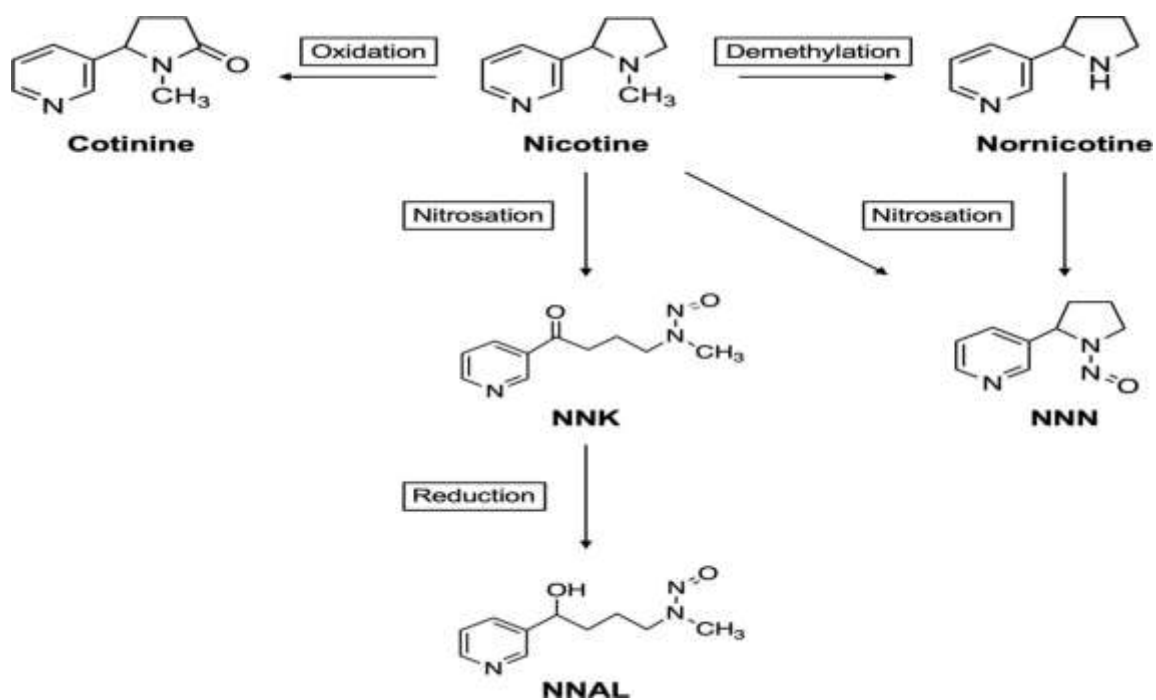


Fig.3.11: Pathways of tobacco alkaloid transformation and TSNA formation. Nicotine undergoes nitrosation to form NNK and, via demethylation to nornicotine, can also yield NNN through nitrosation. Cotinine is formed by oxidation of nicotine, and NNK is reduced to NNAL.

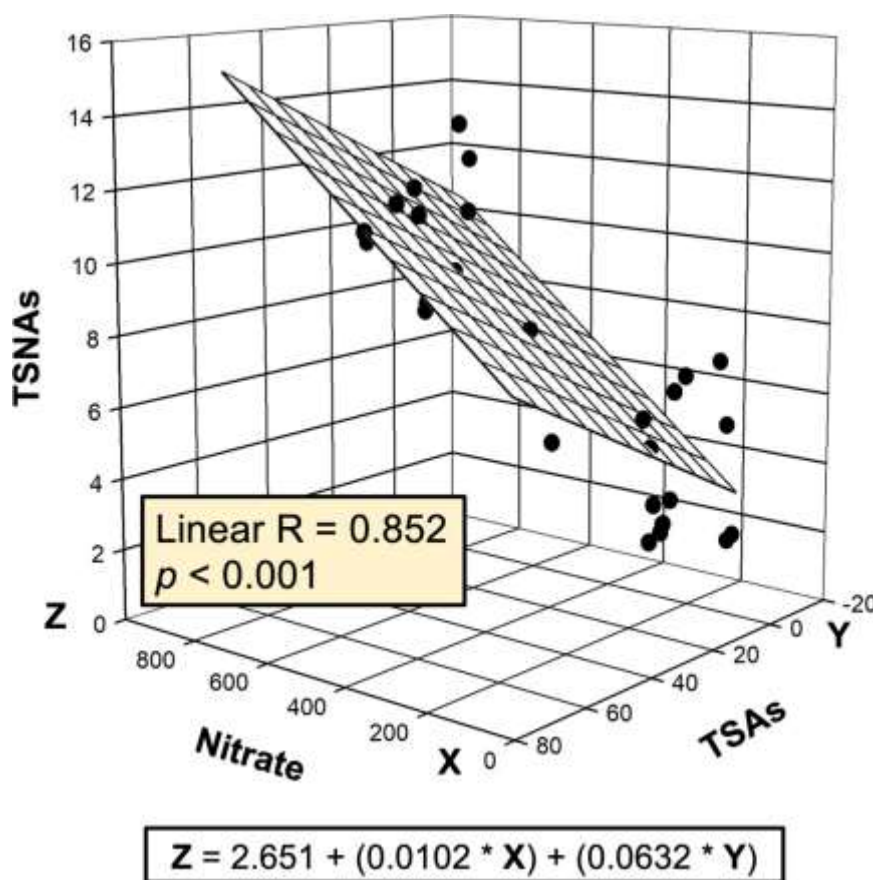


Fig.3.12: Threedimensional mesh plot of multiple linear regression analysis for TSNA formation. This plot illustrates the relationship between TSNA concentrations (Z-axis) and two predictors: nitrate (X-axis) and TSAs (Y-axis), based on Box-Cox transformed values. The regression model yielded a strong linear fit ($R = 0.852$, $p < 0.001$), with the following equation:

$$\text{TSNAs} = 2.651 + (0.0102 \times \text{nitrate}) + (0.0632 \times \text{TSAs}).$$

3.1.7 Discussion on smoking tobacco products

The alkaloids (i.e., nicotine cotinine and nornicotine, GM: 18.2 mg/g, 114 µg/g and 281 µg/g, respectively), and TSNA (i.e., NNK, NNN, and NNAL GM: 654 and 2092 ng/g, respectively), nitrate (10114 µg/g) nitrite (1.1 µg/g) concentrations of 3R4F measured in this study, based on triplicate analyses, are consistent with the previous studies (Lisko *et al.*, 2013; Edwards *et al.*, 2017; Lawler *et al.*, 2017), thus validating the reliability of the present quantitation analysis (Table 3.5).

In accordance with earlier reports, oriental tobacco (zozial) grown without nitrogen fertilizer amendments yielded lower nicotine content, followed by flue-cured bright tobacco and blended cigarette tobacco, whereas higher nicotine content is found in sun-cured beedi tobacco which is grown with abundant nitrogen fertilizer application (Djordjevic and Doran, 2009; Li *et al.*, 2015). The range of nicotine levels in tobacco commensurate with those previously published results (Pakhale and Maru, 1998; Wu *et al.*, 2004). Wu *et al.*, (2002) also demonstrated that differences in blend composition typically yield varied nicotine levels in various international brand cigarettes. Surprisingly, commercial cigarette brands of different countries analysed in our present study have shown similar range of nicotine concentrations, whereas the nitrate levels in cigarette tobacco showed significant variations. Despite the consistent quality control efforts of Philips Morris Inc. (USA) and RJ Reynolds Inc. (USA), in the present study, the USA cigarettes, Marlboro (Philips Morris) and Camel (RJ Reynolds), showed considerably higher nicotine content than previously reported (Malson *et al.*, 2001;; Wu *et al.*, 2002; Djordjevic and Doran, 2009). However, the reasons for the observed higher nicotine content and relatively higher pH of tobacco filler in the USA cigarettes, in our present study, remain unclear.

By contrast, much lower nicotine concentrations detected for water pipe tobacco, in our present study, were consistent with the nicotine content found in different flavoured water pipe tobacco of Netherlands and the USA (Hsieh *et al.*, 2021; Erythropel *et al.*, 2021). Moreover, Hadidi and Mohammed, (2004) also reported that flavored tobacco used for water pipe smoking, more akin to the water pipe tobacco analysed in this study, contained significant quantities of stem, midrib, and other tobacco scrap particles in addition to humectants and flavor additives. It is important to bear in mind that the determination and distribution of nitrosation-related chemical species in tobacco stalk (tobacco stem, midrib, and petioles), as indicated in earlier studies, contained considerably lower levels of alkaloids, higher levels of nitrate, and nitrite, as well as moderate levels of TSNA (Brunnemann *et al.*, 1983; Djordjevic *et al.*, 1989).

Lisko *et al.*, (2013) demonstrated that the minor alkaloid contents vary significantly owing to the differences in blend composition and the differences in the curing of different types of *N. tabacum* cultivars. However, in the present study, the levels of minor alkaloid, nornicotine, in the USA cigarettes and multinational brands available in India are in agreement with earlier studies (Lisko *et al.*, 2013; Edwards *et al.*, 2017). The nornicotine levels (3410 µg/g) in beedi tobacco reported in earlier study (Pakhale and Maru, 1998) was higher than the observed concentrations (737.73-1420.02µg/g) in our present study. Among the analysed tobacco products in this study, beedi tobaccos are found to contain relatively high amounts of nitrate, a precursor of nitrite, and higher amounts of nornicotine, a well-known precursor of NNN (Burton *et al.*, 1988). Beedi tobaccos are typically grown with substantial nitrogen fertilizer amendments (200-220 kg/hectare), sun-cured for 8-12 days and aged for 9 – 12 months, subsequently processed for tobacco filler (Bhide *et al.*, 1987;John, 2008).

Even though absolute concentrations of nitrite in commercial cigarettes were apparently high, which were lower than the nitrate levels, ranging from 3125.76-12248.77, indicative of modest levels of nitrate in tobacco filler,were converted to nitrite by microbial bio-transformations during curing and other processes (Burton *et al.*, 1992; Stanfill, 2020). Similarly, nitrite levels were also lower than nitrate concentrations, albeit lower concentrations of nitrite in beedi tobacco. By contrast, relatively higher amount of nitrate was converted to nitrite in zozial tobacco and water pipe tobacco, where nitrite concentrations were lower than nitrate levels ranging from 450 - 773 and 1140 - 1560 times, respectively. After sun curing, during winter months (December-January), for a brief period of 9-10 days, semi-dry leaves would be crushed under the feet to release the remaining moisture in oriental tobacco leaves thereby further depositing microorganisms on moist tobacco leaves, aiding the microbial actions in the zozial tobacco matter leading to the enhanced conversion of nitrate into nitrite, a potent precursor of carcinogenic N-nitrosamines(Stanfill, 2020; Lalrammawia *et al.*, 2021). Besides, the ratio of nitrite to nitrate and the ratio of NNAL to NNK are possibly indicative of the differences in the post-harvest processing (curing, aging, and fermentation processes) of various tobacco products.

The analysis of TSNA, particularly NNN, NNK, and NNAL, across a diverse range of smoking tobacco products revealed substantial variability in carcinogen content. TSNA concentrations in Marlboro cigarettes were higher than Indian inexpensive brand cigarettes. Ashley *et al.*, (2003) also reported that sum of NNN and NNK in American blend tobacco for the multinational brand, Marlboro, was higher than those in the Indian cigarette brand, Goldflake and the Myanmar brand cigarette brand, London. However, in the present study, a Myanmar brand cigarette tobacco has shown significantly higher value for the sum of TSNA among all the samples Table 3.4.

The TSNA concentrations are higher in American blend cigarettes than in those in FCV (flue-cured Virginia) blended (Indian) and bright Virginia tobacco cigarettes (UK), because burley tobacco is included in the American blend which is known to contain higher nicotine, TSNA and nitrate contents (Wu *et al.*, 2004; Li *et al.*, 2015). However, the tobacco cultivated in Mizoram for the hand-rolled zozial, for economic reasons, only indigenously cured and processed for a brief period of 12-18 days (Lalrammawia *et al.*, 2021). Concomitantly, the 'cured' tobacco leaves would be processed further as tobacco filler rather than stored for aging, which is reflected as the considerably lower TSNA levels more akin to the short-duration cured and aged tobacco in some of the inexpensive Moldovan cigarette brands (Stepanov *et al.*, 2005).

NNN concentrations were higher than NNK concentrations in oriental, sun-cured, water-pipe and blended tobacco, however, the bright Virginia tobacco containing multinational brand cigarette, Dunhill, in which NNK exceed NNN concentrations, indicative of the characteristic feature of Virginia tobacco, and this result is consistent with the earlier reports (Fischer *et al.*, 1989; Wu *et al.*, 2005; Ding *et al.*, 2008). Moreover, variations in TSNA contents of cigarette tobacco can be attributed to tobacco blend, processing, and the storage conditions of tobacco products (Nair *et al.*, 1989; Wu *et al.*, 2004). It should be noted that the oriental-type zozial tobacco contained lower nitrate and TSNA contents as the tobacco crop is cultivated naturally in a nitrate-poor soils without any nitrogen fertilization

(Lalrammawia *et al.*, 2021). Carcinogenic TSNA (NNN and NNK) levels in water pipe tobacco were comparable to those found in flavoured water pipe tobacco available in the USA (Hsieh *et al.*, 2021). Sun-cured beedi tobacco contained the highest TSNA concentrations, which are consistent with previous studies (Wu *et al.*, 2004; Ding *et al.*, 2008; Li *et al.*, 2015).

The observed differences in the alkaloids, TSNA and nitrate levels of different smoking tobacco products can be attributed to variations in tobacco cultivars, agricultural practices, and post-harvest processing such as curing and aging. During curing, aging, and the subsequent processing of leaf mass into tobacco filler, owing to the microbial actions in the presence of nitrite, major and minor alkaloids undergo N-nitrosation, leading to the formation of TSNA (Shi *et al.*, 2013; Law *et al.*, 2016). The variance of alkaloids, TSNA, and nitrate contents may be further influenced by the blending of different tobacco (*N. tabacum*) cultivars according to the specific commercial smoking tobacco products (Wu *et al.*, 2005; Hammond and O'Connor, 2008; Edwards *et al.*, 2021). The significant differences in the alkaloids and TSNA levels in different beedi brands collected from different regions of the India is more likely due to the variation in the blending of different beedi tobacco cultivars. These beedi tobacco varieties are normally cultivated under diverse agro-climatic conditions as well as agricultural practices in different regions of India. Besides, the lack of control over the processing conditions as well as tobacco filler packing during the production of beedis, which is still manufactured under a cottage-industrial conditions using primarily manual labour, further compounded the problem (Bhide *et al.*, 1987; Pakhale *et al.*, 1990; John, 2008).

It is important to note that there are few rare reports on the detection of NNAL in unburned tobacco and tobacco smoke (Brunnemann and Hoffmann, 1983; Wu *et al.*, 2002; Sleiman *et al.*, 2009). Besides, reports on the distribution of NNAL in different smokeless tobacco products are also available (Brunnemann *et al.*, 2017; Prokopczyk *et al.*, 1995; Richter *et al.*, 2008; Stanfill *et al.*, 2011; Hearn *et al.*, 2013; Stanfill *et al.*, 2015; Tyx *et al.*, 2022). However, it is still unclear about the bacteria-mediated generation of NNAL from NNK by reductase activities in unburned tobacco, since microbiota compositions are different for the smoking and smokeless tobacco matter (Zhou *et al.*, 2020; Tyx *et al.*, 2022). Besides, the volatility and

pyrosynthesis of NNAL, unlike NNK and NNN, has not been studied in detail (Hoffmann *et al.*, 1977; Hoffmann *et al.*, 1994; Wu *et al.*, 2002).

It is also important to note that the French dark tobacco contained moderate concentrations of preformed iso-NNAL, while in the tobacco mainstream smoke of non-filtered cigarettes much lower concentrations of iso-NNAL was detected (Tricker *et al.*, 1991). Relatively higher levels of NNN, and NNK were detected, whereas NNAL was present in much lower concentration levels and barely quantifiable in mainstream smoke of 1R4F cigarettes (Wu *et al.*, 2002). NNN and NNK were determined as the predominant particle-bound TSNA albeit trace levels of 4-(methylnitrosamino)-4-(3-pyridyl)-butanal (NNA) and NNAL were detected in the environmental smoke matrix arising from commercial USA cigarette (Sleiman *et al.*, 2009).

Previous studies have reported significant correlations between nitrite and preformed TSNA levels in tobacco during air-curing (Burton *et al.*, 1992; Burton *et al.*, 1994). Besides, TSNA contents in different varieties of tobacco processed with different curing methods also significantly correlated with nitrate levels and the storage conditions (Shi *et al.*, 2013). In our statistical analysis (Fig. 3.9), we observed that nitrite exhibited an insignificant correlation ($r \leq 0.20$, $p > 0.001$) with either the precursors or the products of *N*-nitrosation reactions. Interestingly, nitrate displayed a notably strong correlation with most variables, including alkaloids ($r > 0.64$, $p < 0.001$) and TSNA ($r > 0.72$, $p < 0.0001$). The positive correlations between nitrate and alkaloids or TSNA have been confirmed to exhibit good linearity (Fig. 3.10).

Our findings are in agreement with Fischer *et al.*, (1989) who have reported significant correlations between nitrate contents and the preformed TSNA concentrations in cigarette tobacco including tobacco blends. Additionally, we found a linear correlation ($r > 0.71$, $p < 0.0001$) between alkaloids and their nitrosated products (e.g., nicotine vs. NNK), as shown in Fig. 3.8, however these findings are in contrast to previous studies which reported no associations between alkaloid contents and TSNA levels (Fischer *et al.*, 1989). Besides, previous studies have reported that the conversion of TSAs to TSNA preferably occurs in an acidic environment (Li and Hecht 2022; Pluym *et al.*, 2022). Therefore, tobacco pH did not exert a significant influence over the nitrosation efficiency. However, further studies are needed to

elucidate the correlations between precursor levels and the TSNA concentrations by using higher number of tobacco samples.

In earlier studies, there were conflicting reports of relative contribution from pyrosynthesis to the total TSNA smoke yields. Some studies suggested that approximately one-half of the nitrosamines in tobacco smoke was transferred from the endogenous TSNA content, while the remaining portion was produced through pyrosynthesis during smoking (Hoffmann *et al.*, 1977; Adams *et al.*, 1983). In contrast, other studies have concluded that nearly all of TSNAs were directly transferred from the intrinsic TSNA contents (Wu *et al.*, 2005). Later investigations have shown that minor fractions of TSNA are formed by pyrosynthesis during smoking, whilst significant levels of preformed TSNA are directly transferred from tobacco into the MS smoke (Edwards *et al.*, 2017; Edwards *et al.*, 2021). Furthermore, it was also suggested that the total TSNA contents in mainstream smoke are predominantly derived from the endogenous TSNA wherein NNN is significantly transferred from tobacco and NNK is moderately transferred from tobacco into mainstream smoke (Jaccard *et al.*, 2018; Jaccard *et al.*, 2019).

Previous studies have also suggested that as a vital precursor of nitrogen oxides, nitrate plays a crucial role during the pyrosynthesis of TSNA (Hoffmann *et al.*, 1977; Hoffmann *et al.*, 1994; Wu *et al.*, 2005). The nitrogen oxide species are predominantly generated in tobacco smoke by the thermal decomposition of tobacco nitrate content albeit a secondary fraction is formed from the amino acids and proteins during smoking. In fact, high tobacco nitrate content elevates the nitrogen oxides (NO_x) concentrations and concomitantly promotes the TSNA pyrosynthesis further (Piadé *et al.*, 2013; Edwards *et al.*, 2021; Jin *et al.*, 2022; Mocniak *et al.*, 2022). Pyrosynthesis of polycyclic aromatic hydrocarbons (PAHs) is also suggested to be inversely related to nitrate levels in tobacco filler.

Pyrosynthesis of various carcinogens including polycyclic aromatic hydrocarbons and TSNAs during the combustion of tobacco is the potential manifestation of nitrate content in tobacco. Recently, Mocniak *et al.*, (2022) demonstrated that nitrate content in tobacco filler influences the generation of gas-phase and particulate phase free radicals as well as TSNA. Higher nitrate content in

tobacco more likely augments gas phase free radicals, elevates TSNA content, and impedes particulate phase radicals and PAHs levels, whereas lower nitrate levels in tobacco can curtail TSNA and gas-phase free radicals content, while stimulating particulate phase free radicals and PAHs levels. Thus, nitrate content in tobacco filler may manifest the toxicity and carcinogenicity effects of tobacco smoke (Mocniak *et al.*, 2022; Bitzer *et al.*, 2023). However, higher CO and tar deliveries in beedi could be mainly attributed to the combustion of tendu leaf wrapper which constitutes ~ 55% beedi mass (Watson *et al.*, 2003).

3.2 Smokeless tobacco products

3.2.1 pH, Nornicotine, nitrate, and nitrite concentrations in various Smokeless tobacco products

The analysis of various smokeless tobacco products (STPs) and related preparations revealed notable differences in pH, nornicotine, nitrite, and nitrate content as summarized in Table 3.6. Sahdah exhibited the highest alkalinity with a geometric mean (GM) pH of 10.94 (range: 8.0–12.5), containing 0.16 mg/g nornicotine (0.08–0.28 mg/g), 19.63 mg/g nitrite (4.9–44.9 mg/g), and 4251.21 mg/g nitrate (1379–14025 mg/g). Khaini showed a moderately acidic pH of 6.97 (6.5–7.0), with 0.52 mg/g nornicotine (0.36–0.73 mg/g), 33.90 mg/g nitrite (1.6–80 mg/g), and the highest nitrate content at 26049.51 mg/g (20183.01–31911.66 mg/g). Zarda had a pH of 6.24 (6.0–6.5), 1.14 mg/g nornicotine (0.97–1.27 mg/g), 0.95 mg/g nitrite (0.8–1.1 mg/g), and 7768.49 mg/g nitrate (5406–9659 mg/g). Snus exhibited a nearly neutral pH of 7.16 (7.0–7.5), with low levels of nornicotine at 0.10 mg/g (0.05–0.15 mg/g), 1.58 mg/g GM nitrite (0.9–4.0 mg/g), and 13715.61 mg/g nitrate (12248–15038 mg/g). Supari showed a pH of 7.87 (7.5–8.0), with no detectable nornicotine, but contained 1.85 mg/g nitrite (1.2–2.8 mg/g) and 6605.52 mg/g nitrate (3855–13742 mg/g). CORESTA reference products (CRPs) had a pH of 6.85, with significantly higher levels of nornicotine at 27.5 mg/g (6.2–7.9 mg/g), 3.65 mg/g nitrite (0.6–20.4 mg/g), and 14839.56 mg/g nitrate (7532–39715 mg/g).

Table 3.6: pH, Nornicotine, nitrate, and nitrite concentrations in various Smokeless tobacco products

Category	pH	Geometric Mean and Range		
		Nornicotine (mg/g)	Nitrite (mg/g)	Nitrate (mg/g)
Sahdah	10.94 ^a (8-12.5) ^b	0.16 (0.08–0.28)	19.63 (4.9–44.9)	4251.21 (1379–14025)
Khaini	6.97 (6.5-7)	0.52 (0.36–0.73)	33.90 (1.6–80)	26049.51 (20183.01–31911.66)
Zarda	6.24 (6-6.5)	1.14 (0.97–1.27)	0.95 (0.8–1.1)	7768.49 (5406–9659)
Snus	7.16 (7-7.5)	0.10 (0.05–0.15)	1.58 (0.9–4.0)	13715.61 (12248–15038)
Supari	7.87 (7.5-8)	N.D.	1.85 (1.2–2.8)	6605.52 (3855–13742)
CRPs	6.85 (6.2-6.8)	27.5 (6.2-7.9)	3.65 (0.6–20.4)	14839.56 (7532–39715)

^aGeometricmean

^bRange

N.D. – not detected

The box plots reveal marked variability in nitrite, nornicotine, and nitrate contents-in different smokeless tobacco products. Sahdah exhibited the most alkaline pH (~10.9), which is significantly higher than all other products. CRP [Cooperation Centre for Scientific Research Relative to Tobacco (CORESTA) smokeless reference tobacco products as CRPs] samples, used as the reference product, had a near-neutral pH (~6.8), comparable to Snus, while Zarda and Khaini showed mildly acidic pH profiles (Fig. 3.13). In terms of nitrate levels, Khaini displayed the highest concentrations, exceeding 30,000 mg/g in some samples comparable with CRPs.

Snus and Supari also exhibited considerable nitrate levels (~12,000–15,000 mg/g), whereas Sahdah and Zarda had comparatively lower levels (under 15,000 mg/g) Fig. 3.14. Elevated nitrate levels suggest greater availability of precursors for nitrite formation in Khaini. Nitrite levels were substantially high in Khaini, with a wide range peaking near 80 mg/g, exceedingly high nitrite levels among the analysed STPs. Sahdah contained moderate nitrite levels, while Zarda, Snus, and Supari had lower (NO_2^-) contents, viz., <5 mg/g (Fig. 3.15). The concentration of nornicotine in Zarda showed the highest concentrations (~1.3 mg/g), followed by Khaini. Sahdah, Snus, and especially Supari had much lower nornicotine levels (Fig. 3.16).

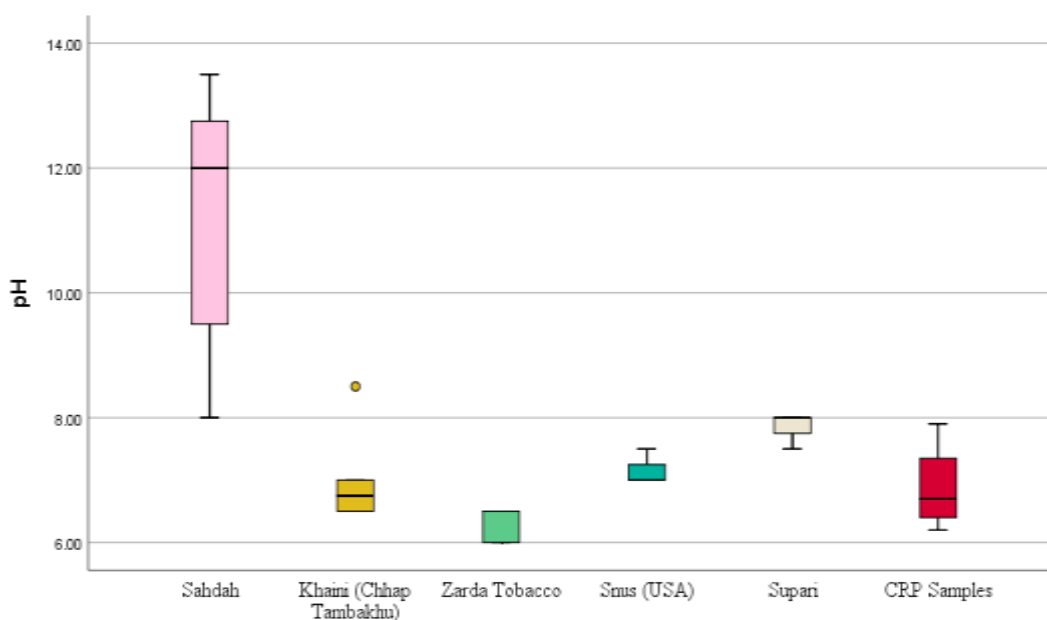


Fig. 3.13: Box plot showing pH variation across smokeless tobacco products.

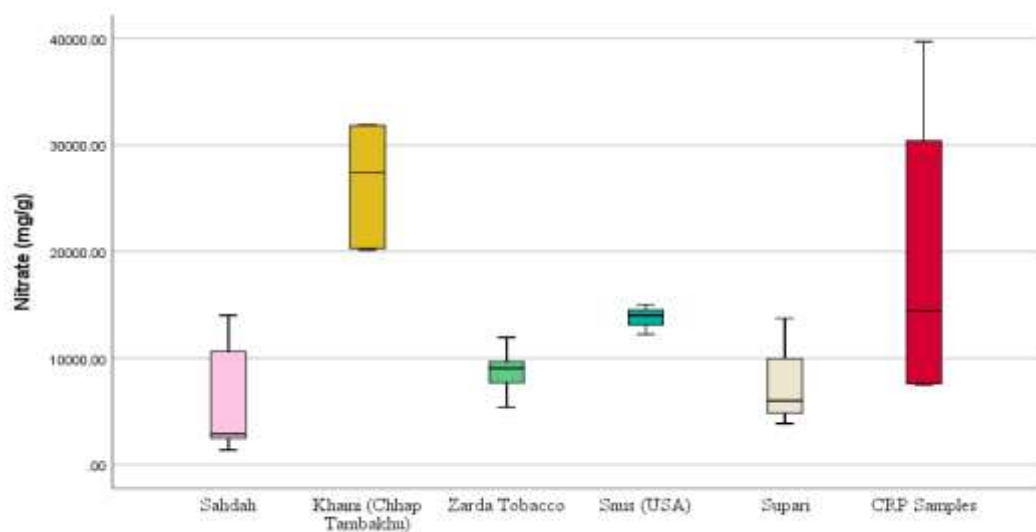


Fig. 3.14: Box plot of nitrate concentrations (mg/g) in smokeless tobacco

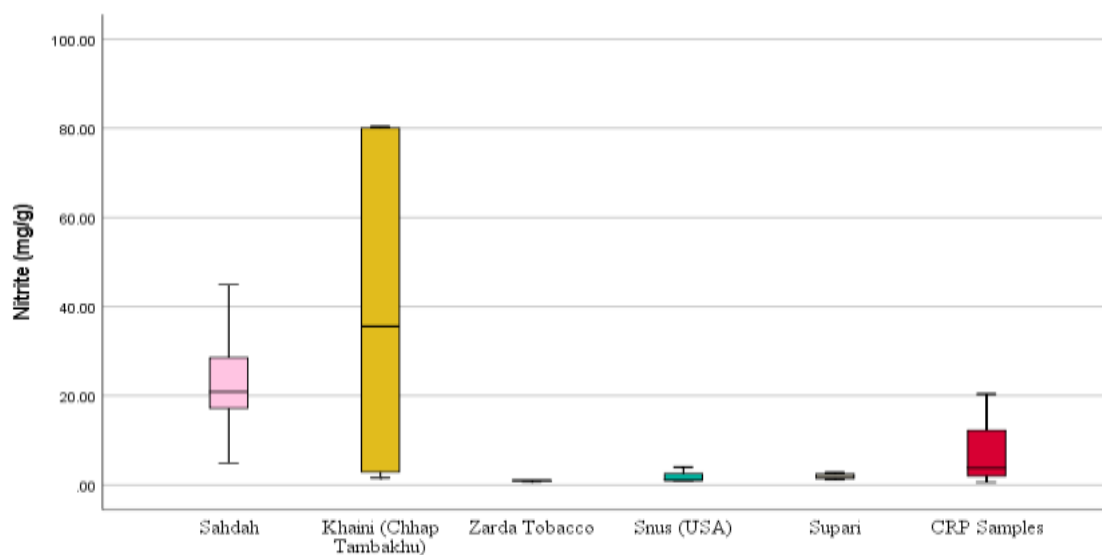


Fig. 3.15: Box plot of nitrite concentrations (mg/g) in smokeless tobacco products.

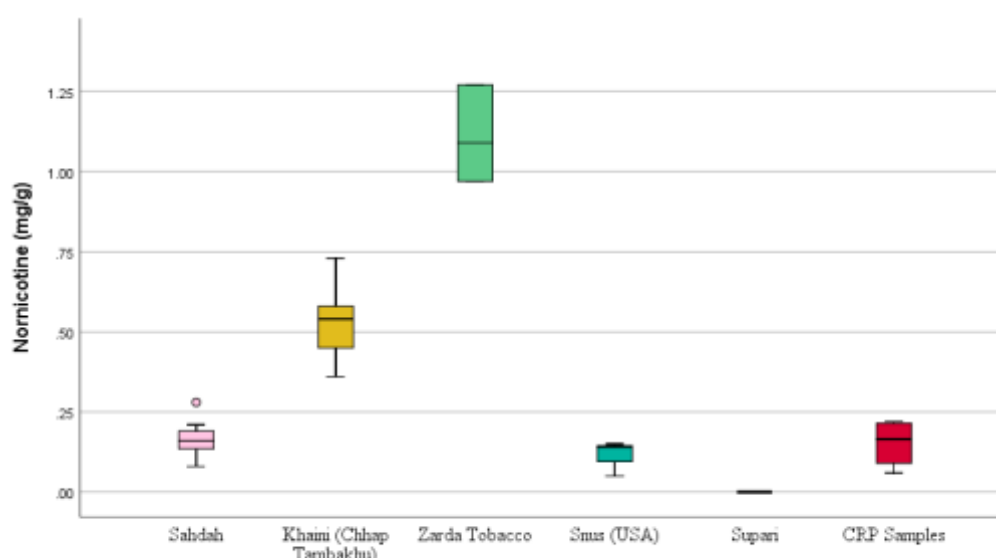


Fig. 3.16: Box plot of nornicotine concentrations (mg/g) in smokeless tobacco products.

3.2.2 Spearman correlation

Spearman correlation was conducted to evaluate the relationships among pH, nornicotine, nitrite, and nitrate levels in various smokeless tobacco products Fig. 3.17. Spearman's Rank Correlation Coefficient (ρ) was employed, and a two-tailed test of significance was used, with statistical significance set at $p < 0.05$. A significant positive correlation was found between pH and nitrite ($r = 0.5825$, $p = 0.0004$), indicating that products with higher pH levels tend to have higher nitrite concentrations. Conversely, pH showed significant negative correlations with both nornicotine ($r = -0.5252$, $p = 0.0017$) and nitrate ($r = -0.4147$, $p = 0.0164$), suggesting that increased alkalinity is associated with lower concentrations of these two constituents. The correlation between nornicotine and nitrate was positive ($r = 0.2970$), but not statistically significant ($p = 0.0933$). Similarly, the correlations between nitrite and either nornicotine ($r = -0.0589$, $p = 0.7449$) or nitrate ($r = 0.0981$,

$p = 0.5869$) were weak and not statistically significant. These results highlight specific pairwise relationships among the chemical constituents, particularly involving pH.

		pH	Nornicotine	Nitrite	Nitrate
pH	Spearman Corr.	1	-0.52518*	0.58254*	-0.4147*
	p-value	--	0.0017	3.75171E-4	0.01641
Nornicotine	Spearman Corr.	-0.52518*	1	-0.05886	0.29695
	p-value	0.0017	--	0.74489	0.09331
Nitrite	Spearman Corr.	0.58254*	-0.05886	1	0.09814
	p-value	3.75171E-4	0.74489	--	0.58691
Nitrate	Spearman Corr.	-0.4147*	0.29695	0.09814	1
	p-value	0.01641	0.09331	0.58691	--

2-tailed test of significance is used

*: Correlation is significant at the 0.05 level

Fig. 3.17: Spearman correlation of pH, nitrate, nitrite, and nornicotine in smokeless tobacco products.

3.2.3 Principal Component Analysis

Principal Component Analysis (PCA) was carried out to explore the variation in chemical composition among different tobacco samples based on three key variables: nornicotine, nitrate, and nitrite. The first two principal components (PC1 and PC2) accounted for 94.63% of the total variance, with PC1 alone explaining 66.67% and PC2 explaining 27.96% (Fig. 3.18). This high cumulative variance indicated that the two-dimensional biplot is sufficient to interpret most of the data structure.

In the biplot, Khaini samples were distinctly separated along the PC1 axis due to their exceptionally high nitrite and nitrate levels. This suggests that these samples may have been subjected to intensive nitrogen fertilization or microbial conversion during curing, leading to elevated nitrate and nitrite accumulation. In contrast, Zarda samples clustered along the PC2 axis, corresponding to their elevated nornicotine content and comparatively low nitrite levels. This pattern may reflect the use of specific tobacco cultivars or limited curing processes that favor the preservation or concentration of minor alkaloids.

Sahdah samples were positioned near the origin, indicating moderate levels of all three analytes, while Snus and Supari samples formed a tight cluster with intermediate values, reflecting chemical profiles that are more balanced or uniform. The direction and length of the variable vectors indicated that nitrate and nitrite contributed predominantly to the variance captured by PC1, whereas nornicotine contributed mainly to PC2.

These patterns reveal that the chemical clustering of tobacco samples is driven by differences in alkaloid content and nitrogen compound levels, likely arising from variations in agricultural practices, tobacco type, and post-harvest processing. The PCA not only confirmed strong differentiation among groups but also provided insight into the relationships between specific analytes and sample groups.

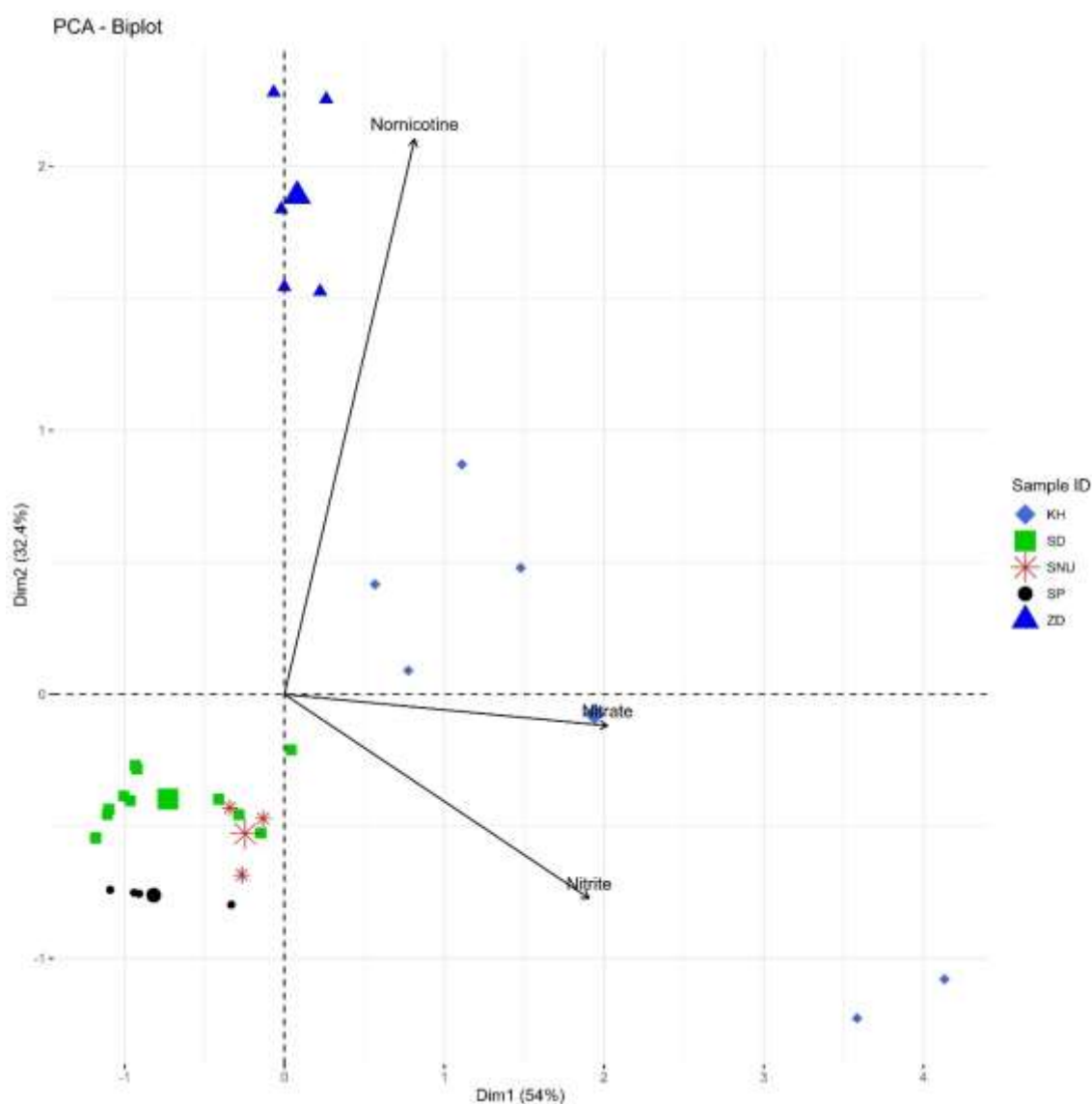


Fig. 3.18: PCA of smokeless tobacco products based on chemical composition

KH: Khaini, SD: sahdah, SNU: Snus, SP: Supari, ZD: Zarda

3.2.4 Discussion on smokeless tobacco products

The comprehensive chemical and multivariate analyses of smokeless tobacco products reveal several important patterns with significant public health implications. The extreme pH variation observed (6.24-10.94) likely reflects fundamental differences in manufacturing processes, with Sahdah's strong alkalinity potentially resulting from the addition of alkaline additives like lime, commonly used to enhance nicotine bioavailability. This finding corroborates previous studies demonstrating

how pH modification influences nicotine absorption rates in oral tobacco products (Pickworth *et al.*, 2014; Wilhelm *et al.*, 2022).

Our correlation analyses revealed three key relationships: (1) the positive pH-nitrite association, (2) negative pH-nitrate and pH-nornicotine relationships, and (3) the non-significant but suggestive positive nornicotine-nitrate trend. These patterns may reflect competing chemical processes during tobacco processing and storage. The pH-nitrite correlation could indicate pH-dependent stabilization of nitrite species, while the inverse pH-nitrate relationship might suggest nitrate reduction favored in acidic conditions. The nornicotine findings are particularly intriguing as they may reflect both varietal differences in tobacco plants and potential pH-dependent alkaloid transformations.

The PCA analysis effectively distinguished tobacco samples based on their chemical composition, particularly nornicotine, nitrate, and nitrite levels. The Khaini and Zarda samples showed unique chemical profiles, with the former characterized by high nitrate and nitrite levels and the latter by high nornicotine content. The clustering patterns suggest that chemical variability in tobacco products is closely linked to differences in cultivation methods, tobacco variety, and processing practices.

This comprehensive study of 29 smokeless tobacco products revealed significant compositional variations with important public health implications. The analysis demonstrated extreme pH differences (6.24-10.94) across products, with Sahdah showing the highest alkalinity likely due to lime additives, while Khaini exhibited exceptionally high nitrate (26,049.51 mg/g) and nitrite (33.90 mg/g) levels that may promote carcinogenic nitrosamine formation. Statistical analyses revealed significant correlations, including a positive association between pH and nitrite ($\rho = 0.5825$) and negative relationships between pH and both nornicotine ($\rho = -0.5252$) and nitrate ($\rho = -0.4147$). Principal Component Analysis clearly distinguished product categories, with Khaini, Sahdah and Zarda forming distinct clusters based on their unique characteristic chemical profiles.

Chapter-4

Chemical

Characterization of

Banana and Black

Gram Ash

4.1 Introduction and Review of Literature

Nitrate and nitrite determination in tobacco products is critical due to their role as precursors for tobacco-specific nitrosamines (TSNAs), IARC Group 1 carcinogens (IARC, 2010). However, nitrate and nitrite are also present in other lifestyle products, including plant-derived ashes used in traditional practices. To gain a better understanding of nitrate/nitrite exposure from diverse lifestyle sources, we extended our analytical approach to include wild banana ash and black gram ash, which are typically used as food additives in some tribal cultural practices albeit poorly characterized.

Northeast India's indigenous culinary and dietary factors reflect the region's ethnic diversity, exhibiting distinctive practices that set them apart from other Indian cuisine. This is notably exemplified through the traditional use of plant-derived alkaline ashes as food additives, a practice that embodies the region's profound indigenous knowledge and the enduring cultural heritage. Among the various food additives, banana ash and black gram ash, commonly known as *Khar* in the Northeastern region, especially in Assam and Arunachal Pradesh, are widely used. This plant-derived ash serves as a vital traditional alkaline food additive used by several ethnic communities across the region (Mazumder *et al.*, 2023). Traditionally, banana ash is prepared by burning various parts of the wild seeded banana plant (*Musa balbisiana* Colla), including its rhizomes and pseudo stems. In contrast, black gram [*Vigna mungo* (L.) Hepper] ash is obtained by burning the entire plant, including the pod covers after harvesting the black grams (Bora *et al.*, 2025). These alkaline additives are normally consumed either as raw ash or as aqueous extracts, represent the rich biodiversity, indigenous knowledge systems, and time-honored culinary traditions. For generations, ethnic communities have utilized plant ash for its multifaceted culinary properties. Its alkaline nature serves as a natural tenderizer, breaking down fibrous textures in ingredients such as bamboo shoots, pulses, and meats. Beyond texture modification, it also functions as a flavor enhancer, imparting a distinctive savory richness while neutralizing bitter compounds in wild-harvested greens, thereby refining the overall taste profile of traditional dishes (Borborah *et al.*, 2016). Its agricultural applications are equally significant, where it is employed as a

natural pesticide, a soil pH regulator to neutralize acidity, and an effective leech repellent in paddy fields, representing an important example of Indigenous Technical Knowledge (ITK) in Northeast Indian farming practices. Additionally, archival reports document its use as a salt substitute in certain regional contexts.

Although ethnomedical records highlight the potential benefits of plant ash in managing digestive disorders, hypertension, and cardiovascular conditions (Kalita, 2022), excessive or improper use may also pose negative health effects. Earlier studies on the physicochemical analyses of banana ash revealed that it primarily contains potassium, sodium, carbonate, and chloride ions, along with some other elements at trace levels (Deka & Talukdar, 2007). Similarly, black gram ash contains essential minerals like potassium, sodium, and calcium ions (Mazumder *et al.*, 2023). The quality of ash varies among banana cultivars, with *Musa balbisiana* Colla (a wild variety of heavily seeded banana) producing superior quality compared to other varieties (Deka & Talukdar, 2007; Bordoloi *et al.*, 2025). With high carbonate contents, the pH levels of banana ash ranged from 10.05 to 11.95 (Sarma *et al.*, 2020). Its alkalinity is attributed to relatively higher alkali and alkaline earth metal ions concentrations (Bordoloi *et al.*, 2025). The pH of black gram was also reported to be 10.94, attributed to high K^+ contents (Mazumder *et al.*, 2023). Banana ash is also known to exhibit antimicrobial properties, more likely due to high pH levels and various metal (including V and Zn) ions contents (Hemanta *et al.*, 2014). While these ashes are valued for their culinary and cultural roles, the increasing burden of cancer in Arunachal Pradesh and the broader Northeastern region underscores the need to critically examine the potential health implications of long-standing dietary practices. According to the Indian Council of Medical Research, National Centre for Disease Informatics and Research (ICMR, NCDIR, 2021), cancer is one of the leading causes of mortality in the region. In Arunachal Pradesh, two key Population-Based Cancer Registries, Pasighat (East) and West Arunachal, report some of the highest cancer incidence rates in the country. The Age-Adjusted incidence Rates (AARs) in Pasighat reach 120.4 per 1,00,000 males and 116.2 per 1,00,000 females, while West Arunachal reports AARs of 109.4 per 1,00,000 males and 95.6 per 1,00,000 females. These figures are considerably higher than the national average AARs of 94.1 for

males and 92.4 for females, as documented in the same report, highlighting a region-specific cancer burden (ICMR, NCDIR, 2021). Dietary patterns, environmental exposures, and lifestyle behaviors may be the potential contributing factors.

Despite earlier studies which predominantly focused on the alkalinity and metal ions contents of plant ashes, a thorough scientific characterization remains elusive, particularly regarding their nitrate/nitrite (belong to IARC Group 2A) contents and morphological/mineralogical characterization. While existing research has characterized several chemical constituents of plant-derived ashes (e.g., carbonates, chlorides, and alkali metals) and basic parameters such as pH and solubility (Deka & Talukdar, 2007; Sarma *et al.*, 2020), critical gaps remain in understanding their potential health risks. Notably, nitrate toxicity has not been systematically evaluated for these plant-derived indigenous food additives. Furthermore, the microstructural properties (e.g., porosity, particle morphology) and surface chemistry (e.g., oxidation states, elemental distribution) of plant ashes remain poorly characterized. The nitrate/nitrite contents analysis and advanced characterization of mineral phases were performed for banana ash/black gram ash samples on an 'as-received' basis in order to:

1. determine nitrate/nitrite concentrations of the above-mentioned biomass ash samples to evaluate their potential exposure risks;
2. explore the structural and compositional attributes of phytolith-derived mineral species in ash samples to gain a better understanding of potential toxicological Implications.

Herein, we employed LC-MS/MS for the determination of nitrate and nitrite levels, for assessing the exposure risk profile of these materials.

Field Emission Scanning Electron Microscope (FE-SEM) was employed to visualize the morphology of the ash particles, simultaneously Energy Dispersive X-ray Spectroscopy (EDX) was also performed to determine the percentage of elemental composition of the ash, with spectra collected at various sites on the sample surface.

X-ray powdered diffraction (PXRD) was employed to identify the crystalline phases in ash specimens scanned over a range of angles to generate diffraction patterns. These patterns are then compared to reference databases to identify the constituent minerals.

X-ray Photoelectron Spectroscopy (XPS) was used to analyze the surface composition of the ‘as received’ ash samples that were mounted in a vacuum chamber and bombarded with X-rays for the evaluation of elemental compositions on the surface. The kinetic energies of ejected electrons are measured to determine the distribution and chemical states of the various elements at the ash surface.

Taxonomic classification of *Musa balbisiana* Colla

Kingdom: Plantae

Phylum: Magnoliophyta

Class: Liliopsida

Order: Zingiberales

Family: Musaceae

Genus: *Musa*

Species: *Musa balbisiana*



Taxonomic classification of *Vigna mungo*

Kingdom: Plantae

Phylum: Streptophyta

Class: Magnoliopsida

Order: Fabales

Family: Fabaceae

Genus: *Vigna*

Species: *Vigna mungo*



4.2 Materials and Methods

4.2.1 Sample Collection and preparation

Banana ash was obtained from *Musa balbisiana* Colla, a wild-seeded banana variety, following traditional preparation methods from Arunachal Pradesh. The plant materials (rhizomes and stems) were washed with water to remove soil and organic debris, then chopped into small pieces and sun-dried for 5-7 days until complete dehydration was achieved. The dried biomass was first subjected to flaming combustion in an earthenware pot over an open flame, followed by the smoldering combustion for an extended period followed by a rather slow cooling period of > 6 h until it reached ambient temperature. The final product was sieved using traditional bamboo sieves without a definite pore size and stored in airtight containers under anhydrous conditions to prevent moisture absorption and contamination. Analyses were performed on the samples as received, without additional treatment to retain their original state.



Fig.4.1: Banana ash

Black gram ash was prepared following traditional methods used in Arunachal Pradesh. Mature black gram plants, including stems, roots, and intact pod covers, were collected post-harvest. The plant material underwent thorough sun-drying for 5-7 consecutive days in the agricultural field until a brittle texture was achieved, indicating complete dehydration. The dried biomass was combusted in an open-air flaming process directly in the agricultural field. The resulting ash was allowed to cool gradually to ambient temperature in a dust-free environment. The final product was sieved using a traditional bamboo sieve without a definite ore size, then stored in containers under anhydrous conditions until analysis to prevent moisture absorption and contamination for further analysis. The samples' Analyses were performed as received, with no additional treatment, to retain their original properties

The methods employed for the quantitative determination of nitrate and nitrite content, as well as the physicochemical characterization of both ash samples are LC-MS/MS method and PXRD/XPS/FE-SEM/SEM-EDX, respectively.



Fig. 4.2: Black gram ash

4.2.2 Chemicals and reagents

All solvents and reagents used in the present study were of analytical grade or higher. Methanol (MeOH) was purchased from J. T. Baker (Center Valley, PA, USA). Ammonium acetate (AA), 2,3-diaminonaphthalene (DAN), nitrate reductase (from *Aspergillus niger*), β -nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt hydrate (β -NADPH), flavin adenine dinucleotide disodium salt hydrate (FAD), hydrochloride (HCl), sodium hydroxide (NaOH), sodium nitrate (NaNO_3), and sodium nitrite (NaNO_2) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The stock solution of each standard and their corresponding stable isotope-labeled internal standard (SIL-IS) was prepared in 10% (v/v) MeOH at final concentration of either 1000 $\mu\text{g/mL}$ or 100 $\mu\text{g/mL}$, then stored at -20°C . For the analyte quantification, a serial dilution of the stock solution was conducted using deionized water to prepare working solutions at different concentrations for establishing the calibration curves. The linear range for each analyte was as following: 1.8 to 230 ng/mL for NO_2^- ; 0.24 to 62 $\mu\text{g/mL}$ for NO_3^- . Each calibrator, depending on the purpose of NO_2^- and NO_3^- quantification, contained corresponding SIL-IS, $^{15}\text{NO}_2^-$: 23 ng.

4.2.3 Sample preparation for nitrate and nitrite analysis

A volume of 5 mL of deionized water was added to 0.1 g of powdered plant ash samples for the aqueous extraction. The resulting mixture was shaken using orbital shaker S101 (FIRSTEK) at 200 rpm for 1 h at room temperature and sonicated for another 1 h using ultrasonic cleaner ELMA LC-130/H (Sumber Aneka Karya Abadi) to achieve complete extraction. After centrifugation at $2790 \times g$ for 10 minutes, the supernatant was filtered using a 13 mm, $0.22 \mu\text{m}$ Nylon syringe filter. The resulting filtrate was then diluted using deionized water to make 2-fold and 10-fold dilution filtrates for the analysis of NO_2^- and NO_3^- , respectively.

For NO_2^- analysis, a 250 μL aliquot of 2-fold diluted filtrates was collected and combined with 250 μL of SIL-IS ($^{15}\text{NO}_2^-$: 92 ng/mL). The resulting 500 μL sample mixture was then subjected to derivatization with DAN (6.25 mM) in 0.5 M HCl at 37°C for 30 minutes to yield nitrite-DAN derivatives (i.e., 2,3-naphthotriazole, NAT) and ^{15}NAT . The reaction was terminated by adding 0.58 M NaOH. The resulting reaction mixture was then diluted 10-fold with a 10% (v/v) methanol containing 1 mM AA solution, and the mixture was ready for injection of 10 μL into the LC-MS/MS system.

For NO_3^- analysis, it was required to convert NO_3^- to NO_2^- by nitrate reductase (from *Aspergillus niger*), as previously described by Chao and co-researchers (Chao *et al.*, 2016) with minor modifications. A 10 μL aliquot of 10-folds diluted filtrates was combined with 10 μL of SIL-IS ($^{15}\text{NO}_3^-$: 15.5 $\mu\text{g/mL}$). The resultant mixture was then incubated at room temperature for 2 h with 95 μL of nitrate reductase solution containing 10 mU of nitrate reductase, 12 mM NADPH, 10 mM phosphate buffered saline (pH 7.5) and 0.1 mM FAD. This allowed for the reduction of NO_3^- to NO_2^- . To continue the derivatization reaction, the mixture containing the total NO_2^- (initial NO_2^- plus NO_2^- reduced from NO_3^-) was diluted 20-fold with deionized water and processed following the same procedure as described for NO_2^- . The concentration of NO_3^- was calculated by subtracting the initial NO_2^- concentration from the total NO_2^- concentration.

4.2.4 Determination of pH

A portion of the water extracts from each plant ash sample, obtained through the aqueous extraction process described earlier, was collected separately for pH measurement. The pH of the aqueous extract was determined using a pH meter (6177M, JENCO). Prior to measuring the pH of the ash samples, the pH meter was calibrated using pH buffer solution at pH 4, 7, and 10 to ensure accuracy. The pH of the ash extract was recorded to assess its alkalinity.

4.2.5 FE-SEM and EDX Analysis

5 g of each ash sample was subjected to FE-SEM analysis. The plant ash specimen was carefully mounted on the sample holder using a double-sided carbon tape. The sample was then sputter-coated with a thin layer of gold. Finally, the gold-coated ash sample was analyzed using a Zeiss Sigma field emission-scanning electron microscope (FE-SEM) equipped with an energy dispersive X-rays Spectroscopy (EDX). Secondary electron (SE) and backscattered electron (BSE) images were acquired at different magnifications, while simultaneous EDX analysis was conducted at 20 kV to determine elemental composition and distribution.

4.2.6 PXRD

The crystallographic structure of ‘as received’ ash samples were characterized using X-ray powder diffraction (PXRD) analysis. Diffraction patterns were recorded using a D8 FOCUS and MINIFIEX diffractometer (Japan) with $\text{CuK}\alpha_1$ radiation ($\lambda = 1.54059 \text{ \AA}$) with diffraction angle (2θ) in the range of 20° – 80° . The obtained diffraction patterns were analyzed using Qualx 2.0 software for phase identification, while peak fitting and pattern refinement were conducted with Origin Pro 2025 software. Phase identification was made by searching the crystallography open database for inorganic compounds.

4.2.7 XPS

The surface elemental composition and its corresponding oxidation states were analyzed using an ESCALAB Xi+ X-ray photoelectron spectrometer (Thermo-Fischer, UK). For binding energy calibration, the C 1s peak at 284.7 eV was used as

an internal reference. The acquired XPS spectra were deconvoluted using Gaussian peak fitting algorithms implemented in Origin Pro 2025 software.

4.3 RESULTS AND DISCUSSION

4.3.1 Ash colour

The ash color is a distinctive attribute that provides an approximate measure of the extent of combustion, based on the flow of air, and the presence of dry plant matter during smoldering combustion (Rodela *et al.*, 2022). The black color of banana ash (Fig. 4.1) suggests it still contains residual organic compounds, resulting from the low-temperature, slow and dull combustion process (Bodí *et al.*, 2011). In contrast, the formation of ash that occurs at relatively higher heating conditions, more likely due to sufficient oxygen supply, in general yields a light gray to white ash (Goforth *et al.*, 2005). Therefore, a gray color ash (Fig. 4.2) suggests that a relatively higher combustion rate of black gram plant matter than banana stem/roots.

4.3.2 pH Determination

The pH of banana ash was determined to be 10.5, indicating a strongly alkaline nature. In contrast, the pH of black gram ash was measured at 9.5, which, while also alkaline, is comparatively lower. The higher alkalinity of banana ash can be attributed to its higher content of alkaline metal oxides and earth alkaline components, such as potassium, calcium, and magnesium oxides, which are commonly present in banana ashes (Bordoloi *et al.*, 2025). On the other hand, the slightly lower pH of black gram ash implies a relatively lower concentration of basic oxides or the possible presence of more acidic constituents. Despite this, it still maintains an alkaline character, indicating its potential suitability for similar applications, albeit with possibly milder effects on pH adjustment when used as an additive.

4.3.3 Nitrate and nitrite determination

The nitrate concentration in banana ash was determined to be 11,908 µg/g, while its nitrite concentration was considerably lower at 14.7 µg/g. In comparison,

black gram ash exhibited a nitrate concentration of 4,590 µg/g and a nitrite concentration of only 0.5 µg/g.

The markedly higher nitrate content in banana ash likely reflects the greater nitrogen uptake and storage capacity of banana plants, which are known to accumulate nitrate as part of their metabolic processes (Marschner, 2012).

The low concentrations of nitrite in both ashes indicate minimal presence of this intermediate nitrogen form, which is often transient and less stable compared to nitrate. The higher nitrite level in banana ash relative to black gram ash is potentially attributable to the differences in thermal decomposition pathways of organic nitrogen during combustion or variations in the oxidation environment during ashing.

Although dietary nitrates can be beneficial in moderate amounts, serving as precursors for nitric oxide production, excessive intake poses health risks. Excessive nitrates can be reduced to nitrites in the human body, which may lead to the formation of carcinogenic N-nitroso compounds. High nitrate consumption has also been associated with methemoglobinemia, particularly in infants, and potential long-term cancer risks in adults (L'hirondel & L'hirondel, 2002). The World Health Organization (WHO) has established an acceptable daily intake (ADI) for nitrate at 3.7 mg per kg of body weight per day, which corresponds to approximately 222 mg of nitrate per day for an adult weighing 60 kg. For nitrite, the ADI is set at 0.07 mg per kg of body weight per day, equivalent to about *4.2 mg per day for a 60-kg individual* (Keller *et al.*, 2020).

In this context, the significant nitrate concentrations observed in banana ash suggests a greater potential for nitrate exposure when used as a food additive, compared to black gram ash. Although the nitrite concentrations in both ashes are relatively low, however even smaller amounts are significant, given the higher toxicity potential of nitrite. Overall, while both banana and black gram ashes may provide certain functional or nutritional benefits when used as traditional food additives, their high nitrate content, particularly in banana ash, necessitates careful evaluation to ensure consumer safety.

4.3.4 PXRD analysis

In this study, detailed X-ray diffraction (XRD) analysis was performed for banana and black-gram biomass ashes to identify and compare their crystalline phases, morphological features, and lattice parameters.

4.3.4.1 Banana ash

The XRD diffraction profile of banana ash in the 2θ range of 20° – 80° revealed distinct and relatively sharp peaks attributable to several crystalline mineral phases (Fig. 4.3). Typical calcium carbonate (CaCO_3) mineral in biomass ash, calcite was identified with characteristic diffraction planes $(1\ 0\ 4)$, $(0\ 0\ 6)$, $(2\ \bar{1}\ 0)$, $(2\ \bar{1}\ 3)$, $(2\ 0\ 2)$, $(1\ 0\ \bar{8})$, $(2\ \bar{1}\ 6)$, and $(3\ \bar{1}\ \bar{2})$. The corresponding trigonal crystal structure (hexagonal), lattice parameters for calcite, $a = 4.9900\ \text{\AA}$, $b = 4.9900\ \text{\AA}$, $c = 17.0615\ \text{\AA}$; $\alpha = 90^\circ$, $\beta = 90^\circ$, and $\gamma = 120^\circ$ (COD ID 9000095). Dolomite shares similar trigonal crystal structure (hexagonal axes) as indicated by diffraction planes $(1\ 0\ 4)$, $(1\ 0\ \bar{5})$, $(2\ \bar{1}\ \bar{3})$, $(2\ 0\ \bar{1})$, $(1\ 0\ \bar{8})$ and $(1\ 0\ 10)$ and lattice parameters $a = 4.8228\ \text{\AA}$, $b = 4.8228\ \text{\AA}$, $c = 16.227\ \text{\AA}$; $\alpha = 90^\circ$, $\beta = 90^\circ$, and $\gamma = 120^\circ$ (COD ID 9001007). Tobermorite was identified with a triclinic structure as indicated by diffraction planes $(3\ \bar{1}\ 0)$, $(2\ 2\ \bar{1})$, $(3\ 1\ 2)$, $(1\ 3\ 0)$, $(4\ \bar{2}\ 1)$, and $(4\ \bar{2}\ 3)$. The corresponding lattice parameters are $a = 11.156\ \text{\AA}$, $b = 7.303\ \text{\AA}$, $c = 9.566\ \text{\AA}$; $\alpha = 101.08^\circ$, $\beta = 92.83^\circ$, and $\gamma = 89.98^\circ$ (COD ID 9005447). Sylvite were identified with cubic crystal system as indicated by diffraction planes $(1\ 1\ 1)$, $(2\ 2\ 0)$, $(3\ 1\ 1)$, $(4\ 0\ 0)$, $(3\ 1\ 1)$, $(4\ 2\ 0)$, $(4\ 2\ 2)$, and $(5\ 1\ 1)$. The corresponding lattice parameters are $a = b = c = 6.4220\ \text{\AA}$; $\alpha = \beta = \gamma = 90^\circ$ (COD ID 9003133). Quartz was identified with a trigonal crystal structure based on diffraction planes $(\bar{2}\ 1\ 0)$, $(1\ 0\ \bar{2})$, $(2\ 0\ \bar{1})$, $(2\ 0\ \bar{2})$, and $(3\ \bar{1}\ 0)$ with the corresponding lattice parameters for Quartz are $a = 4.9209\ \text{\AA}$, $b = 4.9209\ \text{\AA}$, $c = 5.4091\ \text{\AA}$; $\alpha = 90^\circ$, $\beta = 90^\circ$, and $\gamma = 120^\circ$ (COD ID 9005018). Potassium silicate was identified with a monoclinic crystal system structure as indicated by diffraction planes $(1\ 2\ 0)$, $(2\ 1\ \bar{2})$, $(0\ 0\ 6)$, $(0\ 5\ 3)$, $(3\ 0\ \bar{4})$, and $(3\ 3\ \bar{4})$. The corresponding lattice parameters are $a = 6.27\ \text{\AA}$, $b = 12.808\ \text{\AA}$, $c = 16.96\ \text{\AA}$; $\alpha = 90^\circ$, $\beta = 117.38^\circ$, and $\gamma = 90^\circ$ (COD ID 1530995). Taken together, it seems clear that a complex mineral assemblage dominated by Ca, K, and Si-based minerals are present in banana ash.

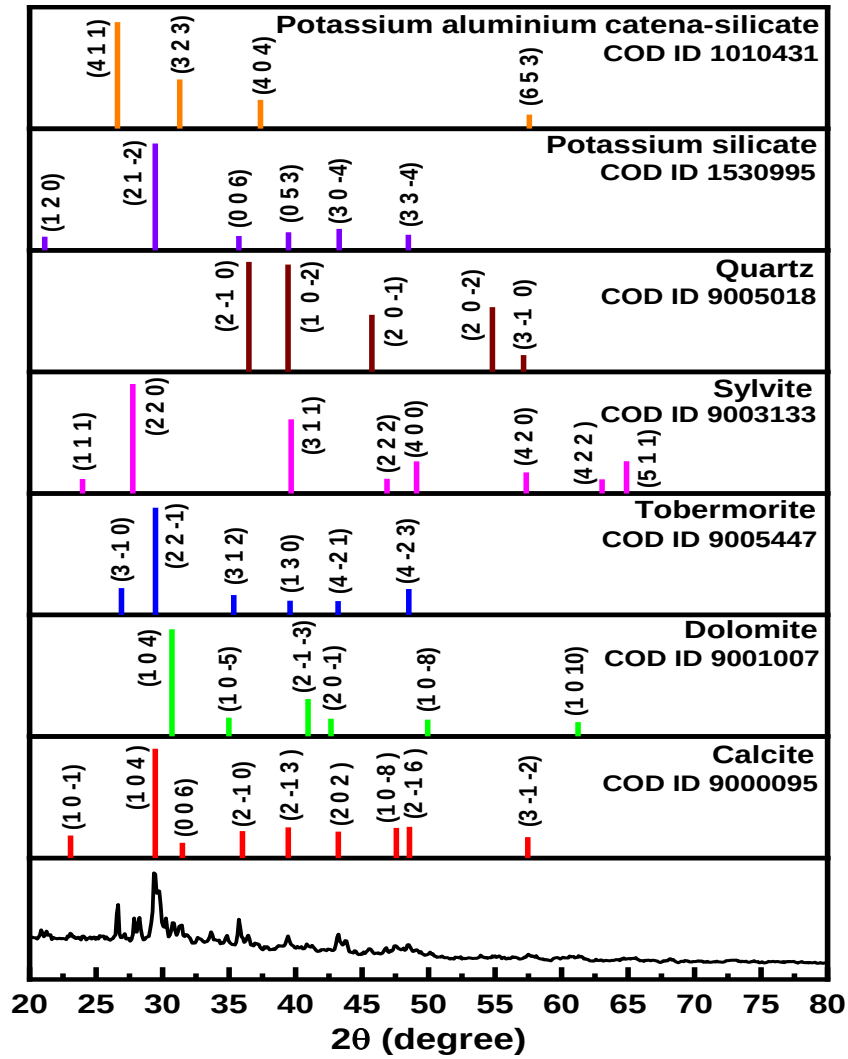


Fig. 4.3: PXRD analysis for Banana ash

4.3.4.2 Black gram ash

The XRD profile of black-gram ash in the 2θ range of 20° – 80° exhibits a diverse assemblage of crystalline phases (Fig. 4.4). Calcium carbonate (CaCO_3) was identified with a trigonal crystal structure (hexagonal axes), as indicated by prominent diffraction planes (104) , $(2\bar{1}0)$, $(2\bar{1}3)$, (202) , and $(2\bar{1}6)$. The corresponding lattice parameters for CaCO_3 , $a = 4.9915 \text{ \AA}$, $b = 4.9915 \text{ \AA}$, $c = 17.088 \text{ \AA}$; $\alpha = 90^\circ$, $\beta = 90^\circ$, and $\gamma = 120^\circ$ (COD ID 7022027) and similarly, calcite was identified with a trigonal crystal structure with a rhombohedral unit cell as indicated by prominent diffraction planes (110) , (211) , (222) , $(10\bar{1})$, (210) , (200) , (330) , and (404) .

2) and (3 2 1) and corresponding lattice parameters $a = b = c = 6.36 \text{ \AA}$; $\alpha = \beta = \gamma = 46.1^\circ$ (COD ID 1010962).

Dolomite was also identified with a trigonal crystal structure (hexagonal axes) as indicated by diffraction planes (1 0 1), (1 0 4), (0 0 6), (0 1 $\bar{5}$), (2 0 $\bar{1}$), (2 $\bar{1}$ $\bar{3}$), (2 0 $\bar{1}$) and (2 0 $\bar{4}$). The corresponding lattice parameters for dolomite are $a = 4.8247 \text{ \AA}$, $b = 4.8247 \text{ \AA}$, $c = 16.1243 \text{ \AA}$; $\alpha = 90^\circ$, $\beta = 90^\circ$, and $\gamma = 120^\circ$ (COD ID 9004931).

Tobermorite exhibits a triclinic structure with diffraction planes (1 1 $\bar{2}$), (3 $\bar{1}$ 0), (1 1 $\bar{3}$), (2 0 3), (1 3 $\bar{2}$), (1 3 $\bar{3}$), and (4 2 2). The unit cell parameters are $a = 11.156 \text{ \AA}$, $b = 7.303 \text{ \AA}$, $c = 9.566 \text{ \AA}$; $\alpha = 101.08^\circ$, $\beta = 92.83^\circ$, and $\gamma = 89.98^\circ$ (COD ID 9005447). This triclinic symmetry, with unequal axes and non-orthogonal angles, reflects a low-symmetry and complex layered structure, typical for hydrated calcium silicate minerals such as tobermorite. Sylvite was identified with a cubic (isometric) crystal system, as confirmed by diffraction planes (2 0 0), (2 2 0), (3 1 1), (2 2 2), and (4 2 0). The unit cell parameters are $a = b = c = 6.2126 \text{ \AA}$; $\alpha = \beta = \gamma = 90^\circ$, consistent with a highly symmetric cubic lattice (COD ID: 9003136). Quartz was identified with a trigonal crystal structure as indicated by diffraction planes (1 0 0), (1 0 $\bar{1}$), ($\bar{2}$ 1 0), (1 0 $\bar{2}$), (2 0 0) and (2 0 $\bar{1}$). The corresponding lattice parameters for Quartz are $a = 4.702 \text{ \AA}$, $b = 4.702 \text{ \AA}$, $c = 5.256 \text{ \AA}$; $\alpha = 90^\circ$, $\beta = 90^\circ$, and $\gamma = 120^\circ$ consistent with a trigonal system in the hexagonal setting (COD ID: 9000780). Finally, potassium sulfate (K_2SO_4) was identified with an orthorhombic crystal structure, as indicated by diffraction planes (1 1 1), (1 2 1), (0 2 2), (1 3 1), (0 3 2), (2 0 2), (1 2 3), and (0 0 4). The corresponding lattice parameters for K_2SO_4 are $a = 5.7503 \text{ \AA}$, $b = 10.0395 \text{ \AA}$, $c = 7.4513 \text{ \AA}$; $\alpha = \beta = \gamma = 90^\circ$, confirming its orthorhombic symmetry (COD ID: 2101319). Hence, a combination of carbonate, silicate, and alkali/alkaline earth mineral assemblages were observed which is consistent with the earlier studies on biomass ashes.

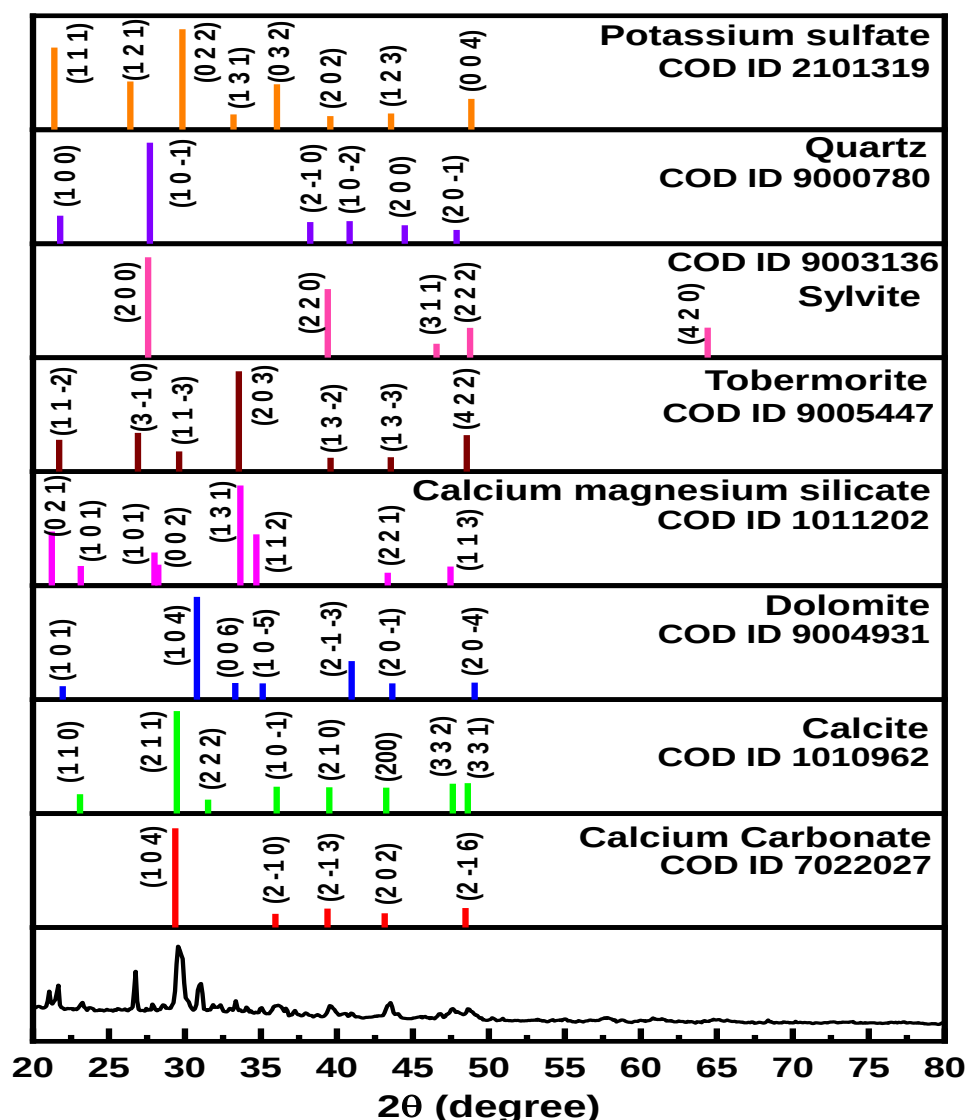


Fig. 4.4: PXRD analysis for Black gram ash

The XRD analysis of banana ash and black-gram ash revealed a diverse and complex chemical composition, reflecting the inorganic minerals uptake and the corresponding thermal decomposition profiles attributed to biomass ashes. Banana ash is dominated by calcite exhibiting a trigonal crystal structure. The coexistence of dolomite ($\text{CaMg}(\text{CO}_3)_2$) points to the significant magnesium concentrations in biomass. The presence of potassium-rich phases, including sylvite (KCl) and potassium silicate, highlights the high potassium content characteristic of banana biomass. Additionally, tobermorite and quartz suggest complex silicate phase transformations and the concomitant recrystallization processes occurring during

smoldering combustion and the subsequent characteristic of slower cooling process. In black-gram ash, calcium carbonate and calcite are prominent, confirming calcium-rich herbaceous biomass ash. Besides, the observed calcium/magnesium silicate phases suggest the extent of interaction(s) between Ca^{2+} , Mg^{2+} , and silica during combustion. The identification of potassium sulfate (K_2SO_4) alongside sylvite indicates the volatilization and re-condensation of K^+ ions during the ash-formation process that more likely causes the random distribution of alkali mineral phases. The presence of quartz in both ashes further confirms that some silica remains unreacted and crystallized after combustion. Taken together, PXRD analysis confirms that biomass ash exhibits several carbonate, silicate, and alkali/alkaline earth mineral phases, which reflect their versatile physico-chemical attributes.

4.3.5 XPS analysis

4.3.5.1 Banana Ash

XPS analysis was performed to elucidate the surface distribution of mineral constituents and their chemical (electronic) states in banana ash. The high-resolution XPS spectral features of various elements, viz., As, C, Ca, Cl, Mg, N, O, P, Si, and Zn located on or migrated to the surface (Fig. 4.5 (a)-(j)).

(a) C1s -K2p

The high-resolution XPS spectrum of banana ash, core level C 1s peaks were deconvoluted into two distinct peaks at 284.8 eV, and 288.4 eV, while XPS peaks attributed to K 2p were present at 292.9 eV, and 295.7 eV, in close proximity to but not overlapping with C 1s peaks. This multi-peak profile indicates the presence of various carbon species and chemical states on the surface of the ash sample.

The XPS peak at 284.8 eV can be attributed to adventitious carbon species in ash, typically arising from surface hydrocarbon contamination during combustion (Biesinger *et al.*, 2022). The presence of this peak indicates residual surface carbon, consistent with the incomplete combustion suggested by the color of banana ash. It is noteworthy that this peak is widely used as an internal reference for charge correction in XPS analysis (Morgan, 2025). The peak at 288.4 eV corresponds to

carbonyl (CO_3)²⁻/COOH with chemical state '+4/+3', indicating surface oxidation (Chen *et al.*, 2020). The prominent K 2p peaks were also observed in close proximity to the core level C 1s XPS peaks. Specifically, the K 2p_{3/2} and K 2p_{1/2} peaks appeared at 292.9 eV and 295.7 eV respectively, indicating the presence of chloride mineral of potassium.

(b) N 1s

The high-resolution XPS spectrum of N 1s shows two distinct peaks. The first peak is observed at 398.0 eV, and the second, more intense peak appears at 399.8 eV. The higher intensity of the peak at 399.8 eV suggests that this chemical state of nitrogen is dominant in the sample.

The peak at 398.0 eV is typically attributed to nitrile ($\text{C}\equiv\text{N}$) functionalities, representing nitrogen in a triple-bonded configuration. The more prominent peak at 399.8 eV is commonly assigned to nitrogen in heterocyclic structures, particularly those containing C–N and C=N bonds, as reported by Kuntumalla and Hoffman (2024). This suggests that nitrogen in banana ash predominantly exists in aromatic or graphitic-like environments, likely derived from nitrogen-containing organic precursors thermally transformed during combustion. The incorporation of such nitrogen species may enhance the alkaline properties and surface reactivity of banana ash, which aligns with its traditional application as a food additive (khar) in Northeastern Indian cuisine, where it is valued for both its functional and sensory attributes.

(c) O 1s

The high-resolution XPS spectrum of the O 1s region in banana ash displays a single, well-defined peak centered at 531.4 eV. This binding energy is consistent with oxygen present in surface-bound functional groups, particularly in carbonyl ($\text{C}=\text{O}$) and hydroxyl ($\text{C}-\text{OH}$) environments. The spectral profile does not exhibit significant peak splitting or broadening, suggesting a relatively homogeneous distribution of oxygen-containing species on the ash surface.

The O 1s XPS spectrum of banana ash shows a main peak at ~531.3 eV, typically attributed to carbonyl (C=O) and hydroxyl (C–OH) groups, as well as chemisorbed oxygen species. Notably, possible contributions from surface carbonate (CO_3^{2-}) and hydroxide (OH^-) species should also be considered, as these can appear around 531–532 eV and influence peak assignment (Stoch and Gablankowska-Kukucz, 1991). The presence of these groups reflects the partial oxidation of organic matter during combustion and the formation of mineral–oxygen bonds on the ash surface.

The incorporation of such oxygen-containing functional groups significantly influences the physicochemical properties of the ash. These groups enhance surface polarity, and solubility. This is particularly relevant to its traditional culinary use as khar, where it functions not only as a flavoring agent but also as a mild base or digestive aid. Furthermore, these functionalities may promote interactions with other food components or minerals, potentially enhancing bioavailability of minerals in indigenous dietary factors.

(d) Mg 1s

The high-resolution XPS peak of Mg 1s occurred at 1304.2 eV. The peak is symmetric and did not exhibit splitting or shoulders, reflecting the most likely chemical environment of Mg^{2+} ions in banana ash.

The Mg 1s peak at 1304.2 eV is characteristic of the oxidized form, such as Mg^{2+} in magnesium carbonate (Chubar., 2018).

(e) Si 2p

The high-resolution XPS spectrum of Si 2p shows a single, prominent peak centered at 102.7 eV. The peak is symmetric and did not show any noticeable splitting or shoulders, suggesting the presence of a single dominant silicate phase located at the surface of biomass ash.

The Si 2p peak at ~102.7 eV is characteristic of silicon in silicate forms (Si–O), typically assigned to Si^{4+} oxidation states in silica (SiO_2) or aluminosilicate minerals (Li *et al.*, 2017). The presence of silicate structures contributes to the

structural stability and thermal resistance of the ash. In traditional culinary use as Khar, silicates are not directly involved in alkalinity but may influence textural properties when the ash is dispersed and is capable of causing abrasions in oral cavity or esophagus when the foodstuff mixed with ash is consumed more frequently.

(f) P2p

The high-resolution XPS spectrum of P 2p shows a single, sharp peak centered at 133.2 eV. The peak appears symmetric and well-defined, suggesting a single dominant chemical state for phosphorus in the sample.

The P2p binding energy observed at 133.2 eV is characteristic of inorganic phosphate species, especially those in the form of pyrophosphate ($[\text{P}_2\text{O}_7]^{4-}$) or orthophosphate ($[\text{PO}_4]^{3-}$). According to Huang *et al.*, (2014), phosphorus in lignocellulosic biomass carbonized under moderate conditions typically presents as phosphate-based species, with P2p peaks ranging from ~132.0 to 134.0 eV. The specific position at 133.2 eV lies within this range and indicates the presence of oxidized phosphate, likely as pyrophosphate, formed during the thermal degradation of plant tissues.

Further support for this assignment is found in the work of Zhang *et al.*, (2024), who reported a P2p peak at approximately 133.6 eV, attributed to PO_4^{3-} groups in corrosion products of magnesium alloys exposed to simulated body fluid. This suggests that P in banana ash is also in a stable, oxidized, and the relevant inorganic phosphate form.

(g) Cl2p

The high-resolution XPS spectrum of the Cl 2p region for banana ash showed a single prominent peak centered at 198.5 eV. The symmetrical and well-defined nature of the peak indicates a stable chemical state. The Cl 2p peak at 198.5 eV is typically corresponds to Cl 2p_{3/2} peak which is indicative of chloride ions (Cl^-) present in simple alkali salts such as NaCl and KCl (Wang *et al.*, 2021). This binding energy falls within the typical range for alkali metal chlorides and suggests that chlorine in banana ash predominantly exists as soluble chloride salts. The presence of

chlorine in banana ash is most likely attributed to the natural mineral content of the plant material where Cl⁻ ion is essential for the photosynthesis (Chen *et al.*, 2010). The retained chloride content can contribute to the overall ionic character of the ash, potentially influencing its solubility when used as a food additive.

(h) Ca2p

The high-resolution XPS spectrum of the Ca2p region in banana ash revealed two distinct and well-resolved peaks at 347.0 eV and 350.7 eV, corresponding to Ca 2p_{3/2} and Ca 2p_{1/2}, respectively. The observed spin–orbit splitting of approximately 3.7 eV is consistent with the expected values for divalent calcium (Ca²⁺) in oxidized forms (Kumar *et al.*, 2020). The peaks are symmetric with no evidence of significant broadening or asymmetry, indicating a chemically stable and uniform calcium environment. These binding energies suggest the presence of Ca²⁺ ion, such as CaCO₃, or hydrated CaCO₃, commonly found in plant-derived ash residues (Park *et al.*, 2020; Lu *et al.*, 2019).

The observed Ca2p binding energies in banana ash suggests the presence of calcite which is in agreement with earlier studies (Ni and Ratner, 2008). These polymorphs of calcium carbonate differ in their calcium coordination geometry and surface chemistry, which can slightly shift the binding energies. The observed Ca 2p BE suggests the presence of calcium in a carbonate-rich environment, possibly influenced by partial hydration or surface structural disorder. However, the absence of peak broadening indicates that calcium exists primarily in a well-ordered, oxidized state, possibly as CaCO₃, CaO, or Ca(OH)₂, rather than as amorphous or surface-hydrated species.

Furthermore, these findings are supported by the work of Park *et al.*, (2020), who reported similar Ca2p_{3/2} and Ca2p_{1/2} binding energies (~346.7 and 350.1 eV) in biomass-derived fly ash, identifying calcium mainly existing as CaCO₃ which also corroborate PXRD data. The binding energy also indicates potential for interaction with chloride, given the Cl2p signal at 198.5 eV in banana ash, which may suggest the presence of CaClOH or related chloride-bearing calcium phases. Taken together,

the XPS data confirm that calcium species in banana ash is predominantly exists in oxidized, stable, and reactive forms, as carbonate (Park *et al.*, 2020).

(i) Zn 2p

The high-resolution XPS spectrum of the Zn2p region in banana ash shows a distinct and symmetric peak centered at 1022.0 eV, which corresponds to the Zn 2p_{3/2} core level. The peak exhibits a narrow profile with no visible shoulder or broadening, indicating that zinc exists predominantly in a single, well-defined chemical state.

The observed binding energy of 1022.0 eV is consistent with the presence of Zn²⁺ in ZnO, as supported by the findings of Biesinger *et al.*, (2010), who systematically analyzed the XPS signatures of first-row transition metals. In their work, ZnO was found to produce Zn2p_{3/2} peaks in the range of 1021.7 eV, distinguishing it clearly from metallic zinc (Zn⁰), which typically appears at a lower binding energy.

Thus, the Zn2p XPS data confirm the presence of zinc in the Zn²⁺ oxidation state, most plausibly as ZnO, in banana ash matrix possibly as an amorphous mineral form.

(j) As 3d -Mg 2p

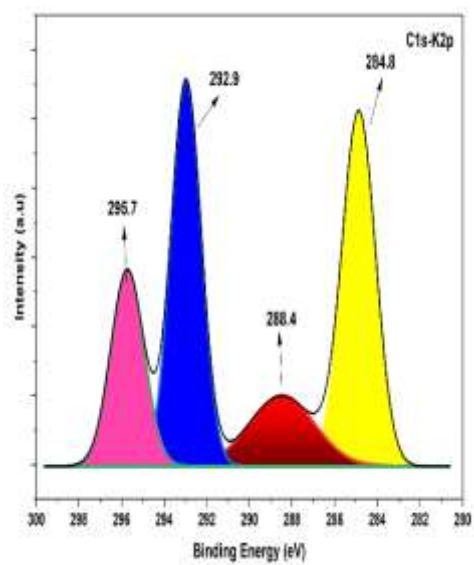
The high-resolution XPS spectrum of the As3d–Mg2p region of banana ash reveals two distinct photoelectron peaks located at 44.5 eV and 50.1 eV. The peak at 44.5 eV is assigned to the As 3d_{5/2} level, while the feature at 50.1 eV corresponds primarily to the Mg 2p core level. The spectral features are sharp and symmetric, suggesting that arsenic exists in chemically well-defined and stable oxidation states.

The As 3d_{5/2} peak at 44.5 eV is characteristic of trivalent arsenic (As³⁺) in an oxidized form, typically associated with compounds such as arsenites (e.g., As(III)–O species). These results corroborate the findings of Feng *et al.*, (2023), who reported binding energies for oxidized As(III) species.

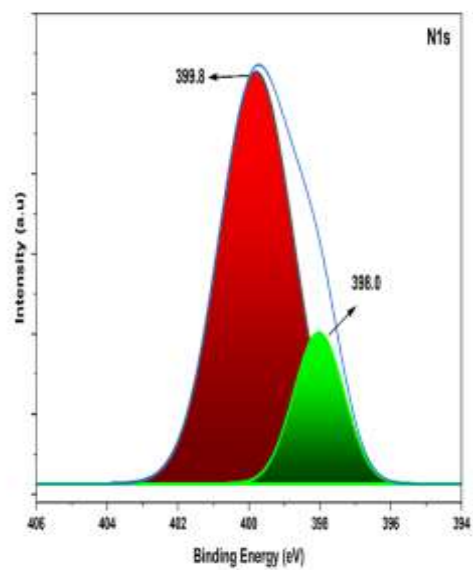
This suggests that arsenic in banana ash exists in an oxidized, but trivalent, form rather than as elemental As. Although As³⁺ is more toxic and mobile than As⁵⁺, the presence of As(III)–O implies partial chemical stabilization within the ash matrix.

The 50.1 eV peak lies within the range of the Mg 2p core level, which reflects the presence of Mg^{2+} in compounds such as MgO , $\text{Mg}(\text{OH})_2$, or MgCO_3 . However, this BE also overlaps with the As 3d_{3/2} peak for As^{5+} , making interpretation more complex. As noted by Ohki *et al.*, (2005), the As 3d_{3/2} component of pentavalent arsenic can appear in the same region as Mg 2p, resulting in spectral interference. Therefore, while the peak at 50.1 eV is most likely dominated by Mg^{2+} , a minor contribution from As^{5+} species cannot be ruled out.

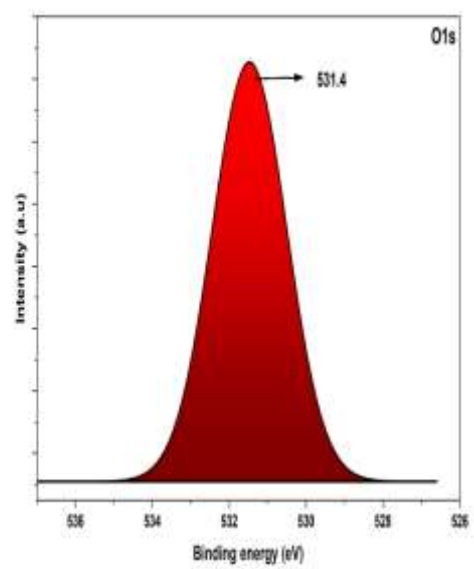
The low signal-to-noise ratios observed for the elements Fe, Mn, Cr, and S hindered the potential identification of the chemical (electronic) states of these species in banana ash, likely due to their lower surface concentrations, which resulted in weak and poorly resolved XPS spectral features. However, their presence cannot be definitively ruled out, as they may exist below the detection limit or in chemical environments not readily detectable under the current experimental conditions. However, their distributions can be assessed using the bulk chemical analysis such as AAS/AES methods.



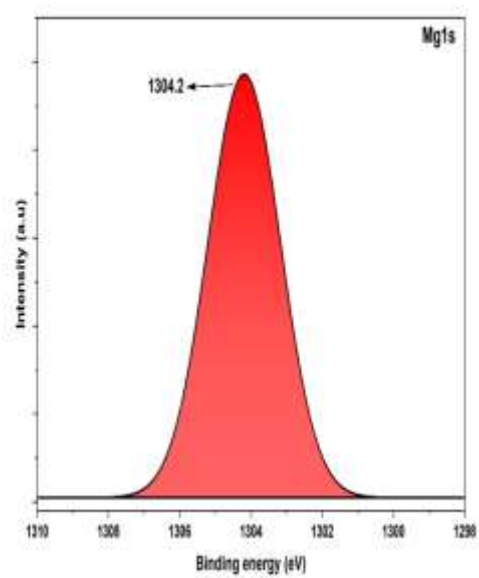
(a)



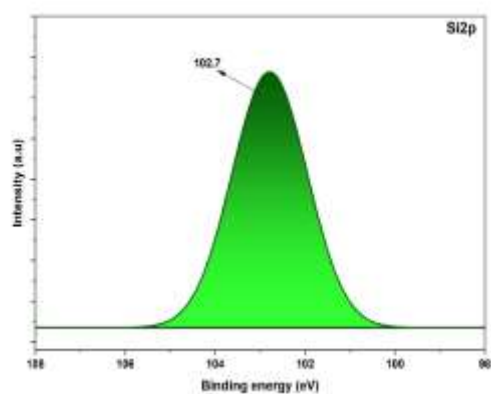
(b)



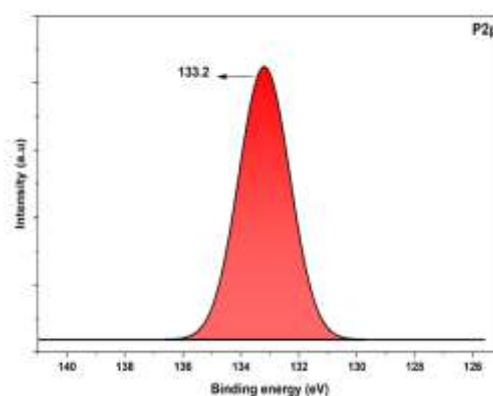
(c)



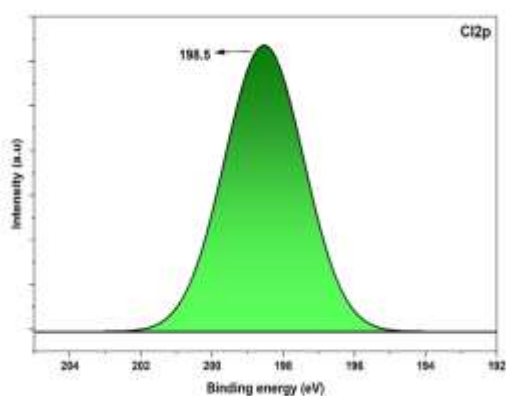
(d)



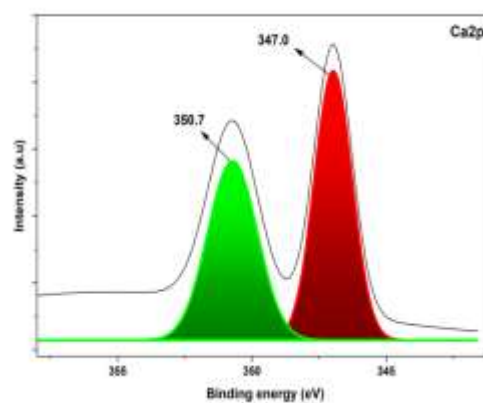
(e)



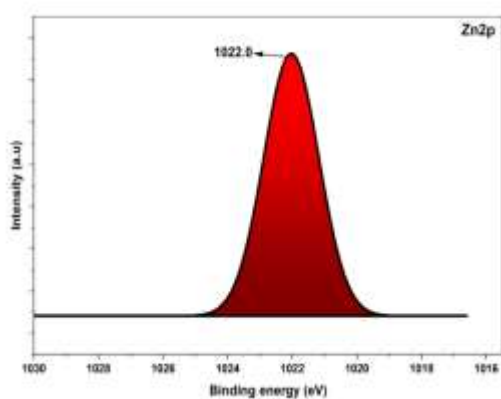
(f)



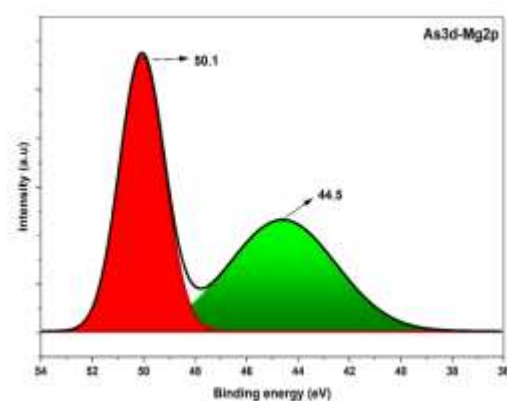
(g)



(h)



(i)



(j)

Fig. 4.5: XPS spectra of various elements present in Banana ash

(a) C1s-K2p; (b) N1s; (c) O1s; (d) Mg1s; (e) Si2p; (f) P2p; (g) Cl2p; (h) Ca2p;
(i) Zn2p; (j) As3d-Mg2p

4.3.5.2 Black gram ash

XPS analysis of black gram ash was also conducted similar to banana ash to elucidate the surface elemental composition and corresponding chemical state. The high-resolution XPS spectra for C, N, O, Mg, Si, P, Cl, Ca, Zn, As and S present on the surface of black gram are presented in Fig. 4.6 (a)- (k).

(a) C1s-K2p

The high-resolution XPS spectrum of C 1s for black gram ash displays four distinct peaks located at BE of 284.8 eV, 288.7 eV, 292.8 eV, and 295.6 eV. These peaks suggest the presence of different carbon-containing functional groups in the sample.

During combustion, incomplete decomposition of organic matter can leave carbon residues, while potassium migrates and enriches at the surface, resulting in strong K 2p peaks appearing near the higher BE side of the C 1s region. The co-existence of strong K 2p and C 1s signals highlights the complex, mixed organic inorganic nature of banana ash surfaces.

The peak at 284.8 eV can be attributed to adventitious carbon species in ash, typically arising from surface hydrocarbon contamination during combustion in the black gram ash (Biesinger, 2022). The peak at 288.7 eV corresponds to carbon in oxygen-containing groups, such as carbonyl (C=O) or carboxyl (O-C=O) functionalities, suggesting partial oxidation of the carbon surface (Lomon *et al.*, 2018).

The peak observed at 292.8 eV is can be assigned to the K 2p_{3/2}. Similarly, peak at 295.6 eV is attributed to K 2p_{1/2} peaks. During combustion, incomplete decomposition of organic matter can leave carbon residues, while potassium migrates and enriches at the surface, resulting in strong K 2p peaks appearing near the higher BE side of the C 1s region. The co-existence of strong K 2p and C 1s signals highlights the complex, mixed organic–inorganic nature of banana ash surfaces, suggesting partial oxidation during open-air combustion.

(b) N1s

The high-resolution XPS spectrum of N 1s for black gram ash displays two distinct peaks located at binding energies of 397.8 eV and 399.7 eV. The peak at 399.7 eV is more intense, while the peak at 397.8 eV is comparatively smaller. These peaks indicate the presence of different nitrogen chemical states in the sample.

The peak at 397.8 eV is commonly assigned to pyridinic nitrogen species, which correspond to nitrogen atoms bonded to two carbon atoms at the edges (Yamada *et al.*, 2021; Kawai *et al.*, 2022; Yamada *et al.*, 2022). The more intense peak at 399.7 eV is typically attributed to pyrrolic or amine-type nitrogen, where nitrogen is bonded to two carbon atoms in a five-membered ring or present as surface amine groups (Yamada *et al.*, 2021).

The presence of both pyridinic and pyrrolic/amine nitrogen suggests incorporation of nitrogen during combustion of the black gram biomass, likely originating from inherent protein and amino acid content. Additionally, the existence of nitrogen functionalities may contribute to the characteristic alkalinity and reactivity of black gram ash when used as a traditional food additive (Khar) in Northeastern cuisine.

(c) O1s

The high-resolution O 1s XPS spectrum of black gram ash exhibits a single, prominent peak centered at 531.3 eV. This peak suggests the presence of oxygen in surface functional groups.

The O 1s XPS spectrum of black gram ash shows a main peak at 531.3 eV, typically attributed to carbonyl (C=O) and hydroxyl (C–OH) groups, as well as chemisorbed oxygen species. Notably, possible contributions from surface carbonate (CO_3^{2-}) and hydroxide (OH^-) species should also be considered, as these can appear around 531–532 eV and influence peak assignment (Park *et al.*, 2020).

The incorporation of oxygen-containing groups can significantly affect the physicochemical properties of the ash. These functionalities improve surface hydrophilicity and enhance solubility, contributing to the ash's characteristic alkalinity and its interaction with other components when used as a traditional food

additive. The presence of these groups may also influence its reactivity and potential as a mild base or mineral supplement in culinary applications.

When compared to banana ash, black gram ash shows a relatively more defined and narrower O 1s peak, indicating a somewhat lower overall surface oxidation. Banana ash, which originates from biomass with higher cellulose and hemicellulose contents, generally exhibits more extensive oxygen functionalities and multiple O 1s components (including contributions from carboxyl and ester groups). In contrast, the oxygen content in black gram ash appears to be more concentrated in fewer types of functional groups, likely due to its higher N content as black gram is leguminous plant species (Koufali *et al.*, 2016). This difference could explain subtle variations in flavor contribution, solubility, and reactivity between the two ashes when used as a flavoring agent/food additive.

(d) Mg 1s

The high-resolution deconvoluted XPS spectrum of Mg 1s for black gram ash exhibits a single, sharp peak at a binding energy of 1304.2 eV, indicating the presence of magnesium species in the sample.

The Mg 1s peak at 1304.2 eV is characteristic of Mg^{2+} in oxidized forms, typically assigned to magnesium oxide (MgO) or magnesium hydroxide ($\text{Mg}(\text{OH})_2$). According to Chubar *et al.*, (2018), similar Mg 1s binding energies (around 1304 eV) have been observed in Mg-Al layered double hydroxides and are indicative of surface $\text{Mg}(\text{OH})_2$ and MgO phases that contribute to surface reactivity and anion exchange capacity. The presence of magnesium suggests partial retention of mineral nutrients from the original biomass during combustion. Magnesium-containing phases can contribute to the overall alkalinity and buffering capacity of the ash, supporting its traditional role as a food additive (Khar). The sharper and more defined Mg 1s peak in black gram ash suggests a more uniform magnesium environment and indicates well-preserved, simpler magnesium phases formed during combustion. In contrast, the slightly broader Mg 1s peak in banana ash implies a more heterogeneous chemical state, possibly due to magnesium being incorporated into mixed mineral phases such as silicates or phosphates, which is consistent with

the higher silica and variable mineral content reported for banana biomass ash. This difference suggests that black gram ash may provide a more stable and consistent magnesium contribution, enhancing its alkalinity and functionality when used as a traditional food additive (Khar). Meanwhile, the variability in banana ash magnesium content could result in different levels of alkalinity, influenced by the biomass source and processing conditions.

(e) Si2p

The deconvolution of the high-resolution Si2p core level reveals single peak centered at 102.5 eV, indicating the presence of silicon species in an oxidized state.

The Si 2p peak at ~102.5 eV is characteristic of silicon in Si⁴⁺ oxidation states, commonly assigned to silica (SiO₂) or silicate structures which corroborate PXRD data. According to Li *et al.*, (2018), similar Si 2p peaks in the range of 101.6–102.6 eV correspond to various hybrid silica environments with different degrees of condensation and local bonding states. This supports the assignment of silicon in black gram ash to oxidized silicate phases.

The presence of silicate species can contribute to the structural stability and thermal resistance of the ash, although they do not directly affect alkalinity. Compared to banana ash, which often contains higher and more complex silicon phases due to its naturally higher silica content, the black gram ash exhibits a simpler and more uniform Si 2p signal, suggesting fewer or less complex silicate structures. This difference may influence the textural or dispersibility properties when used as a food additive. However, silica/silicate minerals in biomass also renders microcrystallinity to the plant ash which may portend negative health consequences when the ash is consumed indiscriminately.

(f) P2p

The XPS spectrum of the black gram ash sample in the phosphorus (P2p) region exhibits a single, well-defined peak centered at a binding energy of 133.0 eV. The peak is symmetrical and narrow, indicating a uniform chemical environment for phosphorus.

The binding energy observed for the P2p peak at 133.0 eV corresponds well to inorganic phosphate species, especially pyrophosphates ($[\text{P}_2\text{O}_7]^{4-}$) or orthophosphates ($[\text{PO}_4]^{3-}$). According to Huang *et al.*, (2014), phosphorus in lignocellulose-derived carbon typically appears in the range of 132–134 eV, corresponding to various phosphate groups such as orthophosphate, pyrophosphate, and metaphosphate. The peak at 133.0 eV specifically matches with pyrophosphate species, which have been commonly observed in biomass-derived materials subjected to thermal treatment. This supports the conclusion that the phosphorus in black gram ash exists predominantly in oxidized phosphate forms, likely derived from the mineralization of organic phosphorus compounds during combustion (Zhang *et al.*, 2024).

(g) Cl2p

The XPS analysis of the Cl2p region revealed a single, symmetric peak centered at a BE of 198.6 eV. The peak exhibits a strong intensity, indicating a significant presence of chlorine in the analyzed sample of black gram ash. The absence of peak splitting suggests that the chlorine species exist in a chemically uniform environment.

The Cl2p peak at 198.6 eV corresponds to the Cl 2p_{3/2} level typically associated with chloride ions (Cl^-) in compounds such as NaCl and KCl. This observation indicates the presence of chlorine in the form of inorganic chloride salts. The absence of significant Cl 2p_{1/2} splitting suggests a chemically uniform environment, implying that chlorine exists mainly in a stable chloride state rather than in oxidized or complexed forms. This is consistent with findings from Wang *et al.*, (2021), where similar Cl2p peaks in MSWI fly ash was attributed to NaCl, KCl, and CaClOH. They noted that binding energies for Cl 2p_{3/2} in KCl fall near 198.6 eV, which matches our spectrum.

(h) Ca2p

The high-resolution XPS analysis of the Ca2p region in black gram ash revealed two distinct peaks at 346.8 eV and 350.4 eV, corresponding to Ca 2p_{3/2} and Ca 2p_{1/2}, respectively. These peaks represent the typical spin–orbit doublet of

divalent calcium (Ca^{2+}). The binding energy range aligns with literature values for calcium oxide (CaO), calcium carbonate (CaCO_3), and possibly calcium silicate (CaSiO_3), all of which are plausible in the ash matrix. The $\text{Ca}2\text{p}$ binding energies observed in black gram ash fall within the expected range for calcium compounds in an oxidized state. The $\text{Ca } 2\text{p}_{3/2}$ peak at 346.8 eV is comparable to those reported for calcite (346.5 eV) as described in the study by Ni and Ratner (2008). Further, the findings correspond well with those of Park *et al.*, (2020), who studied $\text{Ca}2\text{p}$ XPS spectra in biomass-derived fly ash and observed binding energies of 346.7 eV ($\text{Ca } 2\text{p}_{3/2}$) and 350.1 eV ($\text{Ca } 2\text{p}_{1/2}$). Their analysis identified the presence of calcium in forms such as CaO and CaCO_3 , which also played an active role in heavy metal adsorption (e.g., Cd^{2+}), through mechanisms like ion exchange and precipitation. Overall, the $\text{Ca}2\text{p}$ spectrum confirms the dominance of inorganic Ca^{2+} phases in black gram ash, contributing to the ash's alkaline character.

(i) $\text{Zn } 2\text{p}$

The high-resolution XPS analysis of the $\text{Zn}2\text{p}$ region in black gram ash reveals a single, prominent peak at 1022.1 eV, corresponding to the $\text{Zn } 2\text{p}_{3/2}$ core level. The peak is symmetric and narrow, indicating a well-defined zinc chemical state with no evidence of mixed or metallic species. The position and shape of the peak suggest that zinc is present in a fully oxidized state, consistent with the formation of zinc oxide (ZnO) or other Zn^{2+} -containing compounds.

The $\text{Zn}2\text{p}_{3/2}$ binding energy at 1022.1 eV aligns well with values reported for Zn^{2+} in ZnO , as established by Biesinger *et al.*, (2010), who conducted detailed XPS investigations of transition metal chemical states. In their study, ZnO exhibited a $\text{Zn}2\text{p}_{3/2}$ peak between 1021.7 eV. Although Auger parameter analysis (combining XPS and Zn LMM spectra) is ideal for conclusively differentiating zinc species, the absence of peak asymmetry or lower-energy shoulders in this spectrum strongly supports the dominance of Zn^{2+} in the form of ZnO . Zinc in the oxidized state may have originated from the uptake of zinc by the plant during growth and retained in the ash post-combustion. The presence of ZnO in black gram ash is significant, as it

may contribute to surface reactivity, adsorptive potential, and even antimicrobial properties if reused in agricultural or environmental applications.

(j) As 3d – Mg 2p

The high-resolution XPS spectrum of the As3d–Mg2p region for black gram ash exhibits two noticeable peaks: one at 44.0 eV and another at 49.8 eV. The peak at 44.0 eV is assigned to the As 3d_{5/2} orbital, while the one at 49.8 eV is attributed to the Mg 2p level. Both peaks appear symmetric and well-resolved, indicating distinct and stable chemical environments for arsenic and magnesium. 44.8 ev.

The peak at 44.0 eV corresponds to the As 3d_{5/2} binding energy, which is indicative of arsenic in the trivalent oxidation state (As³⁺), specifically in an oxidized As (III)–O configuration. This result is consistent with the findings of Feng *et al.*, (2023), who reported that As³⁺ bonded with oxygen in arsenite-type species typically appears near BE~44.8 eV. While trivalent arsenic (As³⁺) is more toxic and mobile than its pentavalent form (As⁵⁺), its association with oxygen suggests it exists in a chemically stabilized, non-elemental form within the ash matrix.

The second peak at 49.8 eV corresponds to Mg 2p, which generally appears in the 49.5–50.5 eV range for magnesium in oxidized states such as MgO, Mg(OH)₂, or MgCO₃. Magnesium, likely derived from plant uptake and ash mineralization, plays a key role in influencing ash alkalinity and may also contribute to arsenic immobilization through the formation of magnesium arsenites.

However, the interpretation of the higher binding energy region in the As3d spectrum is complicated by peak overlap. According to Ohki *et al.*, (2005), the As 3d peaks for pentavalent arsenic (As⁵⁺) can appear at higher BE, and their spin–orbit pair (As 3d_{3/2}) can overlap with the Mg 2p peak at approximately 49.8 eV. In this spectrum, the peak at 49.8 eV could thus contain contributions from both Mg 2p and As 3d_{3/2} (As⁵⁺), making it difficult to definitively resolve the presence of As⁵⁺ without Auger parameter analysis. This spectral interference is a known limitation in systems where both magnesium and arsenic coexist.

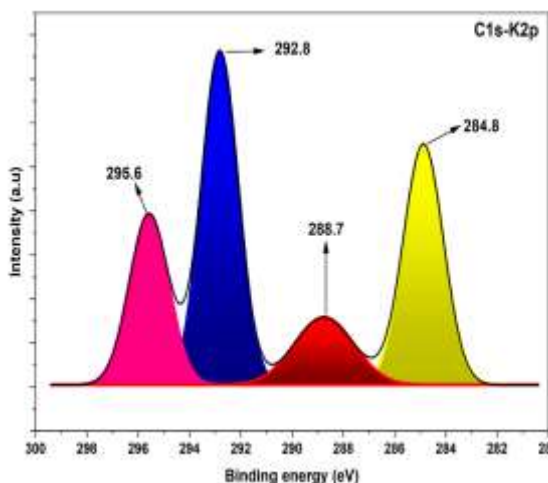
In conclusion, the As3d XPS data for black gram ash confirm the presence of oxidized trivalent arsenic ($\text{As}^{3+}\text{-O}$), while the potential presence of As^{5+} remains uncertain due to its overlap with Mg 2p. Magnesium is present in the form of Mg^{2+} , and its coexistence with arsenic may aid in partial stabilization.

(k) S2p

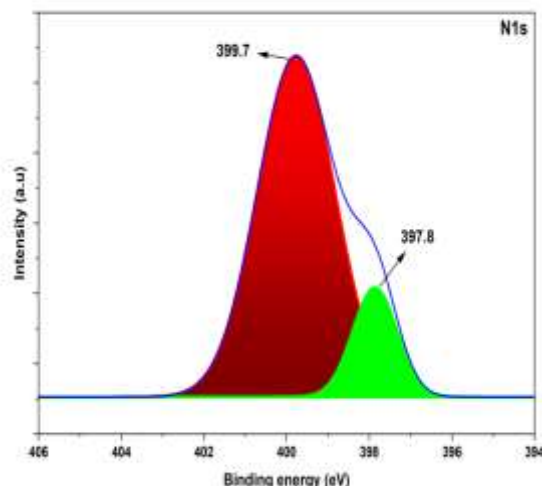
The XPS spectrum of the S 2p region in black gram ash shows a single, well-defined peak centered at 168.8 eV, attributed to the S $2p_{3/2}$ core level. This binding energy is characteristic of sulfur in a fully oxidized form, specifically consistent with the presence of sulfate (SO_4^{2-}) species.

The observed S $2p_{3/2}$ binding energy at 168.8 eV in black gram ash is in close agreement with the values reported for sulfate compounds in the literature. For instance, Gard *et al.*, (2020) identified S $2p_{3/2}$ peaks centered at (169.0 ± 0.1) eV, which were conclusively attributed to SO_4^{2-} species. The minor deviation of 0.2 eV falls within the typical range of experimental variation and chemical environment influence, supporting the assignment of the detected sulfur species in black gram ash as sulfates. The presence of sulfate is likely due to the thermal oxidation of organically bound sulfur or uptake from the plant's natural sulfur content during growth. Sulfate species in ash matrices are generally stable and water-soluble, and their identification has implications for ash reactivity, pH behavior.

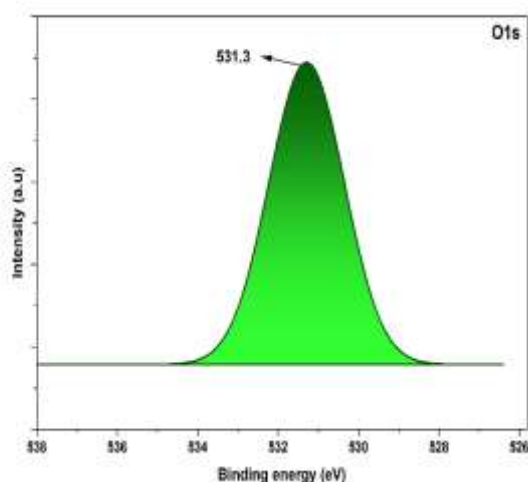
The low signal-to-noise ratios observed in the XPS spectra for Fe, Mn, and Cr hindered accurate determination of their chemical states. This limitation is likely due to their relatively low surface concentrations within the black gram ash matrix, which resulted in weak and indistinct spectral features. However, despite the lack of resolvable signals, the presence of these elements cannot be ruled out, as they may still exist below the detection threshold or in forms not readily captured under the current analytical conditions.



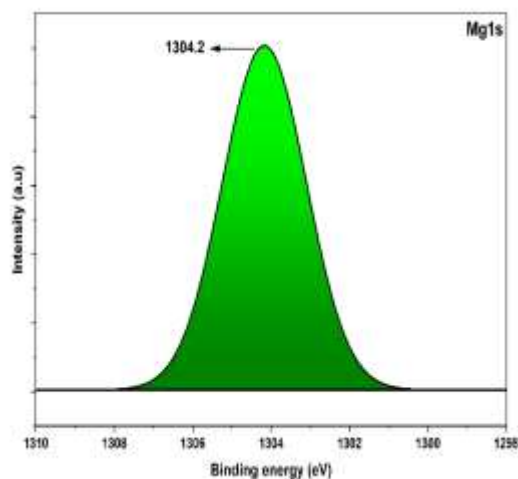
(a)



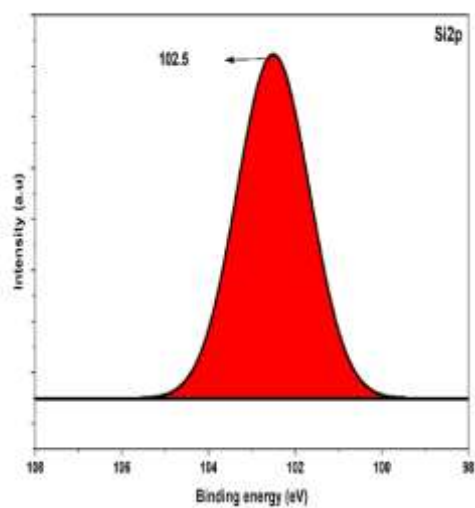
(b)



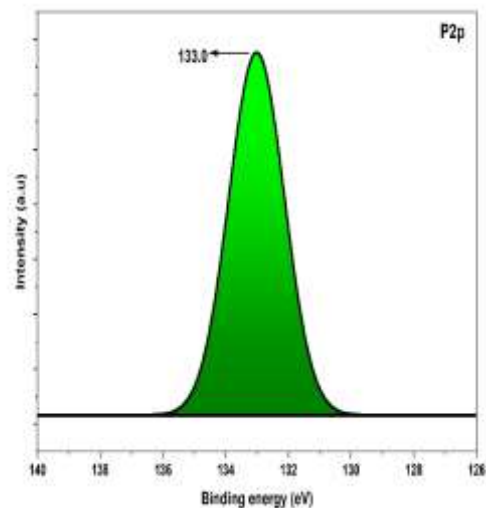
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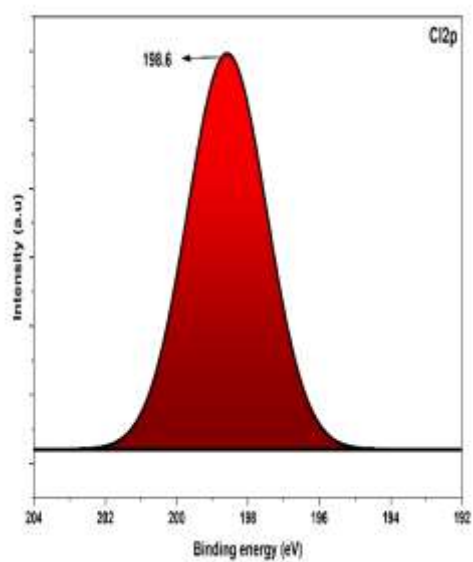
(d)



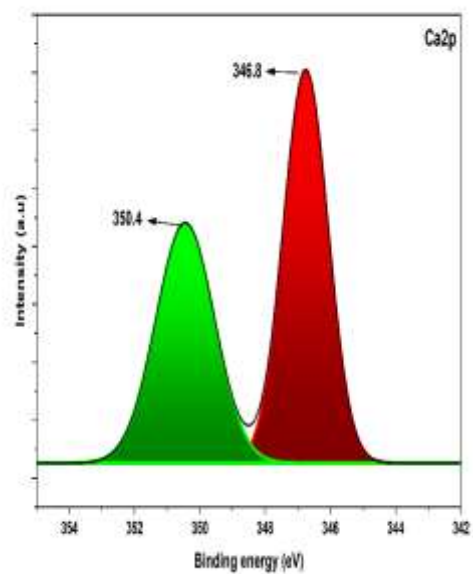
(e)



(f)



(g)



(h)

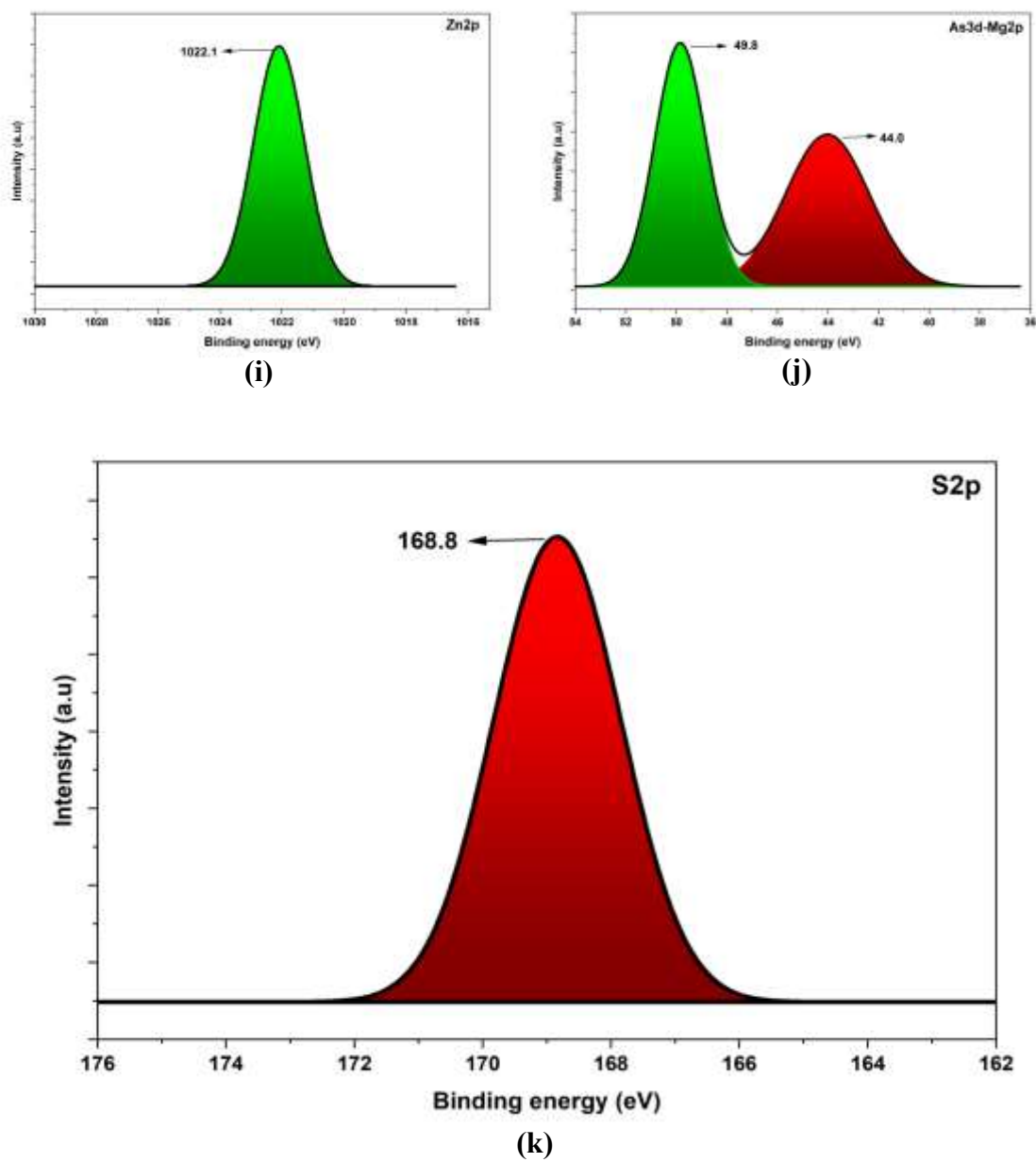


Fig. 4.6: XPS spectra of various elements present in black gram ash

(a) C1s-K2p; (b) N1s; (c) O1s; (d) Mg1s; (e) Si2p; (f) P2p; (g) Cl2p; (h) Ca2p;
(i) Zn2p; (j) As3d-Mg2p; (k) S2p

4.3.5 FE-SEM and EDX Mapping

Field-emission scanning electron microscopy coupled with energy-dispersive X-ray spectroscopy (FE-SEM-EDX) was conducted on multiple randomly selected regions of the collected samples at varying magnifications to evaluate the spatial distribution, morphological characteristics, and chemical composition of the representative inorganic particulates.

In addition to standard SEM-EDX analysis, high-resolution elemental mapping was performed for each sample, revealing a non-uniform distribution of constituent elements. This heterogeneity in elemental dispersion further corroborates the intrinsically complex and heterogeneous nature of the investigated samples. The combined analytical approach provided comprehensive insights into the microstructural and compositional variations across different sample regions.

4.3.6.1 BANANA ASH

In the light of PXRD peaks observed in the banana ash, SEM was applied to probe the morphological features of inorganic species most likely to be present in the banana ash. The morphological study reveals a complex composition of banana ash, where a random distribution of organic particles aggregates along with micro-crystalline phytolith-derived minerals are found. PXRD confirms the presence of crystalline phases, including calcite (CaCO_3), quartz (SiO_2), sylvite (KCl), and modest levels of dolomite, potassium silicate, (**Fig. 4.3**) while EDX data confirms the presence of corresponding elements including carbon (C), oxygen (O), silicon (Si), magnesium (Mg), chlorine (Cl), potassium (K), and calcium (Ca), along with sodium (Na), aluminium (Al), and sulfur (S). The estimated elemental composition by weight percentage, arranged in descending order, is as follows: oxygen (42.78%), carbon (20.75%), potassium (17.39%), silicon (6.07%), magnesium (3.20%), calcium (2.22%), sulfur (1.31%), chlorine (1.28%), sodium (0.88%), and aluminium (0.23%) (**Fig. 4.7 (g)**).

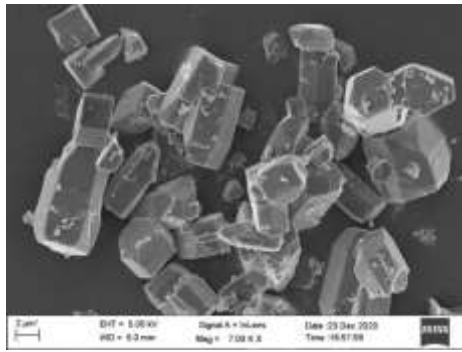
SEM analysis revealed that banana ash particles vary in size, exhibiting various sizes in micrometers. The presence of calcite (CaCO_3), indicated by PXRD data, corresponds to the rhombohedral sub-micron crystallites observed in the SEM

micrograph (Fig. 4.7 (a)) and is further corroborated by EDX spectra showing characteristic peaks for Ca, C, and O. Furthermore, the micrograph illustrates the presence of numerous cubic sylvite (KCl) crystallites (Fig. 4.7 (a) & (b)), as confirmed by compounds and elements detected through PXRD and EDX data, respectively. It is important to note that KCl undergoes volatilization above 770°C (Sharma *et al.*, 2002), suggesting that the smoldering of banana ash in an open-air environment could have occurred below 700°C. Calcite rhombohedra formed during combustion dominate the microstructure and coexist with cubic sylvite in most micrographs. The micrographs reveal the presence of spherical particles, commonly referred to as cenospheres, (Fig. 4.7 (c)) which are typically hollow, as described by Williams *et al.*, (2009). Their porous nature arises from the escape of gases, such as CO₂ or water vapor, during the combustion process. Cenospheres often enclose smaller internal spheres known as plerospheres, which are also hollow (Williams *et al.*, 2009). Agglomeration of soft ash fine grains and tissue fragments, arising from cellulosic or lignocellulosic matter, was present underneath micro-crystallites from possibly soil particles (Fig.4.7 (d)). Elemental mapping and EDX analysis confirmed the presence of silicon (Si), aluminium (Al), oxygen (O), and potassium (K) in the sample. In addition, PXRD analysis identified the presence of quartz (SiO₂), potassium silicates, and potassium aluminium silicates, all of which are typical high-temperature products of mineral transformations involving silicate phases. The presence of agglomerated pseudo-hexagonal, plate-like crystals suggests the formation of an aluminate phase. In addition, cluster of pseudo hexagonal crystallites most likely represent quartz, supported by their geometric morphology, the presence of silicon and oxygen in EDX and PXRD peaks consistent with biogenic silica. The coexistence of these crystalline silicates and the amorphous spherical structures suggests that cenospheres were formed from the melting and rapid cooling of silicate-rich materials during open-air combustion (Fig. 4.7 (c)). Due to the variety of substances adhering to their surfaces, cenospheres exhibit a complex and diverse composition, making precise chemical characterization challenging (Maschowski *et al.*, 2019).

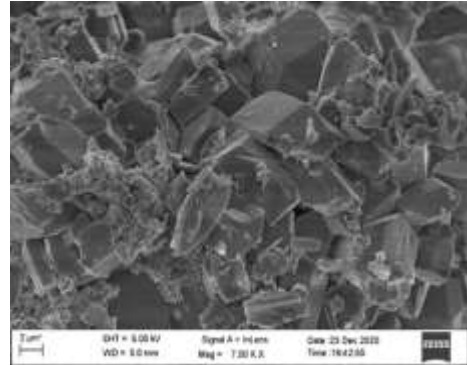
PXRD analysis of the banana ash samples also revealed the presence of tobermorite ($\text{Ca}_5\text{Si}_6\text{O}_{16}(\text{OH})_2 \cdot 4\text{H}_2\text{O}$), supported by EDX detection of Ca and Si, which are the primary elements involved in the formation of calcium silicate hydrates. SEM images showed distinct interlocked platelet-like crystals with sharp edges (Fig 4.7 (e)). These platelets are consistent with typical tobermorite morphologies, which often appear as plate-like crystals (Mostafa *et al.*, 2009). The densely packed interpenetrating platelets, suggesting distorted crystallization derived from the decomposition of biomass during combustion process.

The presence of dolomite ($\text{Ca}_{0.51}\text{Mg}_{0.49}(\text{CO}_3)_2$), a mineral (Tamaddon *et al.*, 2013), in banana ash was identified by applying PXRD, which corroborate the EDX spectral analysis that showed the distribution of several elements Ca, Mg, C, and O at the surface as alkaline earth carbonate forms. Concomitantly, SEM micrographs also exhibiting micron sized trigonal crystallites with relatively smooth surfaces most likely attributed to the mineral, dolomite compared to adjacent calcite exhibiting remarkable morphological difference, most likely attributable to Mg^{2+} substitution leading to alternating layers of Ca^{2+} and Mg^{2+} ions in the lattice during the combustion process.

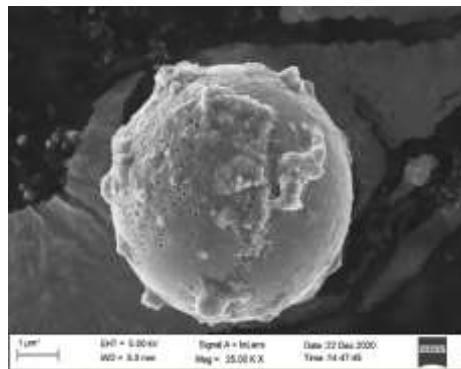
Taken together, the morphological (SEM), elemental (SEM-EDX & XPS), and crystallographic (PXRD) analyses revealed that banana ash is a heterogeneous system comprised amorphous (inorganic and organic phases) and well-defined crystalline (inorganic mineral) phases. The identification of various mineral phases, such as calcite, quartz, sylvite, dolomite, potassium silicate(s), potassium aluminium silicate(s), and tobermorite indicates chemical compositional transformations during combustion. The occurrence of different morphologies arising from rhombohedral calcite, cubic sylvite, trigonal dolomite, and interlinked tobermorite platelets, and besides spherical cenospheres probably made of calcium/potassium aluminum silicates further supports the formation of these minerals possibly under varying thermal conditions during the cooling phase.



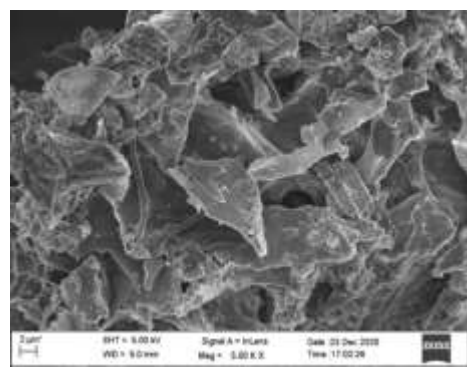
(a)



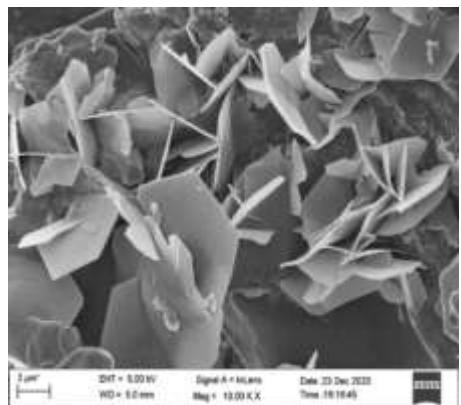
(b)



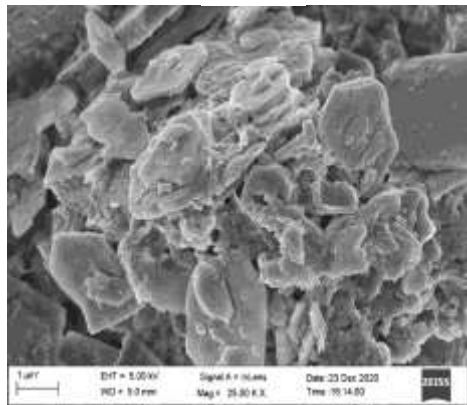
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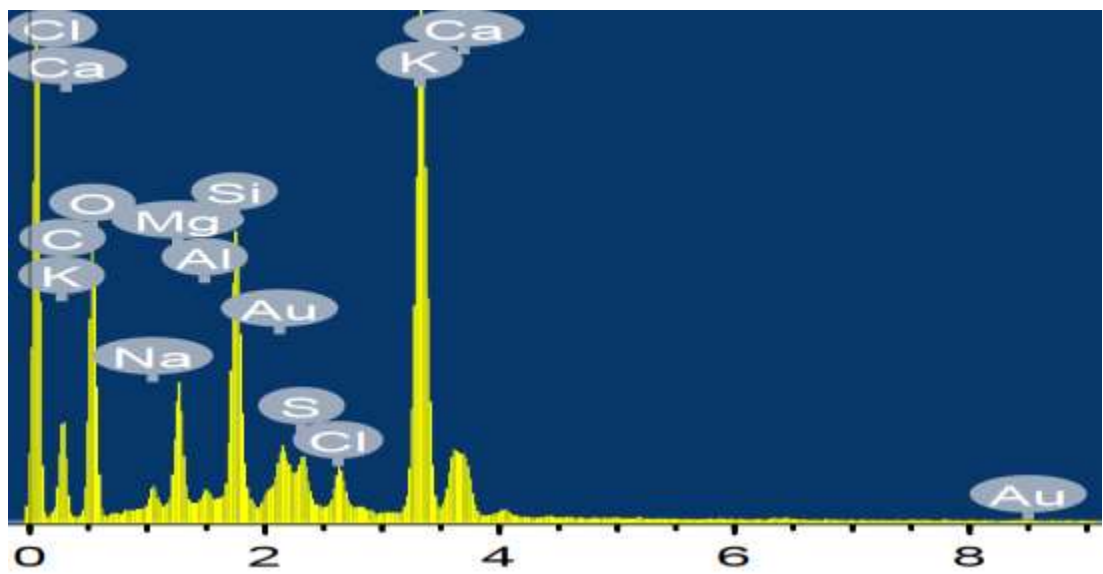
(d)



(e)



(f)



(g)



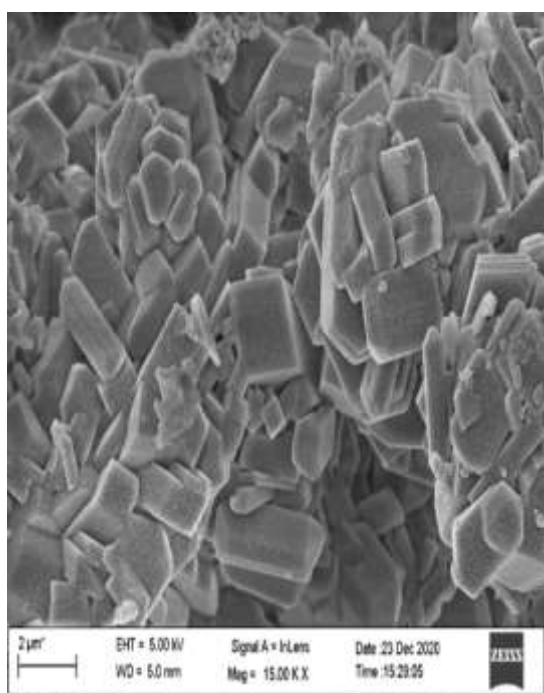
(h)

Fig. 4.7: (a)-(h), FE-SEM-EDX-Mapping micrograph of the surface morphology of banana ash

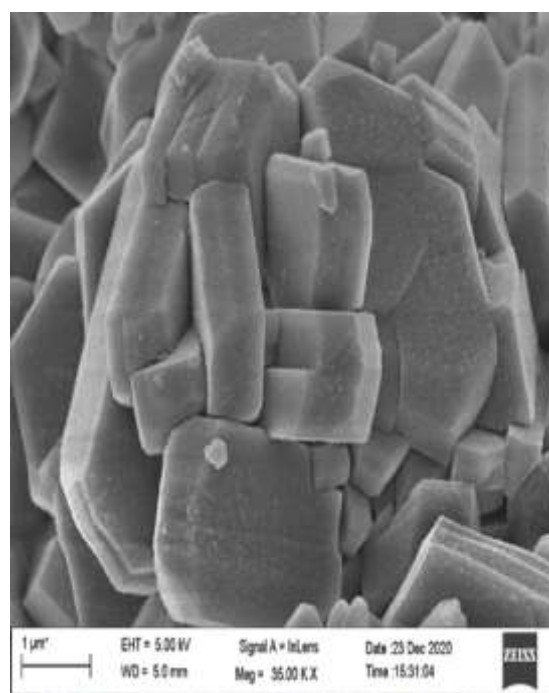
4.3.6.2 Black gram ash

The SEM micrographs of black gram ash exhibited a heterogeneous distribution of various minerals/amorphous phases exhibiting different morphologies in micron scale. The PXRD analysis confirmed the crystalline phases as sylvite (KCl), potassium sulfate (K_2SO_4), calcite (CaCO_3), dolomite ($\text{CaMg}(\text{CO}_3)_2$), tobermorite ($\text{Ca}_5\text{Si}_6\text{O}_{16}(\text{OH})_2 \cdot 4\text{H}_2\text{O}$), and quartz (SiO_2), which corroborate the observed SEM morphologies. The SEM-EDX analysis revealed the corresponding elemental composition of phytolith-derived ash particles with different sizes. The elemental composition at the surface of black gram ash particles, by weight percentage arranged in the descending order, is as follows: O (42.8%), K (21.9%), C (14.6%), Ca (5.43%), Mg (5.35%), Si (2.44%), Cl (1.55%), S (1.38 %), P (1.15%) and Na (0.41%). The morphology varied from dense particle agglomerates, micron-sized mineral crystals to lesser combusted plant tissue fragments, showing evidence of different degrees of combustion through the presence of fused particles alongside fibrous lignocellulose plant tissue remnants. These structural distributions reflect differences in the plant species and their tissues as well as traditional combustion practices during the preparation of plant ash(s) as flavoring agents. The presence of calcite (CaCO_3) in black gram ash, as identified by PXRD method, commensurate with rhombohedral micron scale crystallites in SEM (Fig. 4.8 (a)), more akin to the banana ash particles. This is further supported by EDX spectral data, which has shown the prominent surface distribution of elements, calcium (Ca), carbon (C), and oxygen (O). Additionally, the SEM micrographs reveal numerous cubic-shaped crystallites adjacent to rhombohedral crystallites, which are morphologically similar to sylvite (Fig. 4.8 (b)). The presence of sylvite corroborate PXRD and EDX analyses for potassium chloride. The SEM micrographs also revealed the presence of tobermorite and dolomite, both of which are consistent with the mineral phases previously reported in banana ash (Fig. 4.7). In addition, clusters of pseudo-hexagonal crystallites most likely represent quartz (SiO_2), supported by their geometric morphology, the presence of silicon and oxygen in EDX, and XRD peaks consistent with biogenic silica, a known component in leguminous plants like black gram (Fig. 4.8 (d)).

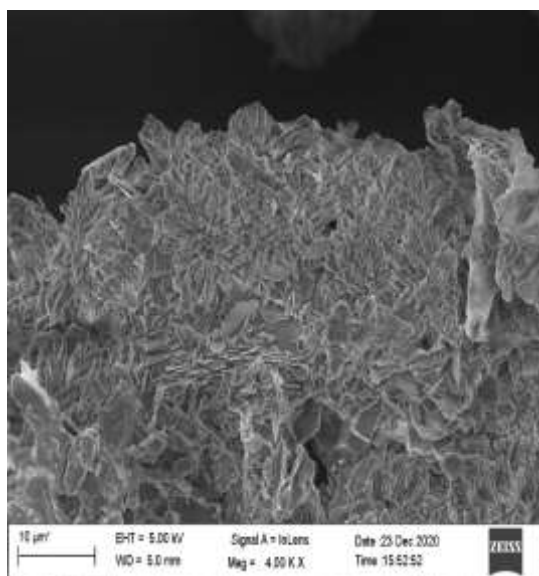
Trigonal crystalline structures were also detected, which may correspond to magnesium-rich calcite phases, formed through the thermal decomposition of calcium–magnesium salts of organic carboxylic acid compounds during combustion (Fig. 4.8 (e)). Fibrous structures were widespread at the ash surface (Fig. 4.8 (f)). These are most likely remnants of lignocellulosic plant fibers that were less combusted, although in some cases which yet reflects different degrees of combustion, including partial melting, gas evolution, and incomplete burnout of organic matter. The combined SEM, EDX, and XRD data suggest that the combustion of black gram biomass likely occurred at temperatures $< 750^{\circ}\text{C}$, allowing for the charring of fibrous organic structures distributed along with amorphous and crystalline mineral phases.



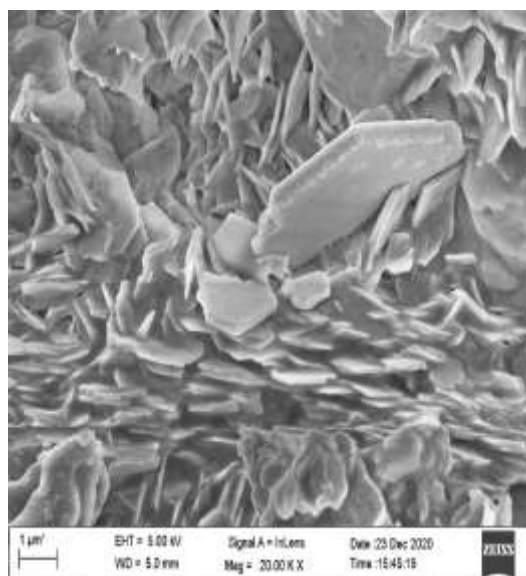
(a)



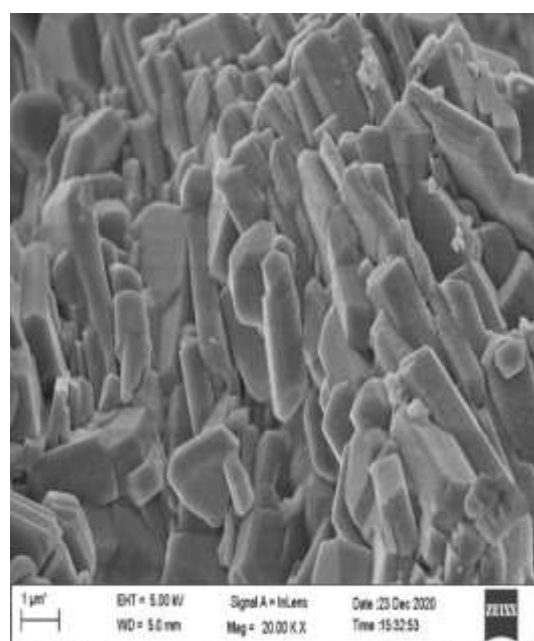
(b)



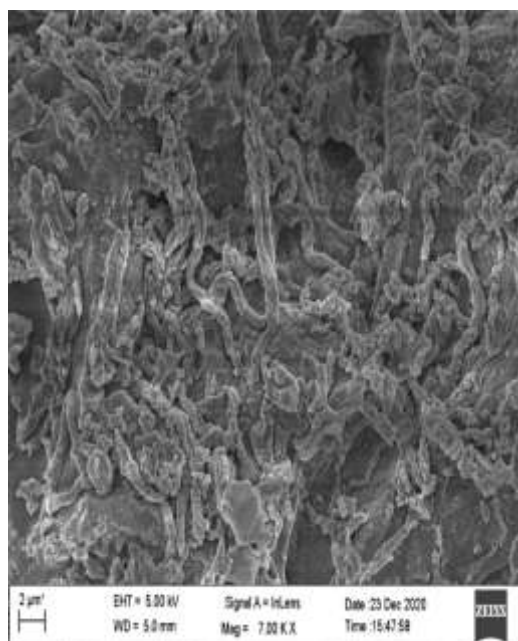
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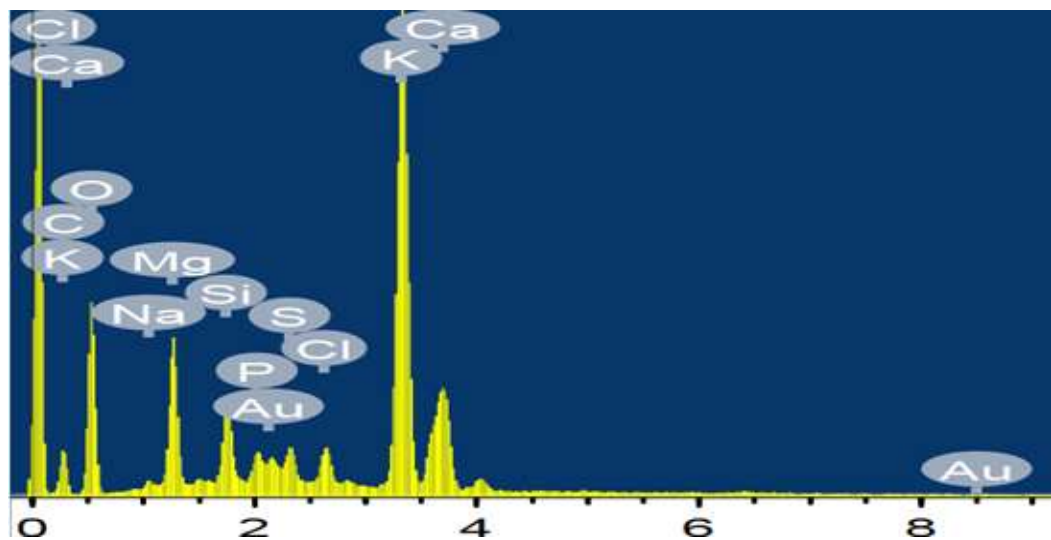
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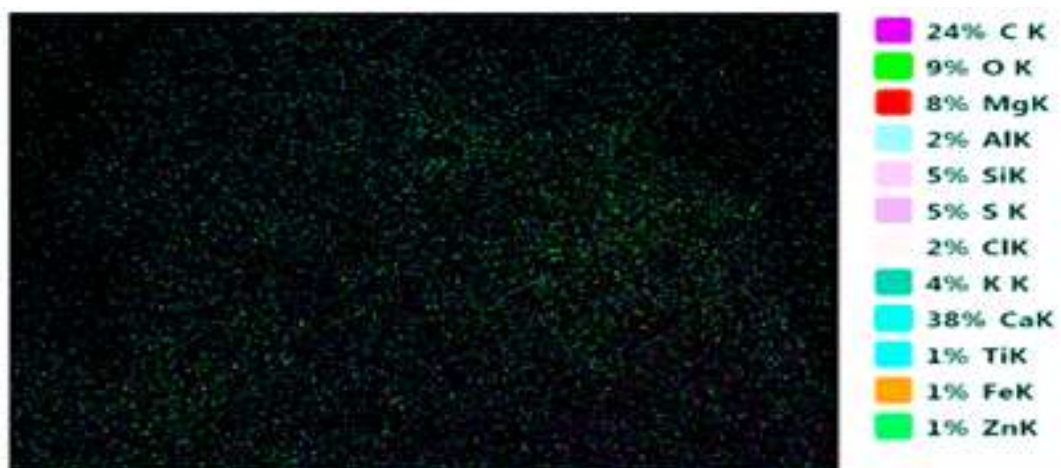
(e)



(f)



(g)



(h)

Fig. 4.8: (a)-(h), FE-SEM-EDX-Mapping micrograph of the surface morphology of black gram ash

Chapter-5

Effect of *Tuibur* on Growth and Extracellular Hydrolytic activity of *Bacillus* *mesentericus*

5.1 Introduction

A distinctive form of tobacco consumption prevalent in Northeast India is the use of tobacco smoke-infused water or tobacco smoke infused alkaline solution [TSIAS], locally known as *tuibur*. This practice, albeit unique, has been an integral part of indigenous Mizo cultures for generations. People make *tuibur* from a variety of tobacco species, including locally grown tobacco and imported tobacco from Myanmar and Shillong. Notably, people generally choose Myanmar-imported tobacco due to its perceived superior quality. The production process only uses tobacco plant stalks, as adding leaves tends to impart a bitter taste to the *tuibur*, detracting from its ideal flavour profile (Devi *et al.*, 2017). It is a cottage-made product produced locally by smoldering tobacco stalk. The smoke released from the smoldering tobacco is made to bubble through an alkaline feedstock (fresh water percolated through tobacco ash) till the solution becomes cognac in color with pungent nicotine smell (Madathil *et al.*, 2018). This tobacco smoke-saturated aqueous solution is then filtered using residual tobacco stalk ash to remove suspended residues, and the filtrate is packaged and marketed as a ‘smokeless’ tobacco product without the required pictorial warnings (Madathil *et al.*, 2018). *Tuibur* quality, while lacking a standard measure, often undergoes distinct grading, which is determined by the amount of tobacco stalk used and the duration of tobacco smoke infusion through water, “the higher the quantity, the better the quality”. Typically, people consume *tuibur* orally by placing it in the buccal cavity for 5–10 minutes or until it retains its nicotinic effects, after which they spit it out and replenish it as needed. Users may take different dosages multiple times a day, depending on their level of addiction. In cases of severe addiction, some users began consuming *tuibur* more frequently and rapidly, while others developed the habit of periodically ingesting it directly into the stomach, often by swallowing it without spitting it out, as is typically done (Phukan *et al.*, 2005). Beyond its documented health impacts from consumption, including associations with chronic and life-threatening health conditions, *tuibur* production poses significant environmental concerns. The traditional manufacturing process in small-scale cottage industries using artisanal facilities generates untreated waste that is routinely discharged into surrounding ecosystems. This improper disposal may introduce bioactive compounds that may disrupt microbial diversity and ecological balance in the surrounding soil and aquatic systems. While research on the adverse

effects of *tuibur* remains limited, its impact on the microbial system remains unexplored in scientific literature so far. To address this breach, we conducted an *in vitro* study evaluating the effect of *tuibur* on the growth and extracellular enzymatic activity by employing the commercially available probiotic bacterium *Bacillus mesentericus* as our model organism. *Bacillus mesentericus* was selected as a model organism for this study due to its ecological ubiquity and relevance to human-associated microbiomes. As a Gram-positive, spore-forming bacterium, it exhibits remarkable environmental persistence, colonizing diverse habitats including soil, aquatic systems, where it plays a crucial role in organic decomposition and nutrient cycling. This bacterium is also known to be present in various food sources and is considered a probiotic bacterium (Nagamine *et al.*, 2019). Of particular significance, it has been identified to be present in the human gastrointestinal tract, where it may contribute to gut microbiota homeostasis. *Bacillus mesentericus* exhibits significant extracellular enzymatic activity, particularly known for secreting amylases, proteases, cellulases, and lipases, which play crucial roles in breaking down complex organic compounds into simpler, absorbable nutrients. The organism's ability to thrive in diverse environments is partly attributed to this versatile enzymatic profile. This dual environmental cum human association positions *B. mesentericus* as an ideal organism for evaluating the growth and enzymatic responses to *tuibur* extract, thus providing a valuable perspective on how such traditional tobacco products might impact both environmental microbial stability and human-associated microbial populations. *B. mesentericus* secretes a diverse range of extracellular hydrolytic enzymes, including cellulase, α -amylase, protease, and lipase, which perform the hydrolysis of carbohydrates, starch, proteins, and lipids, respectively. Cellulases hydrolyze complex plant-based polysaccharides by cleaving β -1,4-glycosidic linkages, thus converting them into simple sugars (e.g., glucose), while another key enzyme, α -amylase hydrolyzes starch by cleaving α -1,4-glycosidic linkages to yield glucose, maltose, and maltotriose (Ejas *et al.*, 2021; Gupta *et al.*, 2003). Proteases hydrolyze complex proteins into their constituent amino acids by breaking the peptide bonds (Barret and McDonald, 1986). Similarly, lipase performs hydrolysis of lipids (fats and oils) to convert them into fatty acids (Kanwar *et al.*, 2005). A large number of *in vitro* studies have revealed that such bacteria-produced hydrolytic enzymes activities are affected by various physico-chemical factors, including different metal ions, pH etc.

(Hetardi and Widhyatudi, 2015; Siti *et al.*, 2015). It was reported that the pH of TSIAS is highly alkaline, and contains different kinds of metal ions including alkali and alkaline earth metal ions in varying proportions (Lalmuanpuui and Muthykumaran, 2016; Gomez *et al.*, 2023). Given such physico-chemical attributes of TSIAS, the present study aims to examine the influence of TSIAS on the extra-cellular hydrolytic enzyme activities of gut bacteria in order to assess its putative effect(s) on bacterial diversity. To the best of our knowledge, there have been no published reports on the effect of TSIAS on *B. mesentericus*.

Objective

This study aimed to investigate the effects of different concentrations of *tuibur* on the growth and extracellular hydrolytic activities of key enzymes, including amylase, cellulase, lipase, and protease.

5.2 Materials and Methods

5.2.1 Isolation of *Bacillus mesentericus*:

A commercial probiotic tablet (VIBACT, manufactured by Allianz Bioscience Pvt. Ltd, Puducherry, India) containing various bacterial species (*Streptococcus faecalis*, *Clostridium butyricum*, *Bacillus mesentericus*, and *Lactobacillus sporogenes*) was the source of *B. mesentericus* in this study. During the isolation process, powder content of a tablet was suspended in sterile phosphate buffer (0.1 M; pH 7.0), and then, heated to 70°C for 15 min. This heat treatment was sufficient to kill all the bacteria, except the spore-forming *B. mesentericus*. Then, the heat-treated suspension was serially diluted and aliquots were spread on Luria-Bertani (LB)-plates to isolate *Bacillus*. The single colonies obtained were repeatedly streaked on LB plates to obtain pure culture of the bacterium, which was further identified as *B. mesentericus* by Gram's staining (Gram-positive), and other biochemical and morphological (spore formation) features.

5.2.2 Preparation of TSIAS for experimental use:

TSIAS (packaged) was purchased from local market. The liquid was subjected to rotary evaporation till it completely dried following which, the concentrated dry form

was dissolved in sterile Millipore water to obtain a stock solution of 200 g L⁻¹ whose measured pH value was pH 9.0.

5.2.3 Growth and generation-time determination

LB medium containing 0.1% tryptone, 0.05% yeast extract and 0.1% sodium chloride was used for growth and generation-time determination of *B. mesentericus*. An actively growing log-phase culture was washed with 0.1 M sterile phosphate buffer (pH 7.0) twice and suspended in the same buffer. An aliquot of the culture was used to measure optical density at 600 nm (OD₆₀₀) using a Shimadzu-make (Japan) spectrophotometer. Then, an equal volume of aliquots was transferred to different conical flasks containing fresh LB-medium. To such flasks, different concentrations of TSIAS was added from the stock (200 gL⁻¹) solution to achieve final concentrations of 0 gL⁻¹ (control), 1gL⁻¹, 2.5 gL⁻¹, 5 gL⁻¹, 10 gL⁻¹ and 20 gL⁻¹. The pH in the medium varied from 6.8 to 8.45 with increasing concentrations of TSIAS and, no attempts were made to bring media pH to a same value. The flasks were incubated at 37°C, and OD₆₀₀ of such cultures was measured at periodic intervals for 40 h. The optical density of cultures was measured at 600 nm to evaluate the proliferation of *B. mesentericus* at different time intervals (up to 40 h) and the corresponding population growth curve was represented by OD₆₀₀. The pH of culture supernatant was measured regularly for any increase or decrease in pH during the growth experiment. To determine generation-time of cultures, OD₆₀₀ values were converted to their respective log₁₀ values, and plotted using semi-log graph as a function of time, and the generation-time was calculated using the following formula: $\text{Generation-time} = \frac{\ln(2)}{\text{Growth rate}}$; Growth rate = Maximum slope value of the kinetic curve.

5.2.4 Determination of cellulase activity:

The cellulase activity was assayed in carboxymethyl cellulose (CMC)-medium containing plates composed of 0.2% NaNO₃, 0.1% K₂HPO₄, 0.05% MgSO₄, 0.05% KCl, 0.2% CMC sodium salt, 0.02% peptone, 2% agar, and Millipore water. After preparation and autoclaving, the medium was amended with TSIAS, the concentration of which ranged from 0-20 gL⁻¹, and poured on plates. An equal volume (3 µL) of actively growing *B. mesentericus* was spotted in the centre of each CMC agar-plate, and incubated for 48 h at 37°C. After incubation, the CMC agar-plates were flooded with

Gram's iodine solution for approximately 3-5 min (Kasana *et al.*, 2008). Once a clear zone around the bacterial spot was visible (an indicator of the presence of cellulase activity), the diameter of the zone was measured using a zone-scale to represent cellulase activity in semi-quantitative term.

5.2.5 Determination of α -amylase activity:

The α -amylase activity was assayed in starch-medium containing plates composed of 0.02% yeast extract, 0.1% K_2HPO_4 , 0.05% $MgSO_4$, 0.05% KCl, 0.2% starch, 0.2% ammonium sulphate, 2% agar, and Millipore water. After preparation and autoclaving, different concentrations of TSIAS (as described in section 2.4) were added to the medium and poured on plates. An equal volume (3 μ L) of actively growing *B. mesentericus* was spotted in the centre of each starch agar-plate, and incubated for 48 h at 37°C, following which the starch agar-plates were flooded with Gram's iodine solution for approximately 5-10 min (Yassin *et al.*, 2021). Once a clear zone around the bacterial spot was visible (an indicator of the presence of α -amylase activity), the diameter of the zone was measured using a zone-scale to represent α -amylase activity in semi-quantitative term.

For quantitative determination of α -amylase activity, the log-phase cultures of *B. mesentericus* were grown in liquid LB-medium for 36 h at 37°C added with different concentrations of TSIAS (0 to 20 gL^{-1}). The cell-free supernatants of 36 h old-cultures obtained by centrifugation for 10 min at 7000 rpm served as crude enzyme for α -amylase activity assay. The α -amylase activity was determined as per the protocol described by Bernfeld (Bernfeld, 1955). A unit of α -amylase activity (U) was defined as amount of enzyme required to liberate 1 μ mol of maltose in 1 min.

5.2.6 Determination of protease activity:

The protease activity was assayed in casein-medium containing plates composed of 0.5% peptone, 0.3% yeast extract, 0.1% casein, 2% agar, and Millipore water. After preparation and autoclaving, the medium was amended with different concentrations of TSIAS, the concentration which was ranged from 0-5 gL^{-1} , and poured on plates. An equal volume (3 μ L) of actively growing *B. mesentericus* was spotted in the centre of each casein agar-plate, and incubated for 48 h at 37°C, following which the casein agar-plates were visualized for appearance of clear zone (an indicator of the presence of

protease activity) around the bacterial spot (Garriga *et al.*, 1996). The diameter of the zone was measured using a zone-scale to represent protease activity in semi-quantitative term.

For quantitative protease activity determination, the crude enzyme was obtained as described in section 2.6. The assay was done as per the method described by Cupp-Enyard (2008). A one unit of protease activity was defined as the amount of enzyme required to liberate 1 μ mole tyrosine/mL per min.

5.2.7 Determination of lipase activity:

Lipase activity was determined as per the protocol of Kanwar *et al.*, (2005). 1 μ m p-nitrophenol produced during the hydrolysis of paranitrophenylpalmitate (pNPP) by 1 mL lipase at 45°C was considered as one unit (U) of lipase activity.

5.2.8 Statistical analysis:

Graphs, calculation of generation time and bar diagrams were prepared by using Microsoft Excel 2010. Unless otherwise indicated, each assay was run in duplicate, and the mean data were noted.

5.3 Results and Discussion:

5.3.1 Effect of TSIAS on *Bacillus mesentericus* proliferation:

In the untreated *B. mesentericus* culture, a generation time of 25.53 min was calculated. It is apparent that, in the 0 - 10 gL^{-1} concentration range, TSIAS did not exert any negative effects on the growth rate of *B. mesentericus* TSIAS (Fig. 5.1). As the TSIAS concentrations were gradually increased, prolonged bacterial generation times were calculated, indicative of the prolonged lag phase. At 20 gL^{-1} of TSIAS, the generation time has increased to 37.72 min, indicative of a significantly retarded growth rate. The pH of each culture was measured to assess the influence of TSIAS on the pH of growth media. The pH of medium was found to be alkaline as the time progressed. At 0 h, the pH was 6.82 which increased to 8.5 in 40 h in the untreated culture. With the increase in TSIAS concentrations over the range of 0-20 gL^{-1} , concomitantly the pH of culture medium incrementally increased. At 20 gL^{-1} TSIAS concentration, the pH level increased from 8.5 at 0 h to 9.4 at 40 h of growth. Therefore, addition of TSIAS engendered the bacterial culture medium alkaline.

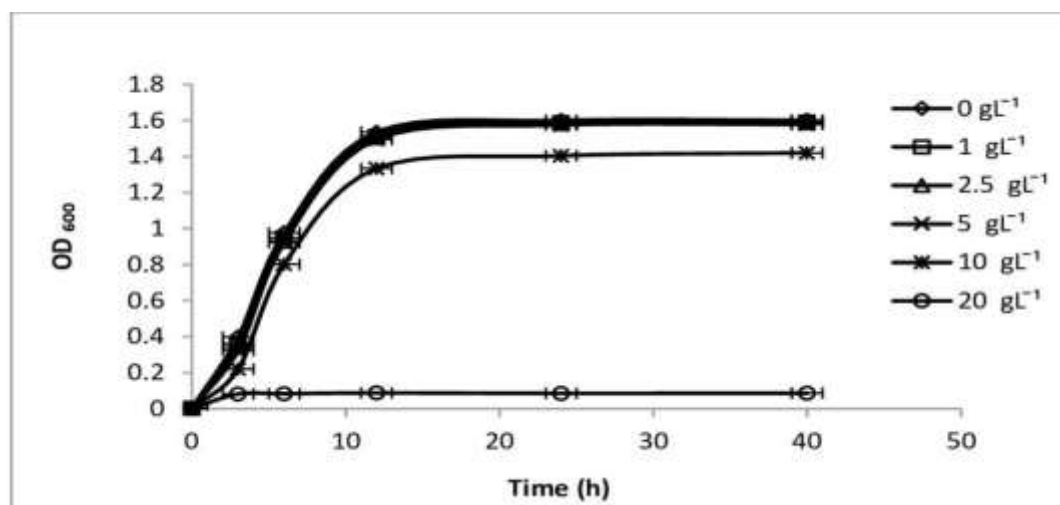


Fig. 5.1: Effect of TSIAS on the growth rate of *Bacillus mesentericus* in the concentration range of 0-20 gL⁻¹ TSIAS. Error bars represent standard deviation of two replicates.

5.3.2 Effect of TSIAS on polysaccharide biodegradation:

Subsequently, we investigated the influence of TSIAS on the hydrolytic enzyme activities of *B. mesentericus*. The qualitative cellulase activity was assessed by using the degradation of CMC in agar-plates. A halo zone diameter of 21 mm was measured in the untreated plate. However, the lytic zone diameters of 19, 17 and 15 mm were measured for the 0.2% CMC-containing agar-plates treated with 1.0 gL^{-1} , 2.5 gL^{-1} and 5.0 gL^{-1} TSIAS, respectively (Fig 5.2).

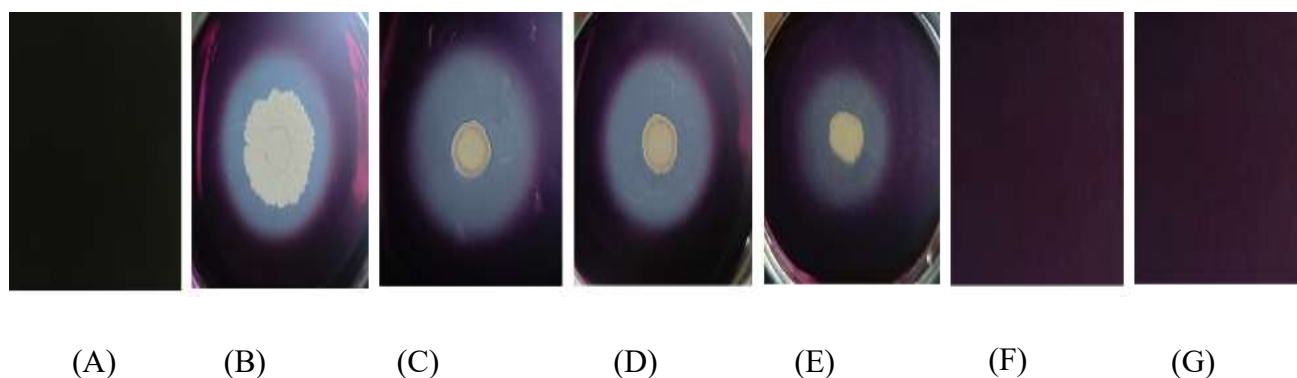


Fig. 5.2: Qualitative Cellulase activity (A) Blank; (B) Control (C) 1.0 gL^{-1} ; (D) 2.5 gL^{-1} (E) 5.0 gL^{-1} ; (F) 10 gL^{-1} ; (G) 20 gL^{-1}

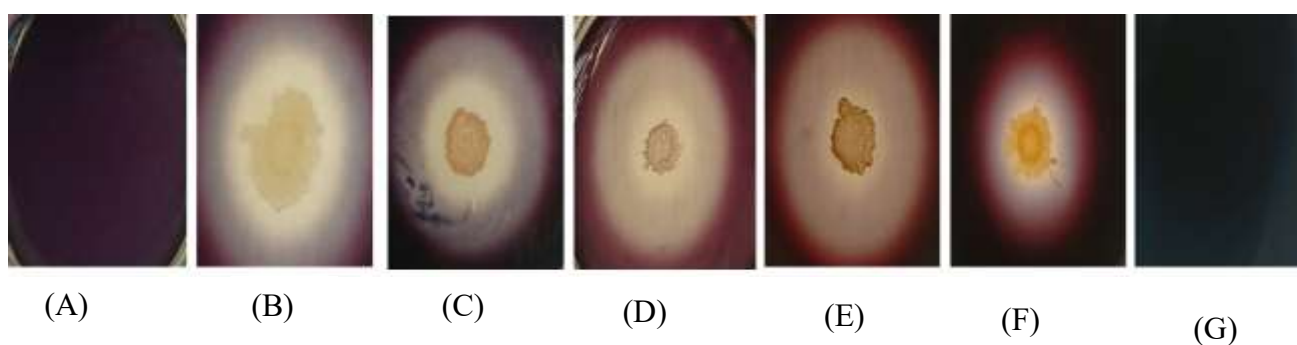


Fig. 5.3: Qualitative Amylase activity (A) Blank; (B) Control (C) 1.0 gL^{-1} ; (D) 2.5 gL^{-1} (E) 5.0 gL^{-1} ; (F) 10 gL^{-1} ; (G) 20 gL^{-1}

Thus, when progressively increasing concentrations of TSIAS were added, correspondingly a considerable reduction in the lytic zone diameters were observed. In $\geq 10 \text{ gL}^{-1}$ TSIAS treated plates, the cellulase activity was suppressed, i.e., neither hydrolytic zone(s) nor *B. mesentericus* colonies were observed. The α -amylase activity was estimated as the varying sizes of lytic zones in agar plates, while in the cell free supernatant, it was quantitatively determined. In contrast to cellulase, α -amylase activity and the formation of *B. mesentericus* colonies were observed even in 10 gL^{-1} TSIAS treated agar-plate. The clear zone diameters of 23, 22, 20 and 15 mm, were measured for 1.0, 2.5, 5.0, and 10 gL^{-1} TSIAS, respectively, in comparison with the untreated plate (25 mm) (Fig. 5.3). However, neither the halo zone nor the colonies of *B. mesentericus* were observed in 20 gL^{-1} TSIAS treated agar-plate. In addition, in the concentration range of 0-10 gL^{-1} TSIAS, the quantitative α -amylase activity was also determined, with the activity of 0.524, 0.5168, 0.5134, and 0.289 UmL^{-1} , respectively. When incubated with $>10 \text{ gL}^{-1}$ TSIAS, the α -amylase activity was imperceptible (Fig. 5.3).

A significant antagonistic effect of TSIAS on the cellulase and α -amylase activities in a concentration dependent manner was observed. It is possible that TSIAS would have, more likely, induced the change in pH (to an alkaline pH) of culture medium and the presence of different heavy metal ions in TSIAS would have inhibited the cellulase and α -amylase activities (Jha *et al.*, 2013; Okonkwo, 2019; Gomez *et al.*, 2023; Gupta *et al.*, 2008).

5.3.3 Effect of TSIAS on protein biodegradation:

The protease activity was tested semi-qualitatively in agar plates, while it was quantitatively determined by using the cell-free supernatant solution. The protease activity in the form of casein degradation was measured as the ‘clear’ lytic zones. A halo zone diameter of 21 mm was measured in the untreated plate, whereas clear zones of 18 and 16 mm were measured for 0.5 and 1 gL^{-1} TSIAS, respectively (Fig 5.4). Interestingly, no halo zones were observed in the agar-plate treated with $\geq 2 \text{ gL}^{-1}$ TSIAS, albeit some colonies of *B. mesentericus* were observed in the plates treated with $\leq 5 \text{ gL}^{-1}$ TSIAS.

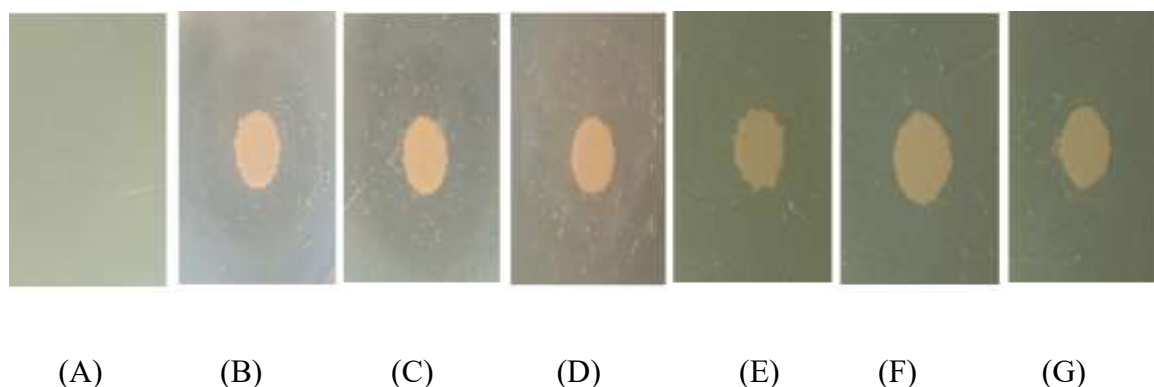


Fig. 5.4: Qualitative Protease activity (A) Blank; (B) Control (C) 0.5 gL^{-1} ; (D) 1.0 gL^{-1} (E) 2.0 gL^{-1} ; (F) 2.5 gL^{-1} ; (G) 5.0 gL^{-1}

The antagonistic effect of TSIAS was more pronounced during the quantitative analysis, i.e., protease activity was substantially inhibited (Fig. 5.5). In untreated culture, the protease activity of 0.052 UmL^{-1} was determined. However, when compared to the untreated culture, a 2-fold decrease in protease activity of *B. mesentericus* culture exposed to 1 gL^{-1} TSIAS ($\sim 0.028 \text{ UmL}^{-1}$), 9-fold decrease for 2.5 gL^{-1} TSIAS exposure (0.0089 UmL^{-1}) and 28-fold decrease in protease activity for 5 gL^{-1} TSIAS exposure (0.00186 UmL^{-1}) was detected. It was previously reported that the protease activity is inhibited by alkaline earth and transition metal ions including Mg^{2+} , Ca^{2+} , Ni^{2+} , etc. (Siti *et al.*, 2015). The observed protease inhibitory effect could be more likely due to the relatively high concentration levels of these metal ions in TSIAS, a tobacco smoke and ash derived product (Lalmuanpuii and Muthukumaran, 2016; Gomez *et al.*, 2023).

5.3.4 Effect of TSIAS on lipid biodegradation:

When *B. mesentericus* colonies were exposed to varying concentrations of TSIAS, the differentially modulated lipase activities were determined. For 1 gL⁻¹ TSIAS, a 9-fold (28.065 UmL⁻¹) increase in the lipase activity was determined compared to untreated culture (3.855 UmL⁻¹). For 2.5 gL⁻¹ and 5 gL⁻¹ TSIAS, only a 4-fold increase and ~ 1.5-fold increase in lipase activity were observed, respectively. At ≥10 gL⁻¹ lipase activity was imperceptible, i.e., lipase activity was suppressed. Thus, lipase enzyme secreted by *B. mesentericus* responded differently to the varying concentrations of TSIAS (Fig. 5.7). It was reported that Ca²⁺ ion can stimulate the activity, whereas some other metal ions (viz., Zn²⁺, Ni²⁺, K⁺ etc.) would exert antagonistic effects on its lipase activity (Hetardi and Widhyastudi, 2015). Tobacco and tobacco derived products contain several metal ions including Ca²⁺, Zn²⁺, Ni²⁺, K⁺ etc. (Lalmuanpuui and Muthukumaran, 2016). Different metal ions present in TSIAS more likely would have engendered the observed abnormal lipase activity. As we have observed for *B. mesentericus* in the present preliminary study, thus, there appears to be a good correspondence between the adverse effects of smokeless tobacco use and the disruption of extracellular enzymatic functions of pro-biotic bacteria. Besides, the experimental data of the present study bring to focus the overall need for a better understanding of the adverse effects of TSIAS and other smokeless tobacco products extracts on the human health.

5.3.5 Metal analysis

Atomic Absorption Spectroscopy (AAS) was employed to quantify selected metal ions in Tuibur, a traditional tobacco-infused preparation. The analysis revealed that manganese (39.4 mg/kg) was present at the highest concentration, followed by magnesium (17.5 mg/kg) and calcium (9.0 mg/kg). Although these metal ions are essential micronutrients for microbial systems, numerous studies have demonstrated their dose-dependent inhibitory effects on key hydrolytic enzymes. For instance, Mg²⁺ has been shown to competitively inhibit cellulase by binding to its active site (Wang *et al.*, 2012), while Ca²⁺ exhibits a biphasic effect, enhancing cellulase and α-amylase activity at low concentrations but becoming inhibitory at higher levels. Similarly, α-amylase activity is significantly suppressed by Mg²⁺ and moderately inhibited by Mn²⁺, whereas lipase and protease activities are negatively affected by

elevated concentrations of divalent cations, including Mg^{2+} , Ca^{2+} , and Mn^{2+} (Uyar *et al.*, 2003; Hetardi & Widhyastudi, 2015; Siti *et al.*, 2015). The concentrations detected in Tuibur suggest that such ions may interfere with the enzymatic systems of environmental or gut microbiota, including *Bacillus mesentericus*, potentially altering microbial metabolism and hydrolytic capacity.

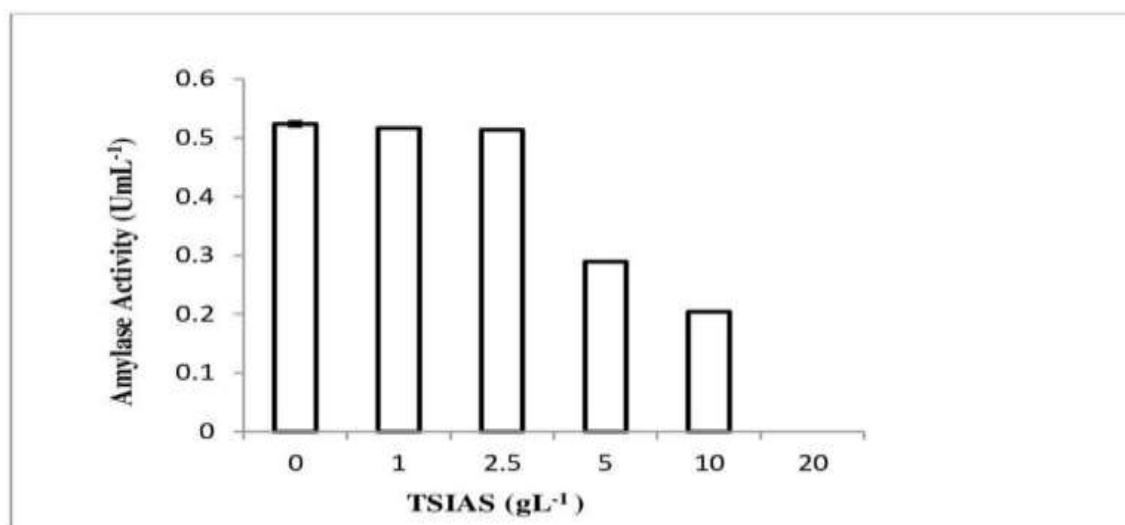


Fig. 5.5: Effect of TSIAS (0-20 gL⁻¹) on α -amylase activity of *B. mesentericus*. Determination of α -amylase activity in *B. mesentericus* extract cultures of 36 h, by using 1% starch solution as the substrate

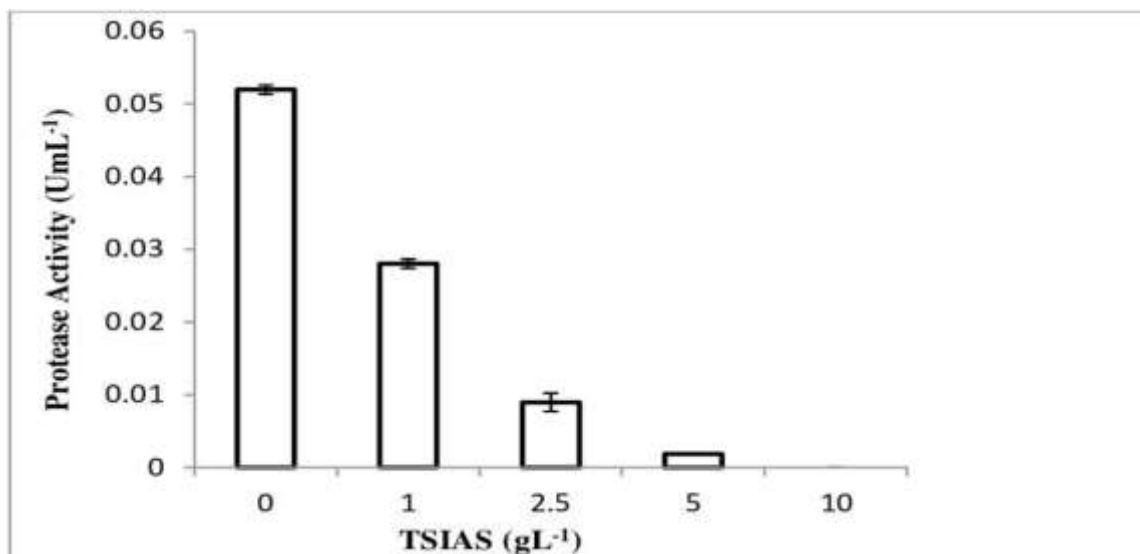


Fig. 5.6: Effect of TSIAS (0-10 gL⁻¹) on the protease activity of *B. mesentericus*. Determination of protease in *B. mesentericus* extract cultures of 36 h, by using casein as the substrate. Error bars represent the standard deviation of two replicates.

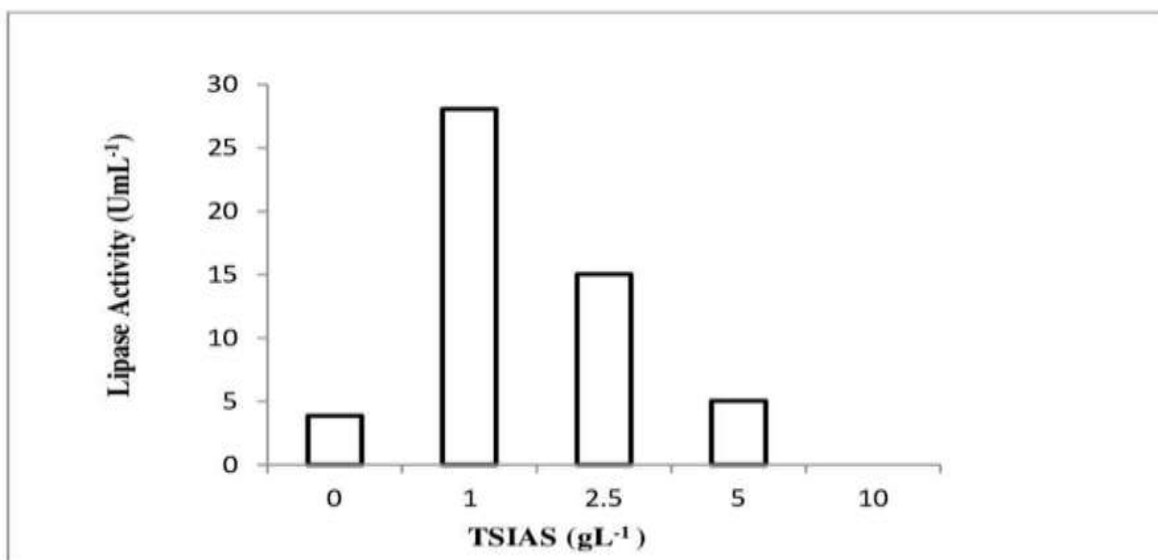


Fig. 5.7: Effect of TSIAS (0-10 gL⁻¹) on the activity of lipase secreted by *Bacill mesentericus*. Determination of lipase activity in the *B. mesentericus* culture extracts 36 h, by using pNPP as the substrate.

5.4 *Tuibur* crystal analysis

During our investigation into the enzyme inhibitory effects of TSIAS on *B. mesentericus*, we encountered an unanticipated yet significant observation of crystal formation in processed *tuibur* samples. This phenomenon was first noted during the preparation of concentrated *tuibur* extracts for enzymatic assays, where the aqueous solution was subjected to gradual evaporation under controlled conditions (40°C, reduced pressure using rotary evaporation). As the solution reached near-complete dryness and was subsequently reconstituted in Milli-Q water (200 g L⁻¹), we observed the gradual emergence of well-defined, crystals along the walls of the glassware after several days of undisturbed storage at ambient temperature.

The crystal formation was particularly noteworthy as it occurred spontaneously without any deliberate crystallization-inducing treatments. Microscopic examination revealed morphologically distinct crystals with apparent cubic and orthorhombic shapes, suggesting the potential presence of multiple crystalline phases. This observation prompted us to systematically investigate the crystalline material, as such spontaneous crystallization in tobacco products had not been extensively reported in prior literature. The crystals were carefully harvested using sterile techniques to prevent contamination and subjected to PXRD analysis.

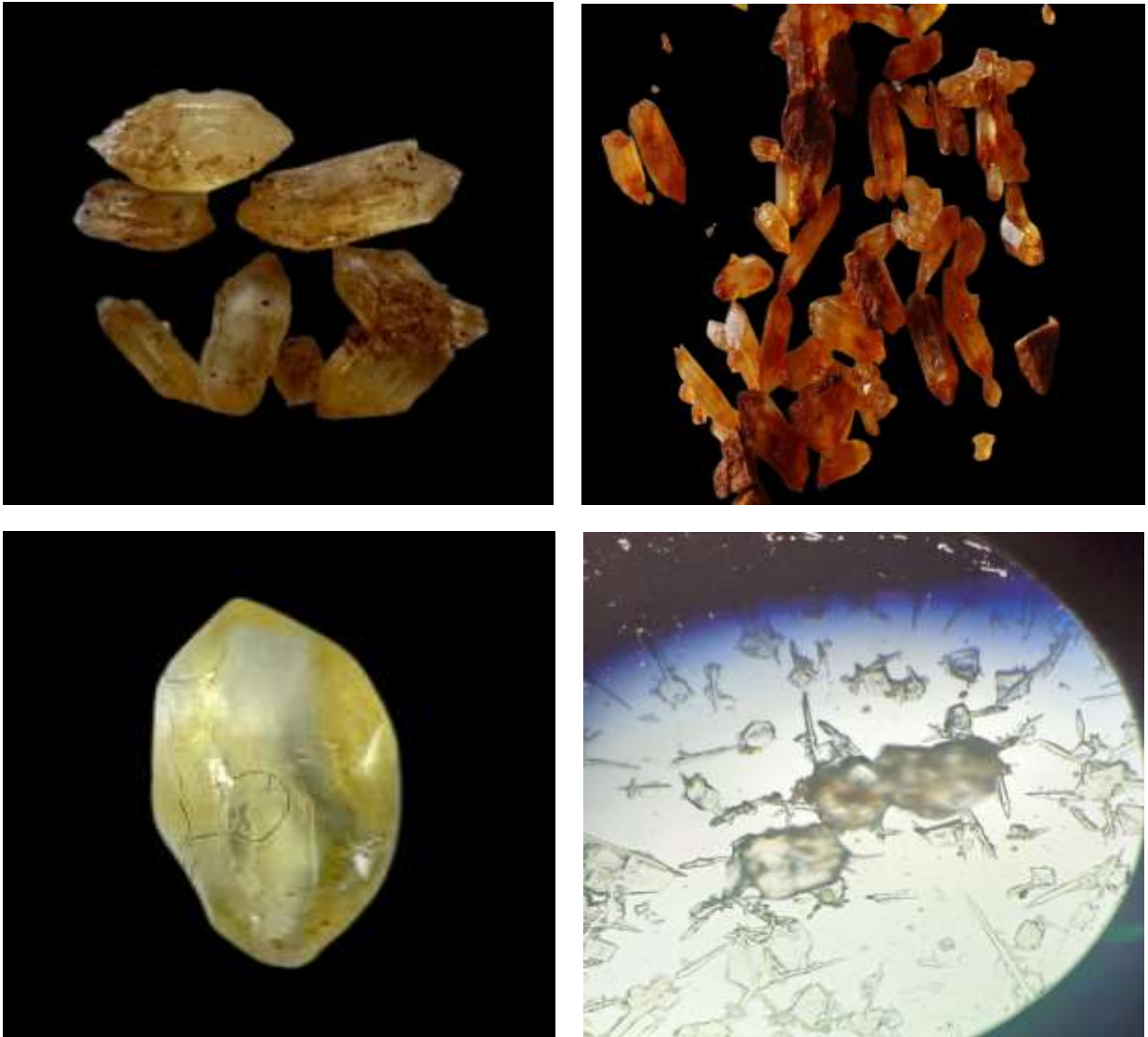


Fig. 5.8: Distinct crystal morphology

5.4.1 RESULTS AND DISCUSSION

The X-ray diffraction (XRD) analysis of the sample confirms the presence of three distinct crystalline phases, Sylvite (KCl), Dipotassium sulfate (K_2SO_4), and Sodium nitrate (NaNO_3); each corresponding to different crystal morphologies observed in the sample. The photographs of the distinct crystal morphologies are shown (**Fig. 5.8**). These phases were identified by matching the experimental 2θ values with reference patterns from the Crystallography Open Database (COD), and their unique sets of Miller indices (hkl) further confirm their identity.

Sylvite (COD ID: 9008651), which crystallizes in the cubic system with space group Fm-3m and unit cell parameter $a = 6.29294 \text{ \AA}$, is the major phase in the sample. Its characteristic diffraction peaks appear at 2θ values of 28.34° , 40.51° , 50.18° , 58.63° , 66.38° , and 73.69° , corresponding to the (2 0 0), (2 2 0), (2 2 2), (4 0 0), (4 2 0), and (4 2 2) planes, respectively (Fig. 5.9)

Dipotassium sulfate (COD ID: 1200012), an orthorhombic phase with space group Pmcn and lattice parameters $a = 5.7704 \text{ \AA}$, $b = 10.0712 \text{ \AA}$, and $c = 7.4776 \text{ \AA}$, is identified as a significant secondary component. Its diffraction peaks are observed at 2θ values such as 21.34° , 29.74° , 30.79° , 35.63° , 37.13° , 37.87° , 43.40° , 54.63° , 58.70° , 65.00° , 72.43° , and 75.45° . These correspond to the (1 1 1), (0 0 2), (0 2 2), (1 3 0), (0 4 0), (0 1 3), (2 2 1), (1 4 1), (1 2 3), (0 6 0), (1 3 4), (0 2 5), (3 5 2), and (0 8 0) planes, respectively (Fig. 5.10)

Sodium nitrate (COD ID: 1010525), also orthorhombic in symmetry (space group Im2m) with unit cell parameters $a = 3.55 \text{ \AA}$, $b = 5.56 \text{ \AA}$, and $c = 5.37 \text{ \AA}$, is present as a minor phase. Its diffraction peaks appear at 2θ values of 23.01° , 29.84° , 44.67° , 47.01° , and 73.49° corresponding to the (0 1 1), (1 1 0), (1 2 1), (0 2 2), and (0 3 3) planes, respectively (Fig. 5.11)

The presence of these three phases, each with distinct hkl reflections and crystal structures, correlates with the observation of three different crystal shapes under microscopy. This confirms that Sylvite, Dipotassium sulfate, and Sodium nitrate originated independently from separate crystalline components within the sample, reflecting a complex multi-phase system with high crystallinity. Notably, the

formation of these crystals occurred under relatively mild and natural conditions without the use of traditional crystallization inducing agents such as seed crystals, temperature gradients, or antisolvents. The presence of Dipotassium sulfate and Sodium nitrate further indicates the coexistence of multiple inorganic salts, possibly arising from leaching of soil minerals or combustion byproducts during the preparation of *tuibur*.

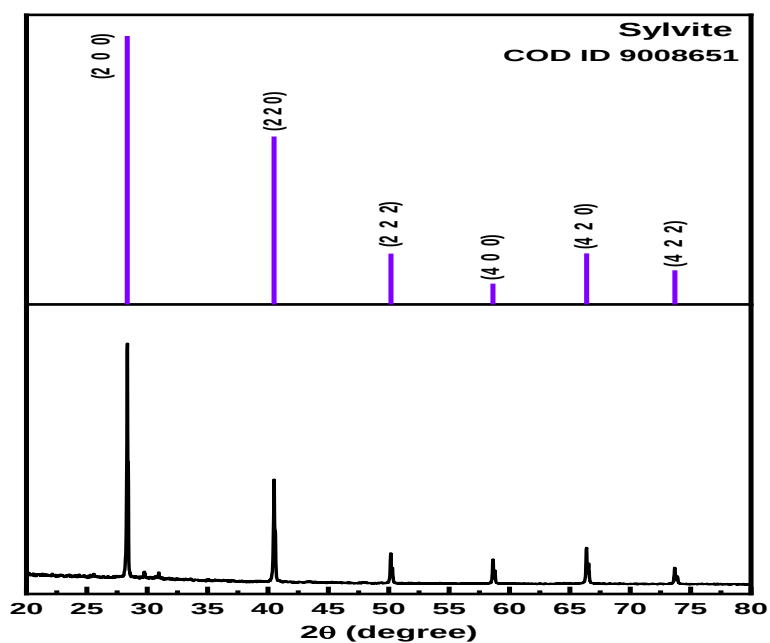


Fig. 5.9: XRD graph showing Sylvite

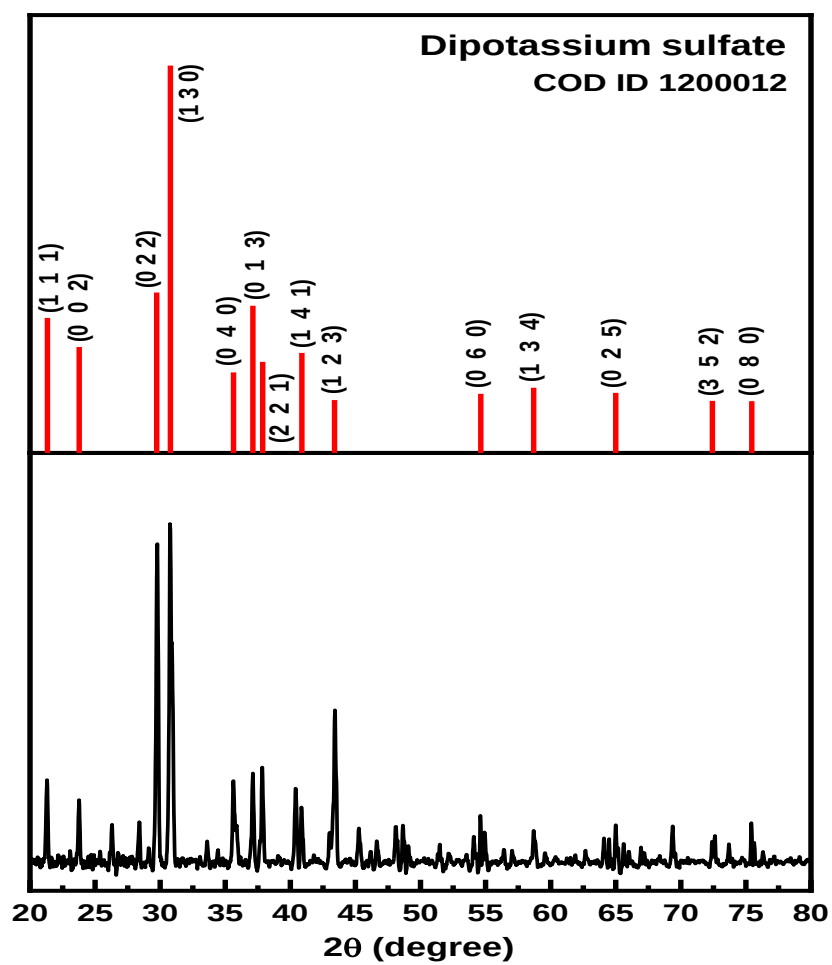


Fig. 5.10: XRD graph showing Dipotassium sulfate

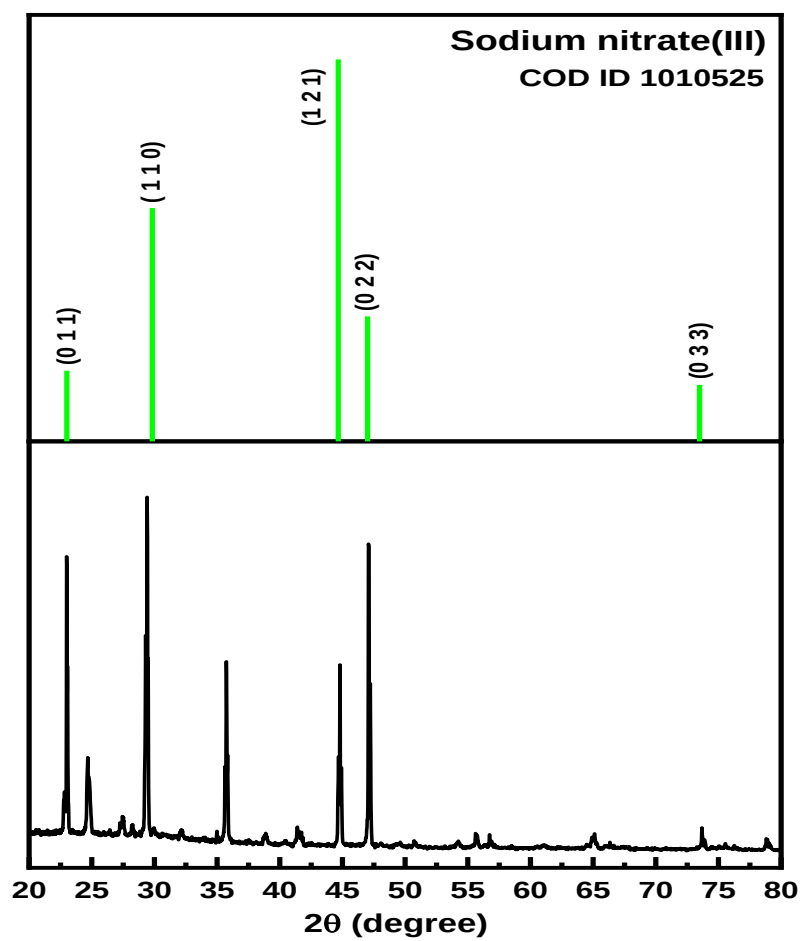


Fig. 5.11: XRD graph showing Sodium nitrate

Chapter-6

Summary and Conclusion

6. Conclusion

The present study provides a comprehensive chemical evaluation of both smoking and smokeless tobacco products, revealing substantial differences in their pH, alkaloid content, nitrite and nitrate levels, and concentrations of carcinogenic tobacco-specific nitrosamines (TSNAs). Among smoking products, water pipe tobacco was found to exhibit the lowest pH, significantly more acidic than commercial cigarettes, beedis, and zozial. This acidic environment may enhance nitrosation reactions, increasing the potential for TSNA formation. The nitrate and nitrite contents varied dramatically across products. Multinational Marlboro cigarettes showed the highest nitrate concentration, while hookah and zozial had much lower levels. Interestingly, despite being a low-cost product, beedi tobacco exhibited one of the highest nitrate levels, along with the highest total alkaloid content, including elevated nornicotine levels. Myanmarrese cigarettes also showed notably high nornicotine and TSNA levels, which may be attributed to agricultural practices such as intensive nitrogen fertilization and extended sun-curing. In contrast, water pipe tobacco and zozial had substantially lower alkaloid and TSNA contents, likely due to the inclusion of stem and midrib tissue, shorter curing durations, and limited nitrogen fertilizer use. These results suggest that the composition of tobacco products is heavily influenced by factors such as the cultivar used, fertilizer application, curing methods, and microbial processing during fermentation and storage.

Furthermore, the study confirmed strong correlations among nitrate, alkaloids, and TSNAs. Spearman correlation analysis demonstrated statistically significant positive associations between nitrate and alkaloids, nitrate and TSNAs, and between alkaloids and TSNAs, reinforcing the concept that both nitrate and alkaloids are key precursors in TSNA formation through N-nitrosation. A robust multiple linear regression model further substantiated this link, indicating that variations in TSNA levels across products can be effectively predicted by their nitrate and alkaloid contents. These findings are consistent with earlier studies and highlight the central role of nitrate not only in chemical transformation processes during curing and storage but also in pyrosynthesis during combustion. Therefore, controlling

nitrate and alkaloid levels during tobacco cultivation and processing could be a critical strategy in minimizing TSNA exposure from smoking products.

In the case of smokeless tobacco products, the study revealed equally concerning disparities. Sahdah exhibited an exceptionally high pH, due to the likely addition of lime, which is known to enhance the free-base form of nicotine and thus increase absorption in the oral mucosa. Khaini demonstrated the highest levels of both nitrate and nitrite, indicating a significant potential for endogenous nitrosamine formation. Zarda contained the highest nornicotine concentration, followed by Khaini, whereas snus and supari exhibited comparatively low levels of both nitrite and alkaloids. Spearman's correlation analysis provided further insight into chemical interactions, revealing a strong positive correlation between pH and nitrite, and significant negative correlations between pH and both nitrate and nornicotine. These trends suggest that alkaline conditions may stabilize nitrite, while acidic conditions may preserve nitrate and alkaloids. The PCA analysis effectively distinguished tobacco samples based on their chemical composition, particularly nornicotine, nitrate, and nitrite levels. The Khaini and Zarda samples showed unique chemical profiles, with the former characterized by high nitrate and nitrite levels and the latter by high nornicotine content. The clustering patterns suggest that chemical variability in tobacco products is closely linked to differences in cultivation methods, tobacco variety, and processing practices.

Taken together, these findings highlight the complex chemical nature of tobacco products and the significant variability driven by agricultural practices, post-harvest processing, additive use, and microbial transformations. Importantly, the strong link between nitrate and TSNA formation, as well as the predictive power of alkaloid levels for nitrosamine concentrations, underscores the urgent need for regulatory interventions targeting fertilizer use, curing conditions, and product formulation. The substantial differences observed among beedi, commercial cigarettes, and hookah tobacco, as well as between Sahdah, Khaini, and snus, point to the necessity of tailored harm-reduction strategies for different tobacco products. Reducing nitrate and alkaloid content in both smoking and smokeless tobacco could

play a critical role in lowering consumer exposure to carcinogenic TSNA's and ultimately mitigating the associated health risks.

The present study reveals significant chemical variability across smoking and smokeless tobacco products, highlighting nitrate and alkaloid content as key precursors to TSNA formation. These findings open important avenues for future research aimed at mitigating the health risks associated with tobacco consumption. Epidemiological studies should be prioritized to establish direct correlations between chemical profiles of tobacco products and the incidence of cancers—particularly oral and esophageal cancers—in high-risk regions like Northeast India. Longitudinal cohort studies and comparative analyses across product types (e.g., beedi, khaini, zarda) and demographics (e.g., gender-specific usage) will strengthen the causal understanding of TSNA-related disease burden. Further mechanistic studies focusing on microbial activity during curing and fermentation processes are necessary to elucidate the pathways of nitrosation and identify potential intervention points. Agricultural field trials exploring the impact of fertilizer use, especially nitrogen-based inputs, on nitrate accumulation and TSNA formation could inform safer cultivation practices. From a public health perspective, chemical surveillance programs and rapid testing technologies for TSNA's and precursors in both raw and processed tobacco would enhance regulatory oversight. Tailored harm-reduction strategies, including modifying curing methods, regulating additives, and enforcing pH control, should be developed based on region-specific tobacco usage patterns.

Building on these findings, we explored alkaline food additives, particularly ash derived from wild banana and black gram. This study provides a comprehensive characterization of traditional alkaline food additives widely used in indigenous culinary practices across Arunachal Pradesh. These additives impart unique sensory qualities and play a functional role in food preparation; however, their chemical profiles raise important public health considerations. Analytical results revealed that banana ash exhibits a higher pH (10.5) and significantly elevated nitrate content (11,908 µg/g) compared to black gram ash (pH 9.5; nitrate 4,590 µg/g), indicating

potentially higher exposure to nitrate toxicity. Moreover, X-ray diffraction (XRD) and X-ray photoelectron spectroscopy (XPS) analyses confirmed the presence of various mineral phases, including calcium carbonate, dolomite, silicates, and potassium salts, as well as trace levels of potentially toxic elements such as arsenite (As^{3+}) and arsenate, along with chlorine, zinc, and phosphate species. These findings suggest that chronic dietary exposure to such elements, particularly inorganic arsenic and excess dietary nitrate, may pose long-term health risks. This concern is supported by regional cancer data. In West Arunachal, the probability of developing stomach cancer is the highest among all cancer types, affecting 1 in every 32 males and 1 in every 62 females, followed by liver cancer, 1 in every 36 males and 1 in every 105 in females. Similarly, in East Arunachal, stomach cancer remains the most probable cancer among males (1 in every 33) reported by ICMR-NCDIR, (2021). When compared with national-level data from the National Cancer Registry Programme (NCRP 2020), these figures are substantially higher. These comparisons reveal that the incidence of stomach and liver cancers in Arunachal Pradesh is three to six times higher than the national average, indicating a region-specific burden likely influenced by environmental, lifestyle, and dietary exposures.

In light of these findings, it is imperative to evaluate the safety of these culturally significant food additives more rigorously. In vivo and in vitro toxicological studies are urgently needed to determine the bioavailability, carcinogenic potential, and cumulative impact of chronic intake of nitrates and arsenic species. Strategies such as improving combustion conditions, modifying preparation techniques, and monitoring elemental content could help minimize harmful constituents without diminishing cultural value. Public health interventions such as awareness campaigns, labeling of traditional food products, and guidance on safe usage levels can play a crucial role in mitigating health risks. Ultimately, while traditional plant-based ash additives are integral to the cultural and culinary heritage of Arunachal Pradesh and other neighbouring states, their long-term health impacts must not be overlooked. Bridging ethnobotanical knowledge with modern toxicological science will be key to ensuring the safe and sustainable use of these practices amid rising cancer burdens in the region.

We further investigated the effects of different concentrations of tuibur on the growth and extracellular hydrolytic activities of key enzymes, including amylase, cellulase, lipase, and protease. TSIAS has differential antagonistic effects on the activities of enzymes such as cellulase, α -amylase, protease and lipase. The cellulase and α -amylase activities of *B. mesentericus* were suppressed by 5 and 10 gL⁻¹ TSIAS, respectively. The protease activity was inhibited significantly when the TSIAS concentrations were incrementally increased. The lipase activity was differentially modulated, viz., lipase activity was stimulated at lower TSIAS concentrations, whereas it was significantly retarded with the increasing concentration of TSIAS. At ≥ 10 gL⁻¹, the lipase activity was suppressed. Thus, there are very distinct differences between cellulase, protease and lipase inhibitory activities of TSIAS. Our findings also indicated that although at lower concentrations, it was moderately growth suppressive, however, at ≥ 10 gL⁻¹ TSIAS, the growth of *B. mesentericus* colonies were substantially retarded. Moreover, the observed results of the present study further indicated that on the basis of limited data available, the role of these hydrolytic enzyme activities in controlling the growth rate of probiotic bacteria is not clearly understood. Overall, the adverse effects of tobacco-smoke extract, TSIAS, on the pro-biotic microbial organism, *B. mesentericus* were observed. The results reported here are of significant interest since they bear on the question of tobacco use.

While the primary focus was on TSIAS's concentration-dependent modulation of hydrolytic enzymes, the serendipitous observation of well-defined crystals during extract processing added a significant dimension to our findings. X-ray powder diffraction (XRD) and microscopy unequivocally identified the crystalline components as Sylvite (KCl), Dipotassium sulfate (K₂SO₄), and Sodium nitrate (NaNO₃), each exhibiting distinct unit cell parameters and morphologies corresponding to cubic and orthorhombic systems. These phases emerged without intentional crystallization treatments, forming under mild conditions during evaporation and storage, thereby highlighting the inherent mineralization potential of tobacco-derived preparations. Importantly, the simultaneous occurrence of crystal formation and enzyme inhibition raises intriguing questions about possible

physicochemical interactions between these inorganic phases and microbial systems. Cl^- and SO_4^{2-} were shown to suppress cellulase activity, demonstrating ion-specific regulation of enzymatic hydrolysis. The presence of ionic species such as K^+ , Cl^- , NO_3^- , and SO_4^{2-} may influence enzyme activity either directly through ionic interference or indirectly by altering the solubility and bioavailability of bioactive organic constituents in tuibur. The study highlights the concentration-dependent inhibitory effects of tuibur on probiotic enzyme activities and microbial growth, along with the unexpected formation of inorganic crystals, pointing to the need for future research to clarify mechanisms of enzyme inhibition, whether due to ionic interference, direct molecular interactions, or oxidative stress; expand microbial scope using metagenomic tools to assess effects on broader probiotic and gut microbiota; study the role of tobacco-derived ionic crystals in microbial inhibition and their interactions with bioactive compounds; and investigate potential health implications, including cytotoxicity, genotoxicity, and dysbiosis, through in vitro and epidemiological approaches.

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BIO-DATA

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Examination passed	Year of Passing	Board/ University	Division	% of marks
SSE(X)	2011	Central Board of Secondary Education	I	89.3
AISSCE(XII)	2013	Central Board of Secondary Education	I	62.2
B.Sc (Chemistry)	2016	Rajiv Gandhi University	II	57.92
M.Sc (Chemistry)	2018	University of Science and Technology Meghalaya	I	66.8

List of Publications

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. PMID: 34820780
2. Zote L, **Kakki B**, Buragohain A, Lalrammawia K, Lalrinhlupuii, Malsawmtluangi, Marak N, Muthukumaran, R. B., ... & Dului, O. G. (2025). Essential and presumably contaminating trace elements in Indian Areca (*Areca catechu L.*) nut and their potential health effect, Romanian Reports in Physics, 77, 701.
3. Zote L, Lalrammawia K, Buragohain A, Lalrinhlupuii, **Kakki B**, Lalmuanpuii R, Pachuau Z, Vanlalhruaia J, Muthukumaran RB, Kumar NS, Jahau L, Sudarshan M, Yushin N, Nekhoroshkov P, Grozdov D, Sergeeva A, Zinicovscaia. Macro-, micro-, and trace element distributions in areca nut, husk, and soil of northeast India. I. Environ Monit Assess. 2021 Jan 15;193(2):65. doi: 10.1007/s10661-021-08859-9. PMID: 33449210
4. Chang, Yuan-Jhe; Rajendra, Muthukumaran; Lalrinhlupuii; Wang, Yi-Jhen; Zote, Lalrinawma; **Kakki, B.**; Hu, Chiung-Wen; Chao, Mu-Rong (2025). Nitrate as an Indicator of Tobacco-Specific Alkaloid and Nitrosamine Content in Smoking Tobacco Products. Journal of Agricultural and Food Chemistry (Communicated).
5. **Bomngam Kakki**, Mintu Medhi, Asahan Ali, Nikrang Marak, Lalrinhlupuii, Jyotirmoy Bhattacharya, Rajendra Bose Muthukumaran (2025). Impact of tobacco smoke-infused aqueous solution on the proliferation and hydrolytic

enzyme activities of a probiotic bacterium, *Bacillus mesentericus*.
(Communicated).

Conference/Symposium

1. **Bomngam Kakki.** Tuibur, an addictive tobacco product, differtially affects the hydrolytic activities of a probiotic bacterium, *Bacillus megaterium*. Proceedings of the International Conference on Science and Technology for Innovative and Sustainable Development (STISD-2023) jointly organised by Department of Chemistry and Department of Industrial Chemistry, Mizoram University, Aizawl during 28-30th June, 2023.
2. **Bomngam Kakki.** Microbial Biodiversity of soil at Tobacco waste contaminated environment. Proceedings of the National Seminar on Conservatoon of Biodicersity (NSCB 2022) held during September 2-3, 2022.
3. **Bomngam Kakki.** Determination of Nitrate and Nitrite in Processed Tobacco. Proceedings of the 4th Mizoram Science Congress, organised by Mizoram Science Technology, and Innovation Council (MISTIC) in collaboration with Mizo Academy of Sciences (MAS), Mizoram Sciences Society (GSM), Mizoram Mathematics Society (MMS), Biodiversity and Nature Conservation Network (BIOCONE) and Mizoram Information & Technology Society (MITS) held during 24-25 November, 2022.

PARTICULARS OF THE CANDIDATE

NAME OF SCHOLAR : Bomngam Kakki
DEGREE : Doctor of Philosophy (Ph.D)
DEPARTMENT : Chemistry

TITLE OF THESIS :Determination of Nornicotine, Nitrate
and Nitrite in Selected Commercial and
Indigenous Tobacco Products available
in Mizoram

DATE OF ADMISSION : 8th August, 2018

APPROVAL OF RESEARCH PROPOSAL:

1. DRC : 26th September 2019
2. BOS : 1st October, 2019
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ABSTRACT

DETERMINATION OF NORNICOTINE, NITRATE AND NITRITE IN SELECTED COMMERCIAL AND INDIGENOUS TOBACCO PRODUCTS AVAILABLE IN MIZORAM

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**DETERMINATION OF NORNICOTINE, NITRATE AND NITRITE IN
SELECTED COMMERCIAL AND INDIGENOUS TOBACCO PRODUCTS
AVAILABLE IN MIZORAM**

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Tobacco consumption remains a major public health concern, primarily due to the presence of carcinogenic compounds such as tobacco-specific nitrosamines (TSNAs). Among the various chemical constituents of tobacco, nicotine, nitrate, nitrite, and nornicotine are of critical importance, as they serve as key precursors in the endogenous formation of TSNAs in users' oral cavity and stomach. This study determined the concentrations of nornicotine, nitrate, and nitrite in a wide range of commercial and indigenous tobacco products available in Northeast India, including beedi, branded cigarettes, zoziyal, and hookah tobacco matter, as well as smokeless tobacco products like khaini, zarda, supari, snus, and sahdah. For comparison purpose, USA cigarette brands along with reference cigarette, 3R4F, were also analysed as these products are regularly analysed on a periodic basis across the world. In addition, levels of nicotine, cotinine, and carcinogenic TSNAs, NNN, NNK, and NNAL were also analyzed to assess the broader chemical risk profile of these products. Tobacco samples were subjected to liquid chromatography–tandem mass spectrometry (LC-MS/MS) for sensitive and specific quantification. The chemical analysis revealed substantial variation in the composition of both smoking and smokeless tobacco products. Among smoking products, beedi and zoziyal showed significantly elevated levels of nornicotine and nitrate, which are key precursors for TSNAs. Nitrite and nitrate concentrations varied markedly across products, with commercial cigarettes showing higher nitrite and beedi displaying the highest nitrate content. Tobacco alkaloid analysis revealed that beedi contained the highest total alkaloid concentration, followed by commercial cigarettes, whereas zoziyal and hookah had the lowest. TSNA levels (NNN, NNK, NNAL) were significantly higher in commercial cigarettes and beedi, particularly those from Myanmar and multinational brands. Statistical analyses revealed strong correlations between nitrate and both alkaloid and TSNA levels. Nornicotine showed a particularly strong association with NNN, highlighting its role in TSNA formation. The observed variance of tobacco alkaloids, nitrate, and preformed TSNA levels could be ascribed to the differences in tobacco agricultural and curing practices.

The analysis of various smokeless tobacco products (STPs) revealed significant differences in nornicotine, nitrate, and nitrite contents. Sahdah was more

alkaline among all products, with moderate nornicotine, nitrite, and nitrate contents. Khaini showed an acidic pH and stood out for having higher concentrations of nitrate and nitrite, along with high nornicotine levels. Zarda had the highest nornicotine content, mild acidity, and moderate levels of nitrate and nitrite. Snus exhibited a near-neutral pH, with low nornicotine and nitrite but relatively high nitrate. Supari (areca nut) showed slightly alkaline pH, no detectable nornicotine, moderate nitrite, and lower nitrate levels. Overall, Sahdah showed the highest pH, while Khaini had higher nitrate and nitrite concentrations. Zarda showed the highest nornicotine content. Similarly, the studied Indian STPs were compared with the reference STPs. PCA revealed clear chemical differences among tobacco products based on nornicotine, nitrate, and nitrite. Khaini is greatly influenced by nitrate and nitrite, while Zarda is influenced by the nornicotine, and sahdah appeared centrally with moderate values. Snus and supari clustered closely, showing balanced profiles. These groupings reflect differences in tobacco type, cultivation, and processing. Collectively, these findings highlight the heterogeneity of tobacco product chemistry and the pivotal role of nitrate and alkaloids in TSNA formation. They underscore the urgent need for region-specific chemical surveillance, regulatory action, and harm-reduction strategies targeting both mainstream and culturally entrenched tobacco products in Northeast India.

Additionally, the nitrate and nitrite contents and the morphological and mineralogical properties of plat-based ashes, considered home-made flavoring agents of Northeast tribal populace, yet most of these ash-based products remains hardly studied in detail. Therefore, we analyzed wild banana ash and black gram ash, both traditionally used as alkaline food additives. Nitrate and nitrite quantification using LC-MS/MS revealed that wild banana ash contained significantly higher levels of both anions compared to black gram ash. Although nitrite levels were low in both, the elevated nitrate concentration in banana ash raises concerns about dietary exposure to alkaloids, amines, and N-nitroso compounds, especially with frequent or prolonged consumption. Powder X-ray Diffraction (PXRD) analysis revealed a structurally diverse array of crystalline mineral phases in both ashes, including calcite, dolomite, quartz, tobermorite, and sylvite, indicating a mixture of carbonate,

silicate, and alkali/alkaline earth-based compounds. Additional phases such as potassium silicate and potassium aluminum silicate were unique to banana ash, while potassium sulfate and calcium carbonate were distinct to black gram ash. X-ray Photoelectron Spectroscopy (XPS) provided further insight into the surface chemistry and elemental speciation. Banana ash exhibited a chemically heterogeneous surface with elements like C, N, O, Mg, Si, P, Cl, Ca, Zn, and As in different oxidation states. Importantly, arsenic was detected as trivalent As(III), implying partial stabilization but still posing potential toxicity. Field Emission Scanning Electron Microscopy (FE-SEM) and Energy Dispersive X-ray Spectroscopy (EDX) confirmed heterogeneous microstructures with rhombohedral calcite, cubic sylvite, pseudo-hexagonal quartz, and platelet-like tobermorite—along with cenospheres, indicating gas evolution during combustion. EDX confirmed that O, K, Si, Ca and C were dominant elements. Collectively, these results highlight the mineralogical complexity, chemical diversity, and potential health risks associated with the use of plant-derived ashes as food additives. The elevated nitrate content, presence of toxic metals like arsenic, and structurally diverse mineral phases underscore the need for toxicological risk assessments and standardized safety guidelines in the continued use of these culturally significant substances in traditional dietary practices.

Similarly, an indigenous tobacco smoke-infused alkaline solution (TSIAS), is widely consumed in Northeast India and various epidemiological studies have also implicated tuibur as a potent risk factor for cancers. While culturally significant, tuibur impose adverse health and environmental risks, yet its putative effects on microbial communities remain largely unexplored. This study, therefore, also investigated the impact of TSIAS on *Bacillus mesentericus*, a probiotic bacterium found in both environmental and human-associated microbiomes, focusing on its growth dynamics and extracellular enzymatic activities, in vitro. Initial growth assays revealed that *B. mesentericus* tolerated TSIAS concentrations up to 10 g/L. However, at 20 g/L, a significant delay in generation time was observed, accompanied by a pH rise in the culture medium, indicating growth retardation and alkaline stress. Enzymatic assays showed a concentration-dependent suppression of

key hydrolytic exoenzymes—cellulase, α -amylase, and protease. Cellulase and protease activities were completely inhibited at ≥ 10 g/L and ≥ 2 g/L TSIAS, respectively, while α -amylase activity persisted up to 10 g/L but was fully suppressed at 20 g/L. These effects are likely linked to elevated alkalinity and the presence of heavy metals such as Mg^{2+} , Ca^{2+} , and Ni^{2+} , which are known enzyme inhibitors. Interestingly, lipase activity displayed a biphasic response: a 9-fold increase at 1 g/L TSIAS possibly stimulated by Ca^{2+} followed by diminished activity at moderate concentrations and complete inhibition at ≥ 10 g/L, potentially due to inhibitory effects of metal ions like Zn^{2+} and Ni^{2+} . These findings suggest that TSIAS can disrupt enzymatic balance in beneficial microbes, with implications for gut and environmental microbiota health. Additionally, we detected crystal formation in aqueous solutions of tuibur extracts left undisturbed for more than 6 weeks. Microscopic and PXRD analyses identified three crystalline phases—Sylvite (KCl), Dipotassium sulfate (K_2SO_4), and Sodium nitrate ($NaNO_3$)—each exhibiting distinct morphologies and high crystallinity. Therefore, these findings highlight the need for a more in-depth investigation of traditional smokeless tobacco products like tuibur, as their complex biochemical and mineral compositions may significantly influence microbial activity in the gut as well as in the environment, with broader implications for both human and environmental health.