Health risk assessment of tobacco users among Mizo population

Thesis submitted in partial fulfilment of the requirements for the degree of Master of Philosophy in Zoology

By

Meesala Krishna Murthy

Registration No: MZU/M.phil/397 of 26.05.2017

Under the Supervision of

Prof. G. Gurusubramanian

Department of Zoology

Department of Zoology
School of Life Sciences
Mizoram University
Aizawl-796004, Mizoram

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CERTIFICATE

This is to certify that ***Health risk assessment of tobacco users among Mizo population*** written by **Meesala Krishna Murthy,** bearing **Registration No: MZU/M.phil/397 of 26.05.2017** has been written under my supervision.

He has fulfilled all the required norms laid down within the M.phil regulations of Mizoram University. The dissertation is the result of his own investigation. Neither the dissertation as a whole nor any part of it was ever submitted by any other university for any research degree.

Prof. G.GURUSUBRAMANIAN
Supervisor
Department of Zoology
Mizoram University

DECLARATION

Mizoram University
Aizawl: Mizoram
December, 2017

I, **Meesala Krishna Murthy**, bearing **Registration No: MZU/M.phil/397 of 26.05.2017** hereby declare that the subject matter of this dissertation is the record of work done by me, that the contents of this dissertation did not form the basis of the award of any previous degree to me or to the best of my knowledge to anybody else, and that the dissertation has not been submitted by me for any University or Institute.

This is being submitted Mizoram University for the study of Master of Philosophy in Zoology.

(Candidate)

(Head) **(Supervisor)**

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CHAPTER-1

INTRODUCTION

1.1. Hematological parameters

Tobacco contains a variety of carcinogenic compounds including PAH (polycyclic aromatic hydrocarbons), irritant compounds, nicotine, CO (carbon monoxide), and other gases that are able to generate reactive oxygen species (ROS) which leads to oxidative damage (Al-Azzawy and Al-Qaicy, 2017). There are about 2.4 billion people consuming tobacco throughout the world in the forms of smoking, chewing, snuffing or dipping (WHO, 2016).Tobacco consuming is the most vital public health concern. Various studies showed its harmful effects on many organ systems mainly liver, kidney, respiratory, reticulo-endothelial system and cardiovascular systems which inturn causes a variety of degenerative pulmonary and cardiovascular diseases as well as cancer (Oztuna, 2004). The effects of tobacco on various metabolic and biological processes and hematopoietic system have been demonstrated. Few studies showed its harmful effects on hematological system, increases in WBC, Eosinophil, and platelet (PLT) counts have been shown. The levels of hematological parameters as Neutrophil, Eosinophil, Monocyte, hemoglobin (Hb) concentration, red blood cell (RBC) and platelet counts have been increased in tobacco users. (Inal *et al*., 2014). Previous studies showed the effect of tobacco on hematological parameters is limited and controversial.

1.2. Liver enzymes (ALT, AST, ALP & LDH)

SGPT (ALT) is found in a variety of tissues but is mainly found in the liver. Increased levels are found in hepatitis, obstructive jaundice and other hepatic diseases. Slight elevation of the enzymes is also seen in myocardial infarction. SGOT (AST) is an enzyme found mainly in heart muscle, liver cells, skeletal muscle and kidneys. Injury to these tissues results in the release of the enzyme in blood stream. Elevated levels are found in myocardial infarction, Cardiac operations, hepatitis, cirrhosis, acute pancreatitis, acute renal diseases,

and primary muscle diseases. Decreased levels may be found in pregnancy, beri-beri and diabetic ketoacidosis. Alkaline Phosphatase (ALP) is an enzyme of the hydrolase class of enzymes and acts in an alkaline medium. It is found in high concentrations in the liver, biliary tract epithelium and in the bones. Normal levels are age dependent and increase during bone development. Increased levels are associated mainly with liver and bone disease. Moderate increases are seen in Hodgkins disease and congestive heart failure. Lactate dehydrogenase (LDH or LD) is an enzyme found in nearly all living cells (animals, plants, and prokaryotes). LDH catalyzes the conversion of lactate to pyruvic acid and back, as it converts NAD+ to NADH and back. A dehydrogenase is an enzyme that transfers a hydride from one molecule to another. LDH exist in four distinct enzyme classes. LDH is expressed extensively in body tissues, such as blood cells and heart muscle. Because it is released during tissue damage, it is a marker of common injuries and disease such as heart failure.

LDH-1 (4H)—in the heart and in RBC (red blood cells), as well as the brain.
LDH-2 (3H1M)—in the reticuloendothelial system
LDH-3 (2H2M)—in the lungs
LDH-4 (1H3M)—in the kidneys, placenta, and pancreas
LDH-5 (4M)—in the liver and striated muscle

Tobacco consuming affects the liver and altering enzymatic and inflammatory pathways in hepatic physiology. A few studies showed the association between tobacco and liver enzymes such ALT (alanine aminotransferase), AST (aspartate aminotransferase), LDH (lactate dehydrogenase) and ALP (alkaline phosphatase) (Mohammed *et al*., 2013). Potential mechanisms for tobacco induced damage include increased platelet reactivity and aggregability, changes in lipids and lipoprotein levels, alteration in hemostasis system, and increased counts and activation of neutrophils (Al-Awadhi *et al*., 2008).

1.3. Kidney parameters (Creatinine & Urea)

Creatinine is the catabolic product of creatinine phosphate which is used by the skeletal muscle. The daily production depends on muscular mass and it is excreted out of the body entirely by the kidneys. Elevated levels are found in renal dysfunction, reduced renal blood flow (shock, dehydration, congestive heart failure) diabetes acromegaly. Decreased levels are found in muscular dystrophy. Urea is the end production of protein metabolism. It is synthesized in the liver from the ammonia produced by the catabolism of amino acids. It is transported by the blood to the kidneys from when it is excreted. Increased levels are found in renal diseases, urinary obstructions, shock, congestive heart failure and burns. Decreased levels are found in liver failure and pregnancy. Diseases like chronic kidney disease (CKD), Cardiovascular and renal are closely associated with tobacco (Orth and Hallan, 2008). Primary hypertension and kidney function decline associated with chronic kidney disease (Warmoth *et al*., 2005). Smoking may be a significant factor in possible changes in urea and creatinine levels in smokers. Very few studies are available on the effects of tobacco on kidney function.

1.4. Lipid profile

Cholesterol is the main lipid found in blood, bile and brain tissues. It is the main lipid associated with arteriosclerotic vascular diseases. It is required for the formation of steroids and cellular membranes. The liver metabolizes the cholesterol and it is transported in the blood stream by lipoproteins. Increased levels are found in hypercholesterolemia, hyperlipidaemia, hypothyroidism, uncontrolled diabetes, nephrotic syndrome and cirrhosis. Decreased levels are found in mal absorption, malnutrition, hyperthyroidism, anaemia and liver diseases. A triglyceride is an ester derived from glycerol and three fatty acids. Triglycerides are the main constituents of body fat in humans and other animals, as well as

vegetable fat .Triglycerides is a type of fat found in your blood. Your body uses them for energy. You need some triglycerides for good health. But high triglycerides might raise your risk of heart disease and may be a sign of metabolic syndrome. Low-density lipoprotein (LDL) is one of the five major groups of lipoprotein which transport all fat molecules around the body in the extracellular water. LDL particles pose a risk for cardiovascular disease when they invade the endothelium and become oxidized, since the oxidized forms are more easily retained by the proteoglycans. A complex set of biochemical reactions regulates the oxidation of LDL particles, chiefly stimulated by presence of necrotic cell debris and free radicals in the endothelium. Increasing concentrations of LDL particles are strongly associated with the development of atherosclerosis over time. High-density lipoproteins are one of the five major groups of lipoproteins. Lipoproteins are complex particles composed of multiple proteins which transport all fat molecules around the body within the water outside cells. Increasing concentrations of HDL particles are strongly associated with decreasing accumulation of atherosclerosis within the walls of arteries. This is important because atherosclerosis eventually results in sudden plaque ruptures, cardiovascular disease, stroke and other vascular diseases. HDL particles are sometimes referred to as "good cholesterol" because they can transport fat molecules out of artery walls, reduce macrophage accumulation, and thus help prevent or even regress atherosclerosis. However, studies have shown that HDL-lacking mice still have the ability to transport cholesterol to bile, suggesting that there are alternative mechanisms for cholesterol removal.VLDL stands for very low density lipoprotein. Lipoproteins are made up of cholesterol, triglycerides, and proteins. VLDL is one of the three main types of lipoproteins. VLDL contains the highest amount of triglycerides. VLDL is a type of "bad cholesterol" because it helps cholesterol build up on the walls of arteries. Very low-density

lipoproteins transport endogenous triglycerides, phospholipids, cholesterol, and cholesteryl esters. It functions as the body's internal transport mechanism for lipids. In addition it serves for long-range transport of hydrophobic intercellular messengers, like the morphogen Indian hedgehog (protein).To directly compare traditional lipid ratios (total cholesterol [TC]/high density lipoprotein cholesterol [HDL-C], non-HDL-C/HDL-C, low density lipoprotein cholesterol [LDL-C]/HDL-C, and triglycerides [TG]/HDL-C), apo lipoprotein B (apoB)/apo lipoprotein A-I (apoA-I) ratio, visceral adiposity index (VAI), lipid accumulation product (LAP), and the product of TG and fasting glucose (TyG) for strength and independence as risk factors for insulin resistance (IR), Cardiovascular disease (CVD), the risk of heart disease etc. Significant rise in lipid profiles [total cholesterol (TC), triglyceride (TG), low density lipoprotein (LDL) and very low density lipoprotein (VLDL)] and decrease in high density lipoproteins(HDL) levels in tobacco users as reported by various studies.

However, there are very few studies on the effect tobacco on hematology, liver, kidney functioning enzymes and lipid profiles respectively. So this study aimed to investigate the effect of tobacco on hematology, liver& kidney functioning enzymes and lipid profiles in population of Mizoram, India.

1.5. Oxidative stress

Tobacco is a main substance to cause bimolecular damage due to its toxic substances, which in turn generates reactive oxygen species (ROS) that leads to modifications in heat and PH of body fluids such as Amniotic fluid, Aqueous humor and vitreous humor, Bile, Blood, Blood plasma, Blood serum, Breast milk, Cerebrospinal fluid, Gastric juice, Lymph, Mucus (including nasal drainage and phlegm), Pericardial fluid, Peritoneal fluid, Pleural fluid, Pus, Rheum, Saliva, Sebum (skin oil), Serous fluid, Semen, Smegma, Sputum, Synovial fluid,

Sweat, Tears, Urine, Vaginal secretion, plasma (Patel *et al*., 2005; Sirisha and Ram Manohar, 2013). Reactive oxygen species, superoxide anion (O2 •), hydrogen peroxide (H2O2) and hydroxyl radical (OH•), malondialdehyde (MDA) and nitric oxide (NO) are involved in DNA damage due to changes in antioxidant enzyme levels in human. So evaluation of antioxidant enzyme levels in human blood is very essential to assess the cellular damages through reactive oxygen species. Smoking and chewing tobacco has been consumed by the millions of people throughout the world, in india, approximately 400 million people have been consuming tobacco (Mehta and Hamner, 1993; Sirisha and Ram Manohar, 2013; WHO, 2016). Free radical generation is associated with tobacco components, resultants higher levels of oxidative stress and lipid peroxidation (Patel *et al*., 2005). This supplies free radicals constantly, verified by antioxidant enzymes. Oxidative damage primarily occurs via production of reactive oxygen species such as superoxide anion, peroxides, and it can damage to lipids, proteins and DNA. Therefore, it may cause to loss of enzymatic activity and structural integrity of enzymes and activate inflammatory processes (Biswas *et al.,* 2015). Oxidative stress plays an important role in the etiology of defective sperm formation, function; sperm count profile and male infertility (Shen and Ong, 2000).

1.6. Antioxidant enzymes

The antioxidant enzymes include SOD, CAT, GSH and GST. These enzymes are also considered as an important indicator of the balance status between the first and second step of the enzymatic antioxidant pathway (Uikey *et al.,* 2003). The SOD catalyzes the conversion of superoxide radicals to hydrogen peroxide; CAT converts hydrogen peroxide into water. Therefore, SOD–CAT system provides the first defense system against oxidative stress and these enzymes work together to eliminate active oxygen species (Sirisha and Ram Manohar,

2013). Glutathione peroxides are antioxidant seleno enzymes that are present in the cytosol of cells. The major function of these enzymes, which use glutathione (GSH) as a substrate, is to reduce soluble hydrogen peroxide and alkyl peroxidases (Biswas *et al.,* 2015). Antioxidants act as a free radical scavengers to inactivate and removal of reactive oxygen species, which are generated from tobacco that causes inequity between pro-oxidants and antioxidants (Marangon *et al.,* 1998; Biswas *et al.,* 2015). SOD, CAT, GST and GSH are first line defense systems to react with reactive oxygen species generated by tobacco components.

Tobacco contains toxic compounds, nicotine, carbon monoxide, polycyclic aromatic hydrocarbons, pyrolysis-derived compounds, and cadmium (Cd) causes health alteration in human. Sperm quality parameters sperm concentration, morphology and motility are decreased in tobacco users, especially in smokers (Klaiber *et al.,* 1984; Hoidas *et al.,* 1985; Jensen *et al.,* 2004; Kapoor and Jones, 2005).

1.7. Reproductive hormones

Follicle-stimulating hormone is a gonadotropin, a glycoprotein polypeptide hormone. FSH is synthesized and secreted by the gonadotropic cells of the anterior pituitary gland, and regulates the development. FSH stimulates primary spermatocytes to undergo the first division of meiosis, to form secondary spermatocytes. FSH enhances the production of androgen-binding protein by the Sertoli cells of the testes by binding to FSH receptors on their basolateral membranes and is critical for the initiation of spermatogenesis. Luteinizing hormone is produced by gonadotropic cells in the anterior pituitary gland. In females, an acute rise of LH triggers ovulation and development of the corpus luteum. In males, where LH had also been called interstitial cell–stimulating hormone (ICSH), it stimulates Leydig cell production of testosterone. It acts synergistically with FSH. LH acts upon the Leydig

cells of the testis and is regulated by gonadotropin-releasing hormone (GnRH).The Leydig cells produce testosterone (T) under the control of LH, which regulates the expression of the enzyme 17β-hydroxysteroid dehydrogenase that is used to convert androstenedione, the hormone produced by the testes, to testosterone, an androgen that exerts both endocrine activity and intratesticular activity on spermatogenesis. LH is released from the pituitary gland, and is controlled by pulses of gonadotropin-releasing hormone. When T levels are low, GnRH is released by the hypothalamus, stimulating the pituitary gland to release LH. As the levels of T increase, it will act on the hypothalamus and pituitary through a negative feedback loop and inhibit the release of GnRH and LH consequently. Androgens (T, DHT) inhibit monoamine oxidase (MAO) in pineal, leading to increased melatonin and reduced LH and FSH by melatonin-induced increase of GnIH synthesis and secretion. T can also be aromatized into estradiol (E2) to inhibit LH. E2 decreases pulse amplitude and responsiveness to GnRH from the hypothalamus onto the pituitary. The effect of estradiol (and estrogens in general) upon male reproduction is complex. Estradiol is produced by action of aromatasemainly in the Leydig cells of the mammalian testis, but also by some germ cells and the Sertoli cells of immature mammals. It functions (in vitro) to prevent apoptosis of male sperm cells. While some studies in the early 1990s claimed a connection between globally declining sperm counts and estrogen exposure in the environment, later studies found no such connection, nor evidence of a general decline in sperm counts. Suppression of estradiol production in a subpopulation of subfertile men may improve the semen analysis. Males with certain sex chromosome genetic conditions, such as Klinefelter's syndrome, will have a higher level of estradiol. Reproductive hormones testosterone, estradiol, luteinizing hormone (LH) and follicle stimulating hormone (FSH) have been changed in tobacco users due to its effect on pituitary gland (Klaiber *et al*., 1984; Vine,

1996; Ramlau-Hansen *et al*., 2007). These hormones play a significant role in maintenance of sperm quality.

1.8. Cotinine

Cotinine is an alkaloid found in tobacco and is also the predominant metabolite of nicotine. Cotinine is used as a biomarker for exposure to tobacco smoke. Cotinine can be detected in blood, saliva and urine. Urine is more responsive to detect low concentrations of cotinine (Avila-Tang *et al.,* 2011). Based on cotinine concentration, one can discriminate no active smokers (<10 ng/mL), light smokers or moderate passive exposure (10 ng/mL to 100 ng/mL) and heavy smokers (>300 ng/mL) (Jarvis *et al.,* 2008). In urine, light smokers or passive exposures (11-30 ng/mL) and active smokers (>500 ng/mL) concentration respectively. In saliva, light smokers or passive exposures (1 -30 ng/mL) and active smokers (>100 ng/mL) concentration respectively (Hewitt, 2011). Cotinine assay is associated with counting the number of cigarettes smoked per day. The amount of nicotine present in the body is revealed by using cotinine concentrations (McNeil *et al.,* 2016). Cotinine is currently being used as a biomarker for treatment of depression, PTSD, schizophrenia, Alzheimer's and Parkinson's diseases. However, very limited reports have examined whether the relationship between tobacco (smoking and smokeless tobacco) and with antioxidant, hormones and cotinine profiles. So the current study focused on antioxidant enzymes, hormones and cotinine levels and their changes in male tobacco consumers with non-users in Mizoram, India.

1.9. Pro apoptotic, pro inflammatory proteins and genotyping markers

Tobacco is associated with number of diseases such as oral cavity lesions, oral cancer leukoplakia, keratosis, sub mucous fibrosis, leukoedema, hairy tongue, tooth decay, cardiovascular disease, peripheral vascular disease, hypertension, diabetes, hyper

cholesterolemia, peptic ulcers and, foetal morbidity and mortality (Gupta and Ray, 2003; Piano *et al*., 2010). Studies revealed that cardiovascular, respiratory and renal diseases have been scrutinized in tobacco users (Piano *et al*., 2010; Teo *et al*., 2006). Tobacco takes every year 6 million deaths and its predicted that 10 million deaths each year by 2020 throughout world (Ezzati and Lopez, 2004; Hossain *et al.,* 2014). SLT and CS both seem to be equally powerful in altering the cellular and metabolic changes. Lombard *et al.,* 2010 reported that Up-regulation of pro-apoptotic and inflammatory genes in male rats due to aqueous extract of tobacco. Glutathione S-transferases (GSTs) mainly involved in detoxification of toxic compounds in phase-II oxidation (Hayes and Strange, 2000). GSTM-1, GSTT-1, and GSTP-1 isoenzymes generally expressed in human gastrointestinal tract (Hayes and Pulford, 1995). GSTM1 and GSTT1 are null genotypes due to homozygosity for an inherited deletion of these genes (Lan *et al*., 2001). Individuals have low detoxification ability with no GSTM1 null genotype expression (Pemble *et al.,* 1994). Reduced GST activity is associated SNPsin the GSTP1 gene at codons 105 (Ile?Val) and 114 (Val?Ala) (Zimniak *et al.,* 1994; DeBruin *et al*., 2000; Cai *et al*., 2001; Tahara *et al*., 2011). An individual's difference in susceptibility to chemically induced carcinomas may possibly be attributed to the variations in the detoxification pathways due to polymorphic varients of GST gene. So we performed a case–control study to evaluate the relevance of GSTM1, GSTT1, and GSTP1 gene polymorphisms and also performed Immunoblots analysis to know the tobacco has been implicated to cause physiological stress leading to extreme toxicological challenge.

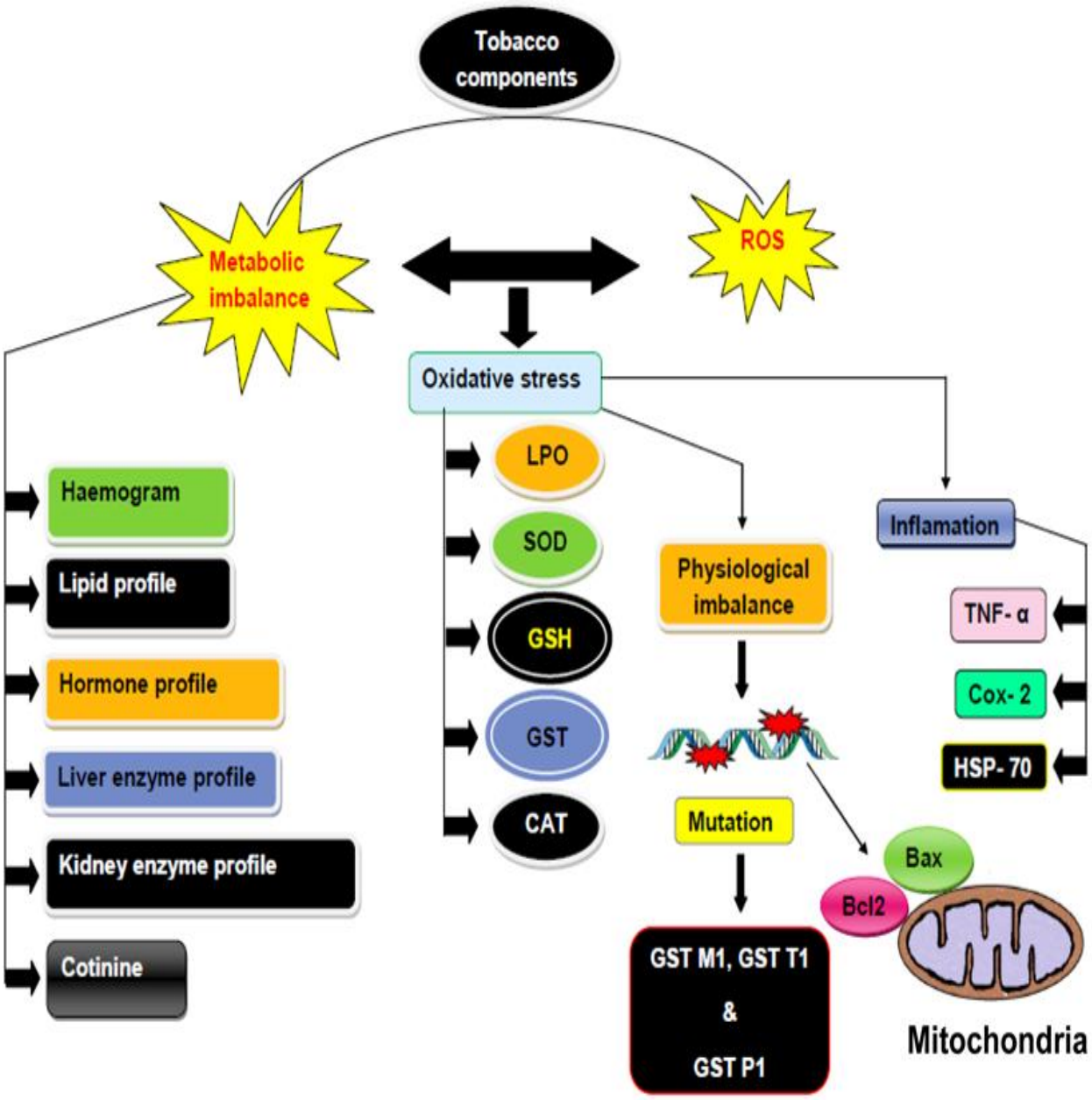


Figure 1. Schematic illustration of possible mechanism of action of tobacco components mediated damage

CHAPTER-2

REVIEW OF LITERATURE

2.1. Hematology parameters

Erythrocyte (RBC), leucocytes (WBC), haemoglobin (Hb) levels are significantly changed in tobacco consumers. Erythrocytes were increased while haemoglobin and leukocytes levels were decreases in smokers compared with the non-smokers. This may lead to various cellular, metabolic and physiological disorders (Zafar *et al.,* 2003). Cigarette and shisha users have significant increase in erythrocyte count (RBCs), hemoglobin (Hb) concentration, Haematocrit (HCT), leukocyte count (TWBC) and mean cell hemoglobin concentration (MCHC) levels. But cigarette and shisha users have no change in mean cell volume (MCV) but few studies showed significant increase in MCV (mean cell volume). However, Cigarette and shisha users have very significant decrease in platelets counts (Nadia *et al.,* 2015; Al-Awadhi *et al.,* 2008; Inal *et al*., 2014). Neutrophil count, Monocyte count, ESR levels were found to be statistically significant between chewers and non-users (Biswas *et al.,* 2015). It is suggested that blood haemotology values should be interpretive with smoking habits.

2.2. Liver enzymes (ALP, AST, ALT & LDH) and Kidney parameters (Creatinine & Urea)

Alkaline phosphatase (ALP), asparatate aminotransferase (AST) and alanine aminotransferase (ALT) levels were significantly increased in smokers compared with non-users respectively (Wannamethee and Shaper, 2010; Breitling *et al.,* 2011; Jang et al., 2012; Abdrabo and Elameen, 2013; Abdul-Razaq and Ahmed, 2013). It showed that liver effectiveness and functions can be affected by smoking. Elevated levels of Plasma creatinine, urea nitrogen and urea levels were found significantly in smoker and chewers group when compared with the control group (Ahmed *et al*., 2015; Akande *et al.,* 2015; Desai *et al.,* 2016; Murtadha, 2017). Previous studies showed that strongest association of tobacco chewing and smoking on renal disorders. Renal disorders are developed due to impairment in renal functions.

2.3. Lipid profile

Lipid profiles such as total cholesterol (TC), low density lipoproteins (LDL), triglycerides (TG) and very low density lipoproteins (VLDL) were increased significantly in smokers and chewers where as high density lipoproteins (HDL) was decreased significantly in smokers and chewers (Mahjub *et al.,* 2010; Murtadha, 2017). Total cholesterol (TC), low density lipoproteins (LDL), triglycerides (TG) and very low density lipoproteins (VLDL) were increased in number of cigarette/bidis smoked where as high density lipoproteins (HDL) had inverse association with cigarettes/bidis smoked per day. The results showed that lipid profile significantly changed by severity of smoking that leads to dislipidaemia in smokers (Joshi *et al.,* 2013). Development of atherosclerosis, myocardial infarction and cardiovascular disease risks significantly linked with lipid profile levels and their ratios (Biswas *et al.,* 2015).This study might be open a new vital insight into tobacco mediated renal and hepatic disorders, atherosclerosis, myocardial infarction and cardiovascular disease risk prevention in mizo population with tobacco chewing and smoking habits.

2.4. Oxidative stress and antioxidant enzymes

It is very well known fact that, tobacco usage causes lipid peroxidation due to formation of reactive oxygen species and free radicals. Tobacco is a significant factor to cause not only oral diseases such as oral cancer, periodontal disease, cleft lip, cleft palate, alveolar bone loss and black hairy tongue but also chronic diseases, including cardiovascular disease, pulmonary disease, and cancer (Khalili, 2008).Tobacco was associated with an increase in GPx, GSH, and CAT. Smokers showed significant increase in GPx, GSH, and CAT than non smokers (Dove *et al*., 2015). Saggu *et al.,* 2012 reported that GPx was decreased; SOD was increased in smokers as compared to non smokers. Previous study showed significant fall in SOD levels in tobacco users

compared with non-users (Sirisha and Manohar, 2013). Sod and GPx were decreased significantly in smokers as compared to non-smokers (Abdolsamadi *et al.,* 2011; Rizvi and Tariq, 2016). The levels of SOD activity was decreased in the gingival crevicular fluid (GCF) and saliva of smokers (Agnihotri *et al.,* 2009). The TBARS level was increased and other antioxidant levels were decreased in tobacco consumers (Bloomer, 2007; Pasupathi *et al*., 2009; Biswas *et al.,* 2015).

2.5. Reproductive hormones

Nicotine is an endocrine disruptor, it can stimulate various hormones such as LH, testosterone, prolactin, growth hormone (GH), adrenocorticotrophin (ACTH), and arginine vasopressin (AVP) without significant changes in thyroid stimulating hormone (TSH) and follicle stimulating hormone (FSH) (Seyler *et al*., 1986; Vine, 1996; Kapoor and Jones, 2005; BlancoMuñoz *et al.,* 2012). Zhao *et al*., 2016, reported that testosterone was significantly increased in smokers compared with non-users in their study. In previous study, testosterone was decreased and increased, based on the number of cigarettes consumed by smokers where as no significance difference in LH and FSH levels (Jeng *et al*., 2014). Blanco-Muñoz *et al.,* 2012 revealed that high levels of testosterone, LH and prolactin observed in smokers. Low serum T levels have been associated with type 2 diabetes (T2D) and cardiovascular disease (Holmboe *et al.,* 2016; Salminen *et al*., 2015; Schipf *et al.,* 2011; Vikan *et al.,* 2010). The risk of diabetes is associated with lower levels of testosterone (Stellato *et al*., 2000).The dose response study of DEHP on 74 male workers, showed significant reduction in testosterone as well as LH,FSH and estradiol were increased significantly in DEHP exposed workers (Pan *et al.,* 2011). Nicotine treated rats showed significant ($p < 0.05$) decrease in

testosterone and FSH where as LH was increased significantly ($p < 0.05$) in nicotine treated rats (Oyeyipo *et al*., 2013).

2.6. Cotinine

Cotinine, a metabolite of nicotine, is widely used to distinguish smokers from nonsmokers in epidemiologic studies and smoking-cessation clinical trials. Quantification of cotinine concentration in bio fluids such as urine, blood, hair, and saliva is a tremendous method to differentiate smokers and non smokers (NCCDPHP, 2006). Self report study from the National Health and Nutrition Examination Survey, 1999–2008, found that high cotinine concentration levels in smokers along with high HbA1c% compared with never smokers (Clair *et al*., 2011). To differentiate smokers from non smokers based on the cotinine cut off value is about 3ng/ml (Benowitz *et al*., 2009).Simultaneous measurement of cotinine and nicotine in Japanese individuals were showed that significant higher levels (1635 (2222) and 3948 (3512) ng/ml) in smokers urine with compared (3.5 (5.3) and 2.8 (4.2) ng/ml) to non smokers (Matsumoto *et al.,* 2013). Yuan *et al.,* 2011 reported that determination of urinary cotinine was a risk factor for development of lung cancer in their study. However, previous studies have showed a lack of comprehensive study in the tobacco users and their health effects, especially in mizo people, Mizoram, India.

2.7. Pro apoptotic and pro inflammatory proteins

Tobacco remains a threat globally especially in India. The oral use of tobacco has been implicated to cause physiological stress leading to extreme toxicological challenge. Tobacco is an age old habit among humans. The effects of tobacco on physiological systems are also well-known. However, the present study is a comprehensive attempt, where tobacco mediated systemic toxicity, physiological perturbation and cellular abnormalities have been investigated in

detail. SLT and its components were responsible for various cellular activities, including perturbing the pro- and anti-apoptotic pathways. The expressions of p53, p21, Bax, Bcl-2, IL-6, TNF- α, Cox-2, iNOS, CDK-4, CDK-6, Cyclin-E and CDK-2 were analyzed from thirteen representative SLT-users and twelve non-users, which in turn leads to the blockage of G0/G1 transition (Satyanarayana and Kaldis, 2009; Biswas *et al*., 2015). P53/p21 pathway is very important in assay the cellular DNA damage (Kahlem *et al.,* 2004). The increased expression of p53 and p21 leads to cell proliferation arrest and keeping time for repairing of damaged DNA (Vogelstein *et al.,* 2000). SLT and its components were responsible for various cellular activities, including perturbing the pro- and anti-apoptotic pathways. Enhanced levels of IL-6, which in turn reduced other cytokines such as IL-1, IL-10 and TNF- α (Hirano, 1998). Fibroblasts and macrophages, induced cyclooxygenase (COX-2) expression during inflammatory processes (Wu,1995). The COX along with NOS associated in pathophysiological conditions (Bassuk *et al.,* 2004). Increasing evidences suggest that there is considerable 'cross-talk' between COX and NOS. In vivo studies revealed that the regulation of COX by NO is a powerful mechanism that is used to magnify the course of the inflammatory response (Vane *et al.,* 1994).

2.8. Genotyping

The G/G, A/G (rs1695), and T/T (rs1138272) were found to be significantly associated with low expression of GSTP1 gene in cancer tissue (Ghatak *et al*., 2016). The gene frequency of GSTM1, GSTT1, and GSTP1 polymorphisms was evaluated. We observed that GSTM1 and GSTT1 null genotype frequencies were 54.3% and 32.6% respectively, while GSTP1 (Ile/Val), (Ile/Ile), (Val/Val) genotype frequencies were 52%, 44%, and 4%, respectively, in the cervical cancer patients. No statistical variation was determined between the control and patient groups in terms of GSTM1, GSTT1, and GSTP1 polymorphisms (p >0.05) (Kiran *et al*., 2010). Previous

study (Cerliani *et al*., 2016) reported that no significant difference between genetic polymorphisms and blood cancer. Consumption of tobacco in various traditional forms such as "tuibur" (tobacco smoke–infused aqueous solution) and Chaini (traditional name "Sada") is common habit that is important risk factor for stomach cancer. Therefore, correlation of consumption of tobacco with the incidence of stomach cancer in Mizoram cannot be ruled out.

CHAPTER-3

OBJECTIVES

The current study confers crucial insight into tobacco use mediated effects on systemic toxicity and stress in males of Mizo population:

- Assessment of plasma cotinine concentration, haematological, lipid, hormone, antioxidant enzymes and biochemical profiles its relationship with reproductive health as well as tobacco use related diseases.
- DNA damage detection by TUNEL assay.
- Expression pattern of apoptosis regulators (BAX and BCl2), pro-inflammatory cytokines (TNF- α and Cox-2) and HSP70 by immunoblotting.
- Polymorphism at GSTM1, GSTT1 and GSTP1 gene loci and susceptibility to tobacco use related diseases.

CHAPTER-4

MATERIALS AND METHODS

4.1. Reagents and chemicals

Alkaline phosphatase (ALP), asparatate amino transferase (AST),alanine amino transferase (ALT), lactate dehydrogenase (LDH), urea, creatinine, total cholesterol, triglyceride, high density lipoproteins (HDL) kits purchased from Coral Clinical Systems, Tulip Diagnostics (P) Ltd. Mumbai. Histopaque-1077, Trichloroacetic acid (TCA), Thiobarbituric acid (TBA), 5, 5′ -dithio-bis (2-nitro benzoic acid) (DTNB) were purchased from Sigma Aldrich (St. Louis, MO, USA). Antibodies (Bax, Bcl-2, TNF-α, COX-2, β- actin) were purchased from Elab science Technology (Danvers, MA, USA). Ethidium bromide (EtBr), hydrogen peroxide (H2O2), ethanol and all other fine chemicals were procured from Merck (Germany).

4.2. Ethical approval

All experiments were performed in accordance with the approved guidelines and regulations of Human Ethics committee (**No.B.12018/1/13-CH (A)/IEC/42**) and the Institutional Bio-Safety Committee of Civil hospital, Aizawl, Mizoram.

4.3. Data collection approach

Questionnaire includes socio-demographic data (age, gender, level of education, marital status, occupation, Family History of tobacco use , Income, religion, Living area, Family members, Numbers of Members used tobacco,. Information about tobacco use, influence on the start of tobacco habit, rationale of the study, individual rights and related risk elements) was collected by interview. Written informed consent was then obtained from each person. Thumb impression was obtained from those who were unable to sign the consent form.

4.4. Inclusion and Exclusion criteria

People lived in Aizawl area those uses tobacco and meet the following inclusion as well as exclusion criteria were chosen for this study. **Inclusion criteria:** Tobacco user age 20-40 years male, physically able and willing to participate. **Exclusion criterion:** very sick or very old person, temporary migrant (guest).

4.5. Study design

The entire study population (N= 300) was subdivided into four groups: **i) Nontobacco-users (N=75)**, individuals do not consume any form of tobacco or who have not used tobacco products for at least past one year, **ii) Smokers (N=75)**, frequency of smoking ranged between 10 piece per day, **iii) Chewers (N=75)**, individuals chewing tobacco products (Zarda, gutkha, sadha and khuva) habitually, at least > 20 times per week for the last 6 months, and **iv) Smokers and SLT users (N=75)**, individuals consume smokeless tobacco products as well as smoking.

4.6. Blood collection

Blood was withdrawn through venipuncture using 5 ml sterile syringe. Blood was transfer to EDTA coated vessels (Fig.2 A, B, C). Blood sample was centrifuged at 3000 rpm for 10 min. After centrifugation, plasma was separate and store at −20 °C until analyze.

4.7. Analysis of hematology parameters

All haematological parameters like, erythrocyte sedimentation rate (ESR), haemoglobin (Hb), erythrocytic count (RBC), leucocytic count, neutrophil count, total lymphocyte count (TLC), monocyte count, eosinophil count, platelet count, packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), total organic carbon in males (TOC M), haematocrit were analysed from all studied individuals by using auto analyzer (Purechem Ltd, Ireland; Fig.2 D).

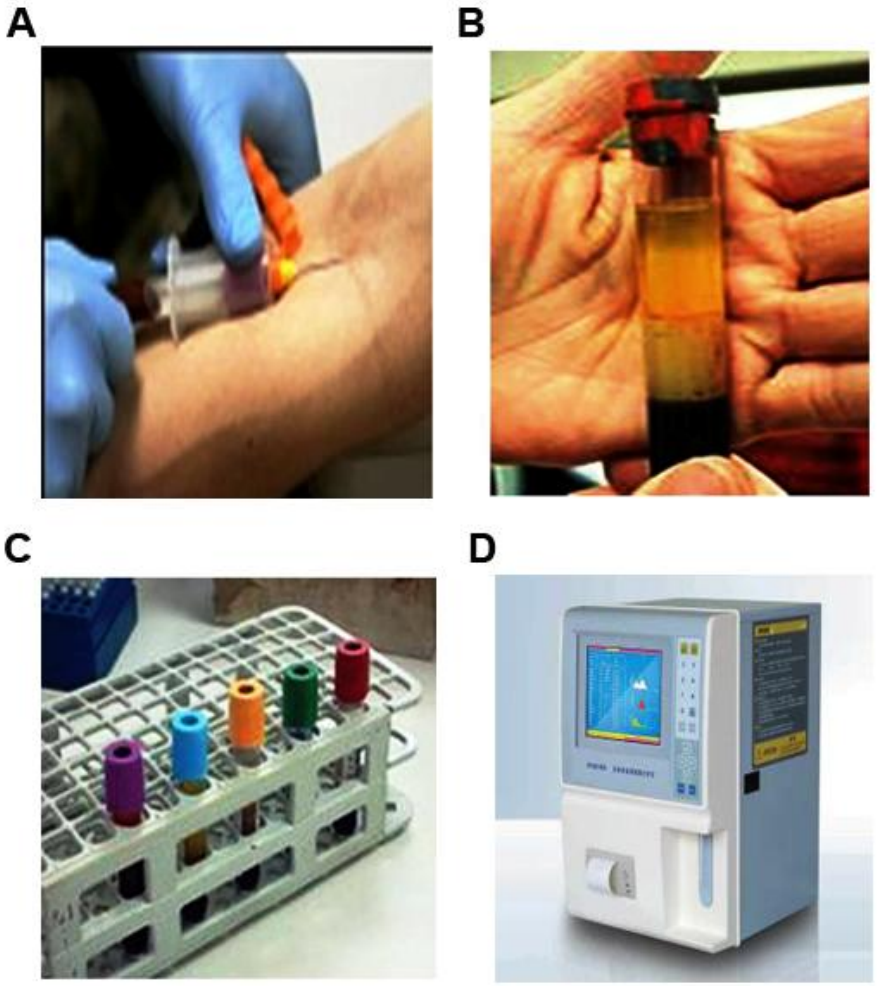


Figure 2. Blood collection and auto analyzer

4.8. Evaluation of biochemical parameters (ALT, AST, ALP, LDH, Creatinine and Urea)

4.8.1. Estimation of SGPT (ALT) activity (Reitman &Frankel's method, 1957)

For the determination of SGPT (ALT) activity in plasma

Principle: SGPT converts L-Alanine and α-Ketoglutarate to Pyruvate and Glutamate. The Pyruvate formed reacts with 2,4Dinitrophenyl hydrazine to produce a hydrazone derivative, which in an alkaline medium produces a brown coloured complex whose intensity is

measured. The reaction does not obey Beer's law and hence a calibration curve is plotted using a Pyruvate standard. The activity of SGPT (ALT) is read off this calibration curve.

Contents	40 assays	80 assays
L1:Substrate Reagent	25 ml	50 ml
L2:DNPH Reagent	25 ml	50 ml
L3:NaOH Reagent (4N)	25 ml	50 ml
S:PyruvateStandard(2mM)	5 ml	5 ml

Storage /stability

Contents are stable at 2-8 ^{0}C at 25 ^{0}C.

Reagent Preparation:

All reagents are ready to use except NaOH Reagent (4N) which has to be diluted 1:10 with distilled/deionised water.

Working NaOH reagent: Sodium Hydroxide was diluted to 250ml or for every 1.0 ml of NaOH.

Reagent (4N) 9.0 ml of water was added. The Working Sodium Hydroxide reagent is stable at 25 ^{0}C.

Sample material: Plasma

Procedure

Wavelength/filter : 505 nm (Hg 546 nm)/Green

Temperature ` : 37 ^{0}C & 25 ^{0}C.

Light path : 1 cm

Plotting of the Calibration curve:

Mixed well and allowed to stand at 25 ^{0}C for 10 min. The absorbances of the test samples against Blank were measured. Plot a graph of the absorbances of sample on the 'Y' axis versus the corresponding Enzyme activity on the 'X' axis.

Addition sequence	**1**	**2**	**3**	**4**	**5**
Enzyme Activity (U/ml)	0	28	57	97	150
	(ml)	(ml)	(ml)	(ml)	(ml)
Substrate Reagent (L1)	0.50	0.45	0.40	0.35	0.30
Pyruvate Standard (S)	-	0.05	0.10	0.15	0.20
Distilled Water	0.10	0.10	0.10	0.10	0.10
DNPH Reagent (L2)	0.50	0.50	0.50	0.50	0.50
Mixed well and allowed to stand at 25 ^{0}C for 20 minutes.					
Working NaOH Reagent (L 3)	5.00	5.00	5.00	5.00	5.00

Mixed well and allowed to stand at 25 ^{0}C for 10 min. The absorbance's of the tubes 2 – 5 against tube 1 (Blank) was measured. Plot a graph of the absorbance's of tubes 2 – 5 on the 'Y' axis versus the corresponding Enzyme activity on the 'X' axis.

Assay

Pipette into clean dry test tubes labeled as Blank (B) & (T)

Addition Sequence	**B (ml)**	**(T) (ml)**
Substrate Reagent (L 1)	250µl	250 µl
Incubate at 37 C for 3 minutes		
Sample	-	50 µl
Mix well and incubate 37 C for 30 minutes	-	-
DNPH Reagent (L2)	250µl	250 µl
Mixed well and allowed to stand at 25 ^{0}C for 20 minutes		
Distilled Water	50µl	-
Working NaOH Reagent (L3)	250µl	

Mixed well and allowed to stand at 25 ^{0}C for 10 min. The absorbance's of the Test (T) were measured against Blank, (Blank) and the activity of the test was read from the calibration curve plotted earlier.

4.8.2. Estimation of SGOT (AST) activity (Reitman & Frankel's method)

For the determination of SGOT (AST) activity in plasma

Principle: SGOT converts L-Aspartate and α Ketoglutarate to Oxaloacetate and Glutamate. The Oxaloacetate formed reacts with 2, 4, Dinitrophenyl hydrazine to produce a hydrazone derivative, which in an alkaline medium produces a brown coloured complex whose intensity is measured. The reaction does not obey Beer's law and hence a calibration curve is plotted using a Pyruvate standard. The activity of SGOT (ASAT) is read off this calibration curve.

Contents	**40 assays**	**80 assays**
L-1 : Substrate Reagent	25 ml	50 ml
L-2 : DNPH Reagent	25 ml	50 ml
L-3 : NaOH Reagent (4N)	25 ml	50 ml
S:PyruvateStandard (2mM)	5 ml	5 ml

Reagent Preparation:

All reagents are ready to use except NaOH Reagent (4N) which has to be diluted 1:10 with distilled/deionised water.

Working NaOH reagent: Sodium Hydroxide was diluted to 250ml or for every 1.0 ml of NaOH Reagent (4N), 9.0 ml of distilled water was added. The Working Sodium Hydroxide reagent was stable at 25 ^{0}C.

Sample material: plasma

Procedure:

Wavelength : 505 nm (Hg 546 nm)/Green

Temperature : 37 ^{0}C & 25 ^{0}C.

Light path : 1 cm

Plotting of the Calibration curve:

Addition sequence	**1**	**2**	**3**	**4**	**5**
Enzyme Activity (U/ml)	0	24	61	114	190
	(ml)	(ml)	(ml)	(ml)	(ml)
Substrate Reagent (L1)	0.50	0.45	0.40	0.35	0.30
Pyruvate Standard (S)	-	0.05	0.10	0.15	0.20
Distilled Water	0.10	0.10	0.10	0.10	0.10
DNPH Reagent (L 2)	0.50	0.50	0.50	0.50	0.50
Mixed well and allowed to stand at 25 ^{0}C for 20 minutes.					
WorkingNaOH Reagent (L3)	5.00	5.00	5.00	5.00	5.00

Mixed well and allowed to stand at 25 ^{0}C for 10 min. The absorbance's of the tubes 2 – 5 against tube 1 (Blank) was measured. A graph of the absorbance's of tubes 2-5 on the 'Y-axis versus the corresponding Enzyme activity on the 'X' axis was plotted.

Assay

Pipette into clean dry test tubes labeled as Blank (B) & Test (T)

Addition Sequence	**(B) (ml)**	**(T) (ml)**
Substrate Reagent (L1)	250 µl	250 µl
Incubated at 37 C for 3 minutes		
Sample	-	50 µl
Mixed well and incubated at 37^{0}C for 60 minutes		
DNPH Reagent (L2)	250 µl	250 µl
Mix well and allow to stand at 25 ^{0}C for 20 minutes		
Distilled Water	50µl	-
Working NaOH Reagent (L3)	250 µl	250 µl

Mixed well and allowed to stand at 25 ^{0}C for 10 min. The absorbance's of the Test (T) against Blank (Blank) was measured and the activity of the test from the calibration curve plotted earlier was read.

4.8.3. ALKALINE PHOSPHATASE (Modified Kind & King's method, 1954)

For the determination of Alkaline Phosphatase activity in plasma

Principle: ALP at an alkaline pH hydrolyses disodium Phenyl phosphate to form phenol. The phenol formed reacts with 4 – Aminoantipyrine in the presence of Potassium Ferri-cyanide, as an oxidizing agent to form a red colored complex. The intensity of the color formed is directly proportional to the activity of ALP present in the sample.

Contents	15 Tests	30 Tests
L1 : Buffer Reagent	60 ml	120ml
L2 : Substrate reagent	6ml	12 ml
L3 : Colour Reagent	60ml	120ml
S : Phenol Standard (10 mg/ dl)	5ml	5ml

Sample material: Plasma

Procedure

Wavelength/ filter: 510 nm (Hg 546 nm) Green

Temperature : 37^0 C

Light path : 1 cm

Assay

Pipette into four clean dry test tubes labelled as Blank (B), Standard (S), Control (C), Test (T).

Addition Sequence	B (ml)	S (ml)	C (ml)	T (ml)
Distilled water	525µl	500 µl	500 µl	500 µl
Buffer Reagent (L1)	500µl	500µl	500 µl	500 µl
Substrate Reagent (L2)	50µl	50 µl	50 µl	50 µl
Mixed well and allowed to stand at 37^0C for 3 minutes				
Sample	-	-	25 µl	25 µl
Phenol Standard (S)	-	25 µl	-	
Mixed well and allowed to stand at 37^0C for 15 minutes and add.				
Colour Reagent (L3)	500µl	500µl	500µl	500µl
Sample	-	-	0.025	-

It was mixed well after each addition. The absorbance's of the Blank (Abs. B), Control (Abs. C) and Test (Abs. T) was measured against distilled water.

Calculations

Total ALP activity in K.A. Units = Absorbance of T- Absorbance C / Absorbance of S – Absorbance of B×10 K.A. Units.

4.8.4. LACTATE DEHYDROGENASE (LDH) (Modified IFCC method, 1982)

For the determination of lactate dehydrogenase in plasma

Principle: LDH catalyzes the reduction of pyruvate with NADH to form NAD. The rte of oxidation of NADH to NAD measured as a decrease in absorbance which is proportional to the LDH activity in the sample.

$$\text{Pyruvate} + \text{NADH} + H^+ \longrightarrow \text{Lactate} + NAD^+$$

Contents	15 Tests	30 Tests
L1 : Buffer Reagent	20 ml	120ml
L2 : Starter reagent	5ml	30 ml

Sample material: Plasma

Procedure

Wavelength/ filter: 340 nm

Temperature : 37^0 C

Light path : 1 cm

Assay
Pipette into four clean dry test tubes labelled as Blank (B), Standard (S), Control (C), and Test (T).

Addition Sequence	B (ml)	S (ml)	C (ml)	T (ml)
Buffer Reagent (L1)	800µl	800µl	800 µl	800 µl
Sample	-	-	25 µl	25 µl
Mixed well and allowed to stand at 37^0C for 1minutes				
Starter Reagent (L2)	200µl	200 µl	200 µl	200 µl

It was mixed well after each addition. The absorbance's of the Blank (Abs. B), Control (Abs.

C) and Test (Abs. T) was measured against distilled water.

Calculations

Total LDH activity in U/L = A/min. × 8095.

4.8.5. Estimation of Creatinine (Alkaline Picrate method)

For the determination of Creatinine in plasma

Principle: Picric acid in an alkaline medium reacts with creatinine to form an orange colored complex with the alkaline picrate. Intensity of the color formed is directly proportional to the amount of creatinine present in the sample.

Creatinine + Alkaline Picrate →Orange Coloured Complex

Contents	15 Tests	35 Tests	70 Tests
L1:Picric Acid Reagent	60 ml	140 ml	2x140 ml
L2:Buffer Reagent	5 ml	12 ml	25 ml
S:Creatinine Standard (2 mg/dl)	5 ml	5 ml	10 ml

Storage/stability

All reagents are stable at 25 ^{0}C.

Reagent Preparation

Sample material: Plasma

.

Procedure

Wavelength/filter	:	520 nm (Hg 546 nm)/Green
Temperature	:	25 ^{0}C
Light path	:	1 cm

Deproteinization of specimen

Pipetted into a clean dry test tube

Picric acid reagent (L1)	2.0 ml
Sample	0.2 ml

Mixed well and centrifuged at 2500 – n3000 rpm for 10 min and a clear supernatant was observed.

Pipette into clean dry test tubes labelled as Blank (B), Standard (S), and Test (T)

Addition Sequence	**B (ml)**	**S (ml)**	**T (ml)**
Supernatant	-	-	550µl
Picric Acid Reagent (L1)	500 µl	500 µl	-
Distilled water	50 µl	-	-
Creatinine Standard(S)	50 µl	50 µl	50 µl
Buffer Reagent (L2)			

Mixed well and kept the test tubes at 25 ^{0}C for exactly 20 minutes. The absorbance of the Standard (Abs. S), and Test Sample (Abs. T) against the Blank was measured.

Calculations

Creatinine in mg% = mg/dl.

4.8.6. Estmation of Urea (Modified Berdthelodt method, 1859)

For the determination of urea in plasma.

Principle: Urease hydrolyses urea to ammonia and CO. The ammonia formed further reacts with a phenolic chromogen and hypochlorite to form a green coloured complex. Intensity of the colour formed is directly proportional to the amount of urea present in the sample.

$$\text{Urea} + H_2O \xrightarrow{Urease} \text{Ammonia} + CO_2$$

Ammonia + Phenolic chromogen → Green coloured complex + Hypochlorite

Contents	75 Assays	3x75 Assays	2x150Assays
L1: Buffer Reagent	75 ml	3x 75 ml	2x150 ml
L2: Enzyme Reagent	7.5 ml	3 x 7.5 ml	30ml
L3:Chromogen Reagent	15 ml	45 ml	60 ml
S : Urea Standard (40 mg/dl)	5 ml	5 ml	5 ml

Working Enzyme Reagent: For the flexibility and convenience in performing large assay series, a working enzyme reagent was made by pouring 1 bottle of L2 (Enzyme Reagent) into 1 bottle of L1 (Buffer Reagent). For smaller series combine 10 parts of L1 (Buffer Reagent) and 1 part of L2 (Enzyme Reagent). 1ml of the working reagent per assay was used instead of 1 ml of L1 and 0.1 ml of L2 as given in the procedure. The working enzyme reagent is stable for at least 4 weeks when stored at 2-8 C.

Cuveties, 1 part of L3 (Chromogen Reagent) was diluted with 4 parts of fresh ammonia free distilled/deionised water. 1ml of working chromogen was used instead of 0.2 ml in the assay. The working chromogen reagent is stable for atleast 8 weeks when stored at 2-8 ^{0}C in a tightly stopper plastic bottle.

Sample material: Serum

Procedure

Wavelength/filter : 570 nm (Hg 578 nm)/Yellow

Temparature : 37 ^{0}C /25 ^{0}C

Light path : 1 cm

Pipetted into clean dry test tubes labelled as Blank (B), Standard (S), and Test (T)

Addition sequence	B (ml)	S (ml)	T (ml)
Buffer reagent (L1)	1ml	1ml	1ml
Enzyme reagent (L2)	100 ml	100 ml	100 ml
D.H_2O	10 ml	-	-
Urea std	-	10µl	-
Sample	-	-	10µl
Mixed well and was incubated for 5 min at 37^0C.			
Chromogen Reagent (L3)	200µl	200µl	200µl

Calculations

Urea in mg/dl = Abs. T/Abs. S × 40 mg/dl

Urea nitrogen in mg/dl = Urea in mg/dl × 0.467 mg/dl.

4.9. Estimation of lipid profiles (TC, TG, HDL, and LDL & VLDL)

4.9.1. Estimation of Cholesterol (CHOD/ PAP method) (Allain *et al*., 1974)

For the determination of Cholesterol in plasma

Principle: Cholesterol esterase hydrolyses esterified cholesterols to free cholesterol. The free cholesterol is oxidised to form hydrogen peroxide which further reacts with phenol and 4-animoantipyrine by the catalytic action of peroxidase to form a red coloured quinoneimine dye complex. In tensity of the colour formed is directly proportional to the amount of cholesterol present in the sample.

$$\text{Cholesterol esters} + H_20 \xrightarrow{\text{Cholesterol Esterase}} \text{cholesterol} + \text{Fatty acids}$$

$$\text{Cholesterol} + 0_2 \xrightarrow{\text{Cholesterol Oxidase}} \text{cholesterol} + H_20_2$$

$$\xrightarrow{\text{Peroxidase}}$$

H_20_2 + 4 Aminoantipyrine + Phenol ⟶ Red Quinoneimine dye + H_20

Contents	**75 ml**	**2 X 75ml**	**2 X 150ml**	**2 X 250ml**
L1:Enzyme reagent1	60 ml	2 X 60ml	2 X 120ml	2 X 200ml
L2:Enzyme reagent 2	15 ml	2 X 15ml	2 X 30ml	2 X 50ml
S:Cholesterol Standard (200 mg/ dl)	5 ml	5ml	5ml	5ml

Storage/ stability

Contents are stable at 2 -8^0 C.

Reagent Preparation:

Working Reagent: The contents of 1 bottle of L2 (Enzyme Reagent 2) was poured into 1 bottle of L1 (Enzyme reagent 1). This working reagent is stable for at least 8 weeks when stored at 2-8^0 C. Upon storage the working reagent may develop a slight pink colour however this does not affect the performance of the reagent.

Alternatively for flexibility as much of working reagent may be made as and when desired by mixing together 4 parts of L1 (Enzyme Reagent 1) and part 1 of L2 (Enzyme Reagent 2).Alternatively 0.8 ml of L1 and 0.2ml of L2 may also be used instead of 1 ml of the working reagent directly during the assay.

Sample material: Plasma

Procedure

Wavelength/ filter : 505 nm (Hg 546 nm) / Green

Temperature : 37^0C / 25 ^{0}C

Light path : 1 cm

Then pipette into clean dry test tubes labeled as Blank (B), Standard (S) and Test (T):

Additon Sequence	**B (ml)**	**S (ml)**	**T (ml)**
Working reagent	1.0	1.0	1.0
Distilled water	0.01	-	-
Cholesterol Standard(S)		0.01	-
Sample			0.01

Mixed well and incubated at 37^0C for 5 min. or at R.T. (25 ^{0}C) for 15 min. The absorbance of the Standard (Abs. S), and Test Sample (Abs. T) was measured against the Blank, within 60 Min.

Calculations

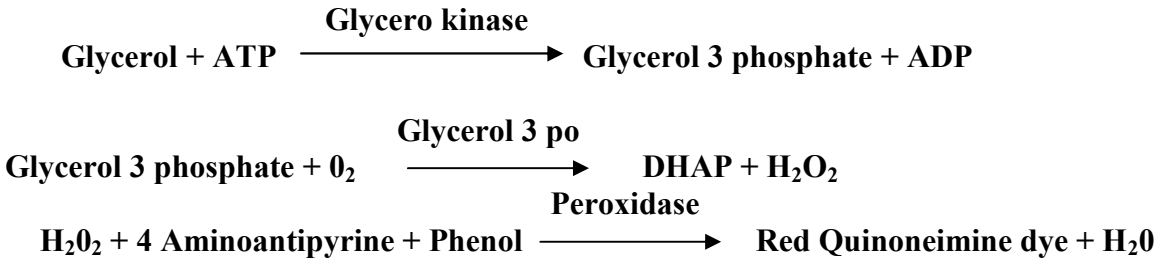
Cholesterol in mg/dl = Abs. T/ Abs. S× 200 mg/dl

4.9.2. Estimation of triglyceride (GPO/ PAP method) (Fossati &Prencipe, 1982)

For the determination of triglyceride in plasma

Principle: Lipoprotein lipase hydrolyses triglycerides to glycerol and fre fatty acids. T he glycerol formed with ATP in the presence of glycerol kinase forms glycerol 3 phosphate which is oxidized by the enzyme glycerol phosphate oxidase to form hydrogen peroxide. T he hydrogen peroxide further reacts with phenolic compound and 4- aminoantipyrine by the catalytic action of peroxidase to for a red coloured quinoneimine dye complex. Intensity of the colour formed is directly proportional to the amount of triglycerides present in the sample.

Triglycerides $\xrightarrow{\textbf{Lipoprotein lipase}}$ **Glycerol + Free fatty acids**



Contents	**75 ml**	**2 X 75ml**	**2 X 150ml**	**2 X 250ml**
L1:Enzyme reagent1	60 ml	2 X 60ml	2 X 120ml	2 X 200ml
L2:Enzyme reagent 2	15 ml	2 X 15ml	2 X 30ml	2 X 50ml
S:Triglyceride Standard (200 mg/ dl)	5 ml	5ml	5ml	5ml

Storage/ stability

Contents are stable at 2 -8^0 C.

Reagent Preparation:

Working Reagent: The contents of 1 bottle of L2 (Enzyme Reagent 2) was poured into 1 bottle of L1 (Enzyme reagent 1). This working reagent is stable for at least 8 weeks when stored at 2-8^0 C. Upon storage the working reagent may develop a slight pink color however this does not affect the performance of the reagent.

Alternatively for flexibility as much of working reagent may be made as and when desired by mixing together 4 parts of L1 (Enzyme Reagent 1) and part 1 of L2 (Enzyme Reagent 2).Alternatively 0.8 ml of L1 and 0.2ml of L2 may also be used instead of 1 ml of the working reagent directly during the assay.

Sample material: Plasma

Procedure

Wavelength/ filter : 505 nm (Hg 546 nm) / Green

Temperature : 37^0C / 25 ^{0}C

Light path : 1 cm

Then pipette into clean dry test tubes labeled as Blank (B), Standard (S) and Test (T):

Additon Sequence	**B (ml)**	**S (ml)**	**T (ml)**
Working reagent	1.0	1.0	1.0
Distilled water	0.01	-	-
Triglyceride Standard(S)		0.01	-
Sample			0.01

Mixed well and incubated at 37^0C for 5 min. or at R.T. (25^0C) for 15 min. The absorbance of the Standard (Abs. S), and Test Sample (Abs. T) was measured against the Blank, within 60 Min.

Calculations

Cholesterol in mg/dl = Abs. T/ Abs. S× 200 mg/dl

4.9.3. Estimation of HDL (PEG Precipitation method) (Grillo *et al*., 1981)

For the determination of HDL in plasma

Principle: When the plasma is reacted with the polyethylene glycol contained in precipitating reagent, all the VLDL and LDL are precipitated. The HDL remains in the supernatant and is then assayed as a sample for cholesterol using the cholesterol reagent.

Contents	75 ml	2 X 75ml	2 X 150ml	2 X 250ml
L1:Precipitating Reagent	60 ml	2 X 60ml	2 X 120ml	2 X 200ml
S:cholesterol Standard (25 mg/ dl)	5 ml	5ml	5ml	5ml

Storage/ stability

Contents are stable at 2 -8^0 C.

Sample material: Plasma

Procedure

Wavelength/ filter : 505 nm (Hg 546 nm) / Green

Temperature : 37^{0}C / 25 ^{0}C

Light path : 1 cm

Then pipette into clean dry test tubes labeled as Blank (B), Standard (S) and Test (T):

Mixed well and incubated at 37^{0}C for 5 min. or at R.T. (25^{0}C) for 15 min. The absorbance of the Standard (Abs. S), and Test Sample (Abs. T) was measured against the Blank, within 60 Min.

Additon Sequence	B (ml)	S (ml)	T (ml)
Working reagent	1.0	1.0	1.0
Distilled water	0.05	-	-
HDL Standard(S)		0.05	-
Sample			0.05

Calculations

HDL Cholesterol in mg/dl = Abs. T/ Abs. S× 25 × 2mg/dl

LDL Cholesterol in mg/dl = (Total cholesterol) – (Triglycerides/5) – (HDL Cholesterol)

4.10. Determination of oxidative stress (lipid peroxidation) and Antioxidant enzymes

4.10.1. Estimation of MDA level (Lipid Peroxidation) (Satoh *et al*., 1978**)**

Principle: This test is a calorimetric test which takes advantage of the ability of hydro peroxide to generate free radicals after reacting with transitional metals, when buffered chromogenic substance is added; a colored complex appeared. This colored complex was measured spectrophotometrically. Lipid peroxidation levels in the testis was measured using thio-barbituric acid reactive substances (TBARS).The testis was then homogenized in ice cold 1X PBS (10%) and the concentration of TBARS was expressed as nmol of MDA per mg protein. The absorbance was read at 540 nm.

Procedure

1. Homogenized tissue in 1X PBS (10%) if weight of tissue is 50 mg, 500µl of PBS was added. (wt of tissue × 10).

2. 500µl of homogenate tissue and PBS amount taken µl of 10% TCA in one eppendorf tube and centrifuged.

3. The supernatant was taken out carefully.

4. The amount of supernatant taken should be known.

5. 0.8%TBA was added in 1(protein):2 (TBA) ratios.

If more pink is observed, it contains more malondialdehyde

6. 1 ml of supernatant and 2 ml of TBA (1:2) was taken.

7. Boiled for 45 min and were observed for the formation of colored complex.

8. Slight pink color is expected to come.

9. O.D. at 540 nm was taken.

4.10.2. Estimation of Superoxide Dismutase (SOD) enzyme activity (Asada *et al*., 1974**)**

Principle: SOD is an enzyme that catalyses dismutation of two superoxide anion into hydrogen peroxide and molecular oxygen.

SOD is one of the most important enzymes in the front line of defense against oxidative stress.

Reagents

1. Phenazenemethosulfate (PMS): 0.06 mg dissolved in 1 ml of distilled water.

2. Nitrobluetetrzolium (NBT): 2.5 mg dissolved in 1 ml of distilled water.

3. NADH: 0.6 mg dissolved in 1 ml of distilled water.

4. Acetic acid

5. n-Butanol

Procedure

Reagents	**Volume**
PMS (Phenazinemethosulfate)	50µl
NBT(Nitrobluetetrzolium)	15µl
NADH	100µl
Sample	50µl
Incubated for 90 sec at 30 ^{0}C, stopped the reaction by adding Acetic acid	500µl
n-Butanol	2ml
The absorbance was taken at 560nm	

Calculation

% inhibition = {Blank-Test/Blank} x 100

50% inhibition = 1 unit

1% inhibition = 1/50

SOD unit = 1/50 × % inhibition

4.10.3. Estimation of Catalase activity (Aebi, 1984)

Principle: The assay is performed as described by Aebi *et al*, (1974). Catalase catalyses the dismutation of H_2O_2 and thus competes with GSHPs for the common substrate. It is considered to be the primary scavenger of intracellular H_2O_2. In the UV range H_2O_2 absorbs maximally at 240nm.Catalase rapidly breaks down H_2O_2 leading to a decrease in absorbance. A difference in the absorbance at 240 nm per unit time is measured of Catalase activity.

Reagents

1.50 nM phosphate buffers, pH 7 was made by mixing A and B in a 1:1.5 v/v ratio.

A) 6.8 g----KH_2PO_4

B) 8.9 g----Na_2HPO_4

2. 30 mM H_2O_2 made by diluting 340µl 30% H_20_2 to 100 ml with Phosphate buffer pH-7.

3. Sample used: 10% (w/v) homogenates of tissue, cell line.

Procedure

Pipette successively into the cuvettes	**Blank**	**Sample**	**Concentration in assay mixture 50 m mol/l**
Phosphate buffer	1 ml		
Sample	2 ml	2 ml	
H2O2	-	1ml	10 m mol/l
The reaction was started by adding H2O2.The O.D was taken at 10 sec intervals up to 30 sec.			

Calculation

$0.23 \times \log A_1/A_2$

A_1 is A_2 at 0 sec

And A_2 is A_2 at15 sec.

4.10.4. Estimation of Glutathione (Rahman *et al*., 2007**).**

Principle: Glutathione is measured by its reaction with DTNB 5, 5 dithio 2-nitobenzoic acid (Ellmans reaction) to give a compound that absorbs light at 412 nm.

Reagents

1.0.2 M Na2HPO4:28.392 g was weighed and dissolved in 1l distilled water. 0.425 mg in 15 ml.

2.10 mM DTNB:39.63 mg DTNB was dissolved in 10 ml of 0.2M Na_2HPO_4. Dithionitrobenzene 4 mg in 1 ml.

Procedure

Reagents	**Blank**	**Test**
Na_2HPO_4	900µl	900 µl
DTNB	20 µl	20 µl
Distilled water	80 µl	-
Sample	-	80 µl
Absorbance taken at 412 nm within 5 min		

4.10.5. Estimation of Glutathione-S-Transferase (GST) (Habig *et al.,* 1974)

Principle: Glutathione-S-Transferase catalyses the reaction of CDNB with the SH group of glutathione.

GSH (reduce) + CDNB ⟶ DNB-S-Glutathione

Reagents

1. Phosphate Buffer: 0.1M, pH 6.5.

a) K_2HPO4---0.1M. (Solution A)

8.709g of $K_2HPO_4$was dissolved in 500 ml of distilled water.

b) KH_2PO_4---0.1 M.(solution B)

6.084g of $KH_2PO_4$was dissolved in 500 ml of distilled water.

164 ml of Solution A was mixed with 336 ml of Solution B and adjust the pH to 6.5.

2. CDNB (F.W=202.6)-20mM.

20.26 mg of CDNB was made upto 5 ml in 95% ethanol. Stored in refrigerator.

3. GSH (F.W=307.3)-20mM.

3.073 mg of reduced GSH was dissolved in 0.5 ml of distilled water. It was prepared fresh.

4. Sample was then added.

Procedure

Reagents	**Volume**
Distilled water	4.4 ml
Phosphate buffer	250µl
CDNB	50µl
Incubate at 37^0C for 10 mins	
GSH	250µl
Extract	50µl
Read absorbance at 340 nm at 1 min interval	

For blank distilled water was taken.

Calculation

$$\text{GST activity} = \frac{\text{Absorbance of test-absorbance of blank} \times 1000 \text{IU/L}}{9.6 \times \text{vol. of test } (\mu l)}$$

(Where 9.6 is the molar extinction coefficient of GST)

4.11. Evaluation of reproductive hormones (testosterone, estradiol, leutinizing hormone and follicle stimulating hormone)

4.11.1. Estimation of testosterone concentration in plasma (Joshi, 1979**)**

Principle: Testosterone in the blood is bound to SHBG (60%) and in lower quantity to other proteins (for example albumin); the unbound Testosterone (< 1% of total Testosterone) is known as "free Testosterone". The chemical formulation of this assay allows to release completely the Testosterone from bound proteins; thus Diametra Testosterone kit allows to measure the concentration of total Testosterone (bound + free) in the sample. For the measurement of free Testosterone only, ELISA Diametra "Free Testosterone" kit is available. Testosterone (antigen) in the sample competes with the antigenic Testosterone conjugated with horseradish peroxidase (HRP) present in the Conjugate for binding to the antibodies antitestosterone coated on the microplates (solid phase). After the incubation, the bound/free separation is performed by a simple solid-phase washing. The enzyme HRP in the bound-fraction reacts with the Substrate (H2O2) and the TMB Substrate and develops a blu color that changes into yellow when the Stop Solution (H2SO4) is added. The colour intensity is inversely proportional to the Testosterone concentration in the sample. Testosterone concentration in the sample is calculated through a calibration curve.

Reagents

Calibrators (CAL0, CAL1, CAL2, CAL3, CAL4), Control**,** Conjugate (Testosterone conjugated with horseradish peroxidase (HRP)), Coated Microplate , Anti Testosterone antibody adsorbed

on microplate, TMB Substrate (H2O2-TMB 0.26 g/L),Stop Solution (Sulphuric acid 0.15 mol/L),10X Conc. Wash Solution (Phosphate buffer 0.2M, Proclin < 0.0015%).

Procedure

Reagent	Calibrator	Sample/ Control	Blank
Sample/ Control		25 µL	
Calibrator C0-C4	25 µL		
Conjugate	100 µL	100 µL	
Incubate at 37°C for 1 hour. Remove the content from each well. Wash the wells 3 times with 300 µL of diluted wash solution.			
TMB Substrate	100 µL	100 µL	100 µL
Incubate at room temperature (22-28°C) for 15 minutes in the dark.			
Stop Solution	100 µL	100 µL	100 µL
Shake the micro plate gently. Read the absorbance (E) at 450 nm against a reference wavelength of 620-630 nm or against Blank within 5 minutes.			

Calculation: Interpolate the values of the samples on the calibration curve to obtain the corresponding values of the concentrations expressed in ng/mL.

4.11.2. Estimation of estradiol concentration in plasma (Joshi, 1979**)**

Principle: 17β-Estradiol (antigen) in the sample competes with the antigenic 17β-Estradiol conjugated with horseradish peroxidase (HRP) for binding to the limited number of antibodies anti 17β-Estradiol coated on the microplate (solid phase). After incubation, the bound/free separation is performed by a simple solid-phase washing. Then the enzyme HRP in the bound-fraction reacts with the Substrate (H2O2) and the TMB Substrate and develops a blu color that changes into yellow when the Stop Solution (H2SO4) is added. The colour intensity is inversely

proportional to the 17β-Estradiol concentration in the sample. 17β-Estradiol concentration in the sample is calculated through a calibration curve.

Reagents: Calibrators (CAL0, CAL1, CAL2, CAL3, CAL4, CAL5), Control**,** Conjugate (Estradiol conjugated with horseradish peroxidase (HRP)), Coated Microplate , Anti Estradiol antibody adsorbed on microplate, TMB Substrate (H2O2-TMB 0.26 g/L), Stop Solution (Sulphuric acid 0.15 mol/L),10X Conc. Wash Solution (Phosphate buffer 0.2M, Proclin < 0.0015%).

Procedure

Reagent	**Calibrator**	**Sample/ Control**	**Blank**
Sample/ Control		25 µL	
Calibrator C0-C4	25 µL		
Conjugate	200 µL	200 µL	
Incubate at 37°C for 2 hour. Remove the content from each well. Wash the wells 3 times with 300 µL of diluted wash solution.			
TMB Substrate	100 µL	100 µL	100 µL
Incubate at room temperature (22-28°C) for 15 minutes in the dark.			
Stop Solution	100 µL	100 µL	100 µL
Shake the micro plate gently. Read the absorbance (E) at 450 nm against a reference wavelength of 620-630 nm or against Blank within 5 minutes.			

Calculation: Interpolate the values of the samples on the calibration curve to obtain the corresponding values of the concentrations expressed in pg/mL.

4.11.3. Estimation of LH concentration in plasma (Kosasa, 1981)

Principle: In this method, the LH calibrators, the patient specimens and/or the controls (containing the native antigen) are first added to streptavidin coated wells. Then monoclonal biotinylated and enzyme labeled antibodies are added and the reactants mixed: these antibodies have high affinity and specificity and are directed against distinct and different epitopes of LH. A reaction between the various LH antibodies and native LH occurs in the microwells without competition or steric hindrance forming a soluble sandwich complex. The interaction is illustrated in the following equation:

$$\text{EnzAb} + \text{AgLH} + \text{BtnAb(m)} \underset{\text{k-a}}{\overset{\text{ka}}{\leftrightarrow}} \text{EnzAb - AgLH-BtnAb(m)}$$

BtnAb(m) = biotinylated monoclonal antibody (excess quantity)

AgLH = native antigen (variable quantity)

EnzAb = enzyme labeled antibody (excess quantity)

EnzAb-AgLH-BtnAb(m) = antigen-antibodies sandwich complex

ka = rate constant of association

k-a = rate constant of dissociation

Simultaneously, the complex is deposited to the well through the high affinity reaction of streptavidin and biotinylated antibody. This interaction is illustrated below:

$$\text{EnzAb -AgLH-BtnAb(m)} + \text{StreptavidinC.W.} \Rightarrow \text{Immobilized complex}$$

Streptavidin C.W. = streptavidin immobolized on well.

Immobilized complex = antibodies-antigen sandwich bound.

After equilibrium is attained, the antibody-bound fraction is separated from unbound antigen by a washing step. The enzyme activity in the antibody bound fraction is directly proportional to the native antigen concentration. The activity of the enzyme present on the surface of the well quantitated by reaction with a suitable substrate to produce colour. By utilizing several different calibrators of known antigen values, a dose response curve can be generated from which the antigen concentration of an unknown can be ascertained.

Reagents: Calibrators (CAL0, CAL1, CAL2, CAL3, CAL4, CAL5), Control**,** Conjugate (LH conjugated with strepavidin), Coated Microplate , Anti LH antibody adsorbed on microplate, TMB Substrate (H2O2-TMB 0.26 g/L),Stop Solution (Sulphuric acid 0.15 mol/L),10X Conc. Wash Solution (Phosphate buffer 0.2M, Proclin < 0.0015%).

Procedure

Reagent	**Calibrator**	**Sample/ Control**	**Blank**
Sample/ Control		20 µL	
Calibrator C0-C4	20 µL		
Conjugate	100 µL	100 µL	
Incubate at 37°C for 1 hour. Remove the content from each well. Wash the wells 3 times with 300 µL of diluted wash solution.			
TMB Substrate	100 µL	100 µL	100 µL
Incubate at room temperature (22-28°C) for 15 minutes in the dark.			
Stop Solution	100 µL	100 µL	100 µL
Shake the micro plate gently. Read the absorbance (E) at 450 nm against a reference wavelength of 620-630 nm or against Blank within 5 minutes.			

Calculation: Interpolate the values of the samples on the calibration curve to obtain the corresponding values of the concentrations expressed in mIU/mL.

4.11.4. Estimation of FSH concentration in plasma (Odell *et al.,* 1981**)**

Principle

The essential reagents required for an immunoenzymatic assay include high affinity and specificity antibodies (enzyme-linked and immobilised) with different and distinct epitope recognition, in excess, and native antigen. In this procedure the immobilization takes place during the assay at the surface of a microplate well through the interaction of streptavidin coated on the well and exogenously added biotinylated monoclonal anti FSH antibody. Upon mixing monoclonal biotinylated antibody, the enzyme labeled antibody and a serum containing the native antigen, reation results between the native antigen and the antibodies without competition or steric hindrance to form a soluble sandwich complex. The interaction is illustrated by the following equation:

$$\text{EnzAb(p)} + \text{AgFSH} + \text{BtnAb(m)} \underset{\text{K-a}}{\overset{\text{Ka}}{\rightleftharpoons}} \text{EnzAb(p)- AgFSH- BtnAb(m)}$$

BtnAb(m) = biotinylated monoclonal antibody (Excess quantity)

AgFSH = native FSH antigen (variable quantity)

EnzAb(p) = enzyme labeled policlonal antibody (Excess quantity)

EnzAb(p)- AgFSH- BtnAb(m) = antigen-antibodies sandwich complex

Ka = rate constant of association

K-a = rate constant of disassociation

Simultaneously the complex is deposited to the well through the high affinity reaction of streptavidin and biotinylated antibody. This interaction is illustrated below:

EnzAb(p)-AgFSH-BtnAb(m) + streptavidincw $\rightarrow$ immobilized complex

Streptavidincw = streptavidin immobilized on well Immobilized complex = antibodies-antigen sandwich bound. After equilibrium is attained, the antibody-bound fraction is separated from unbound antigen by a washing step. The enzyme activity in the antibody-bound fraction is directly proportional to the native antigen concentration. By using several different serum references of known antigen values, a dose response curve can begenerated from which the antigen concentration of an unknown can be ascertained.

Reagents: Calibrators (CAL0, CAL1, CAL2, CAL3, CAL4, CAL5), Control**,** Conjugate (FSH conjugated with HRP), Coated Microplate , Anti FSH antibody adsorbed on microplate, TMB Substrate (H2O2-TMB 0.26 g/L),Stop Solution (Sulphuric acid 0.15 mol/L),10X Conc. Wash Solution (Phosphate buffer 0.2M, Proclin < 0.0015%).

Procedure

Reagent	**Calibrator**	**Sample/ Control**	**Blank**
Sample/ Control		50 µL	
Calibrator C0-C4	50 µL		
Conjugate	100 µL	100 µL	
Incubate at 37°C for 1 hour. Remove the content from each well. Wash the wells 3 times with 300 µL of diluted wash solution.			
TMB Substrate	100 µL	100 µL	100 µL
Incubate at room temperature (22-28°C) for 15 minutes in the dark.			
Stop Solution	100 µL	100 µL	100 µL
Shake the micro plate gently. Read the absorbance (E) at 450 nm against a reference wavelength of 620-630 nm or against Blank within 5 minutes.			

Calculation: Interpolate the values of the samples on the calibration curve to obtain the corresponding values of the concentrations expressed in mIU/mL.

4.12. Estimation of cotinine concentration in plasma (Mamsen *et al.,* 2010**)**

Principle

Tobacco contains nicotine which metabolizes to form a toxic alkaloid i.e. cotinine. It stimulates autonomic ganglia and central nervous system. Cotinine is the best indicator of tobacco smoke exposure.

Reagents

Microwell coated with polyclonal Ab to cotinine	12×8×1
Standard Set (ready to use)	0.5 ml
Cotinine HRP Enzyme Conjugate (ready to use)	12 ml
TMB Substrate (ready to use)	12 ml
Stop Solution (ready to use)	12 ml

Procedure

All reagents must be brought to room temperature (18-26° C) before use.

1. Pipette 10 µl of standards, controls and specimens into selected well in duplicate.

2. Add 100 µl of the Enzyme Conjugate to each well. Shake the plate, 10-30 seconds, to ensure proper mixing.

3. Incubate for 60 minutes at room temperature (18-26° C) preferably in the dark.

4. Wash the wells 6 times with 300 µl distilled water using either a suitable plate washer or wash bottle taking care not to cross contaminate wells.

5. Invert wells and vigorously slap dry on absorbent paper to ensure all residual moisture is removed. This step is critical to ensure that residual enzyme conjugate, does not skew results. If using an automated system, ensure that the final aspiration on the wash cycle aspirates from either side of the well.

6. Add 100 µl of Substrate reagent to each well.

7. Incubate for 30 minutes at room temperature, preferably in the dark.

8. Add 100 µl of Stop Solution to each well. Shake the plate gently to mix the solution.

9. Read absorbance on ELISA Reader at 450nm within 15 minutes after adding the stopping solution.

Calculation: Interpolate the values of the samples on the calibration curve to obtain the corresponding values of the concentrations expressed in ng/mL.

4.13. Isolation of PBMC

PBMC were immediately isolated from fresh whole blood by density gradient centrifugation according to the method of Boyun *et al*., 2002 using equal volume of blood and Ficoll (Histo-paque 1077). Briefly, 5 ml blood was layered carefully over equal volume of Histopaque 1077 and subjected to centrifugation for 30 min at 400 × g. PBMC were collected from the buffy layer formed at the plasma‐ Histopaque 1077 interface and the pellet was re-suspended in PBS (50 mM, pH 7.4). This process was repeated twice or thrice to remove the extraneous platelets.

4.14. Estimation of protein (Lowry *et al*., 1951**)**

Principle: The principle behind the Lowry method of determining protein concentrations lies in the reactivity of the peptide nitrogen[s] with the copper [II] ions under alkaline conditions and the subsequent reduction of the Folin Ciocalteau phosphomolybdic phosphotungstic acid to hetero polymolybdenum blue by the copper-catalyzed oxidation of aromatic acid.

The Lowry method is sensitive to pH changes and therefore the pH of assay solution should be maintained at 10 - 10.5. The Lowry method is sensitive to low concentrations of protein. Dunn (1992) suggests concentrations ranging from 0.10 - 2 mg of protein per ml while Price (1996) suggests concentrations of 0.005 - 0.10 mg of protein per ml. The major disadvantage of the Lowry method is the narrow pH range within which it is accurate. However, we have used very small volumes of sample, which have little or no effect on pH of the reaction mixture. A variety of compounds had interfered with the Lowry procedure. These include some amino acid derivatives, certain buffers, drugs, lipids, sugars, salts, nucleic acids and sulphydryl reagents (Dunn, 1992).

Price (1996) notes that ammonium ions, zwitter ionic buffers, non-ionic buffers and thiol compounds may also interfere with the Lowry reaction. These substances should be removed or diluted before running Lowry assays.

Reagents

A) 4 g of Na_2CO_3 +4 g of NaoH dissolved in 100 ml.

B) i) 53 mg of sodium potassium tartarate dissolved in 500 µl of distilled water.

ii) 200 mg of $CuSO_4$ dissolved in 500 µl of distilled water.

50 ml of reagents A and 1 ml of reagent B were mixed.(analytical reagent)

C) Lowry's reagent---Folin (2 ml in each sample).

Procedure

1. 990 µl of distilled water was added in each test tube.
2. 2 ml of analytical reagent was added in each test tube.
3. 10µl of sample was added to each test tube.
4. It was then incubated in dark for 40 min
5. 500 µl of Folin reagent was added to each test tube.

6. Then mixed and incubated for 20 min.
7. The O.D was taken at 750 nm.
8. Blank was set at 0.00
9. Protein concentration of the tissues was calculated based on the standard graph using bovine serum albumin.

4.15. Western blot analysis

For western blotting analysis, 5 samples were selected randomly from each groups, non-users, smokers, chewers and combinations (N= 5/each group).Immunblots were performed from cell lysate to analyze the expression of different apoptotic markers, such as, Bax, Bcl-2 and the level of different pro-inflammatory cytokines, such as, TNF-α , Cox-2 and HSP-70. β –actin was used as loading control respectively. Concentration of proteins was determined by the protocol of Lowry et al.,1951 Equal amount of protein (50 μ g) was loaded on each lane followed by separation using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted in PVDF membrane (Millipore, Massachusetts, USA). The membrane was blocked for 1.0 h at 37 °C with 5% bovine serum albumin (BSA) solution. Then the membrane was incubated with anti-rabbit polyclonal antibody (1:1000) for overnight at 4 °C, followed by incubation with alkaline conjugated anti rabbit antibody (1:2500) for 4.0 h. After washing, the membrane was developed using a ECL (BIO-RAD, Cat. # 170-5060, USA). For each result three independent set of western blot experiments were performed for different subjects (Burnette, 1981). For each result three independent set of western blot experiments were performed for different subjects. The current result is the representative of one western blot data. All western blots were performed under the same experimental conditions. The densitometry analysis was done by using a Image J software, NIH.

4.16. Isolation of DNA

Lymphocytes from whole blood were separated by lysing the red blood cells (RBCs) using a hypotonic buffer (ammonium bicarbonate and ammonium chloride; Himedia) with minimal lysing effect on lymphocytes. Three volumes of RBC lysis buffer was added to blood sample and mixed by vortexing and inverting thoroughly for 5 min and centrifuged (Eppendorf 5415R) at 20,00 g for 10 min. The supernatant was mostly discarded, leaving behind 1ml to prevent loss of cells. To the pellet, 3 vol RBC lysis buffer was added, and vortexing, inverting, and centrifuging steps were repeated two to three times until a clear supernatant and a clean white pellet were obtained. After the final wash, the supernatant was discarded completely, and the pellet was resuspended in 500 _l PBS, followed by addition of 400 µl cell lysis buffer (10 mM Tris-HCl, 10 mM EDTA, 50 mM NaCl, 10% SDS, pH 7.5) and 10 µl proteinase K (10 mg/ml stock; Himedia). The sample was vortexed to dissolve the pellet completely and incubated for 2 h at 56°C in a water bath (CW-30G; Jeio Tech) for lysis. An equal volume of phenol (equilibrated with Tris, pH 8) was subsequently added to the tube and mixed well by inverting for 1 min. The tube was centrifuged at 10,000 g (at 4°C) for 10 min, and the aqueous upper layer was transferred to a fresh tube containing equal volumes (1:1) of phenol and chloroform: isoamyl alcohol (24:1). The tube was mixed by inverting for 1 min and centrifuged for 10 min at 10,000 g (at 4°C). The supernatant was then transferred to a fresh tube, and 10 µl of 10 mg/ml RNase A (Fermentas, Thermo Scientific) was added. The sample was incubated at 37°C for 30 min before an equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed by inverting the tube for 1 min and centrifuging at 10,000 g (at 4°C) for 10 min. The supernatant was transferred to a fresh tube, and twice the volume of absolute alcohol (Merck) was added and inverted gently a few times and chilled at 20°C, followed by centrifugation at 10,000 g at (4°C) for 20 min. The supernatant was discarded, 250 µl 70% ethanol was added, and the pellet was tapped gently,

followed by centrifugation at 10,000 rpm for 10 min and decanting the supernatant gently. The pellet was air-dried in a laminar air flow, and the dried pellet was resuspended in 50 µl nuclease-free water or 1µlTE buffer and frozen at 20°C or 80°C for storage (Sambrook *et al.,* 1989).

4.17. Genotyping of GST M1/T1/P1 Gene Polymorphisms

GSTM1 and GSTT1 null polymorphisms were detected by multiplex polymerase chain reaction (PCR) (Table. 1) (Arand *et al*., 1996). The reaction mixture (10 µL) contained 50–100 ng of genomic DNA in 1X Taq buffer, 200 µmol/L of each dNTP, 0.15 µmol/L of each primer, and 1 U of Taq DNA polymerase. Amplified products were analyzed by electrophoresis on 12% polyacrylamide gels resulting in a 219-bp fragment for GSTM1, 459-bp fragment for GSTT1, and a 349-bp fragment of the albumin gene (as an internal control). The absence of the specific GSTM1 and/or GSTT1 fragments indicated the corresponding null genotype, whereas the presence of the 349 bp albumin band ensured that the null genotype was not documented due to failure of PCR. In addition, two SNPs in the GSTP1 gene for amino acid substitution at codons 105 (Ile?Val) and 114 (Val? Ala) were genotyped by PCR-RFLP method (Table. 1) (Ali-Osman *et al*., 1997; Correa, 2002; Garcıa *et al*., 2012). The fragment containing the GSTP1 Ile105- Val (rs1695) polymorphic site was amplified according to the following parameters: 94 °C for 5 minutes, followed by 35 cycles at 94 °C for 30 seconds, 58 °C for 30 seconds, 72 °C for 1 minutes, and a final elongation at 72 °C for 7 minutes. Ten µL of PCR product was digested with 1 unit of BsmAI (New England Biolabs, Barcelona, Spain) for 3 hour at 55 °C. Digests were electrophoresed on 12% polyacrylamide gels resulting in three fragments of 305, 135, and 128 bp (allele A) or in four fragments of 222, 135, 128, and 83 bp (allele G). PCR cycling conditions were 5 minutes at 94 °C followed by 35 cycles at 94 °C for 30 seconds, 52 °C for 30 seconds, and 72 °C for 30 seconds with a final elongation step at 72 °C for 7 minutes for GSTP1

Val114Ala (rs1138272) polymorphism. Ten µL of PCR products was digested with 1 unit of AciI (New England Biolabs, Barcelona, Spain) for 3 hour at 37 °C and electrophoresed on 12% polyacrylamide gel. The T allele as defined by the presence of an intact fragment of 170 bp and the C allele by the presence of two fragments of 143 and 27 bp.

Table 1: Primers tested for polymorphism pattern in the present study.

Gene	Primer 5'-3'	Annealing Temp.	Type of polymorpm	Amplified products/Alleles
GSTM1	F:GAACTCCCTGAAAAGCTAAAGC R:GTTGGGCTCAAATATACGGTGG	60 °C–1 minutes	Gene deletion	Nullgenotype + 219 bp
GSTT1	F: TTCCTTACTGGTCCTCACATCTC R: TCACCGGATCATGGCCAGCA	60°C–1 minutes	Gene deletion	Null genotype+ 459 bp
Albumin	F: GCCCTCTGCTAACAAGTCCTAC R:CCCTAAAAAGAAAATCGCCAAC		-	Internal control 349 bp
GSTP1 I105V (rs1695)	F: AATACCATCCTGCGTCACCT R: TGAGGGCACAAGAAGCCCCTT	59 °C–40 seconds	BsmAI RFLP	A - Digested fragment (308, 258 bp) G - Digested fragment (258, 219and 89 bp)
GSTP1V114A (rs1138272	F: ACAGGATTTGGTACTAGCCT R:AGTGCCTTCACATAGTCATCCTTG	50 °C–40 seconds	AciI RFLP	C - Digested fragment (143, 27 bp) T - Intact fragment (170 bp)

4.18. Statistical analysis

Data was collected, arranged and reported as mean ± standard deviation (SD) of four groups, and was analyzed using the statistical programme for social science (SPSS/version15.0). The statistical method was one way analyzes of variance ANOVA test, and if significant differences between means will be found, Duncan's multiple range test (Whose significant level was defined as $p < 0.05$) according to (Snedecor and Cochran, 1982) to estimate the effect of different groups.

CHAPTER-5

RESULTS

5.1. Analysis of hematology parameters

A significant increase in the levels of leukocyte count, Neutrophil count, platelet count, erythrocyte sedimentation rate (ESR), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) in tobacco user groups (smokers, SLT users as well as smokers & SLT users) compared to the non-user group ($p < 0.05$). However hemoglobin, erythrocyte count, lymphocyte count, Monocyte count, Eosinophil count, packed cell volume (PCV), mean corpuscular volume (MCV) levels were decreased significantly in tobacco user groups (smokers, SLT users as well as smokers & SLT users) compared to the non-user group ($p < 0.05$) (Table.2).

5.2. Evaluation of biochemical parameters (ALT, AST, ALP, LDH, Creatinine and Urea)

The plasma levels of four markers ALP, AST, ALT, and LDH were compared in tobacco user groups (smokers, SLT users as well as smokers & SLT users) and to the non-user group (Mean ± SD). ALP, AST, ALT, and LDH levels increased as daily tobacco amount increased. Non-user group ALP, AST, ALT, and LDH levels were (8.41 ± 3.32), (29.70 ± 12.03), (20.30 ± 7.32) and (352.31 ±18.33) respectively. Smoker were (25.10 ± 9.59), (34.60 ± 15.51), (26.53 ± 8.99) and (473.97 ± 16.44) respectively, SLT users were (37.07 ± 17.55), (34.47 ± 9.66), (527.32 ±15.88) and (3.17 ± 1.09) respectively where as smoker and SLT users were (37.99 ± 17.29), 39.60 ± 19.55), (44.88 ± 13.72) and (4.57 ± 2.05) respectively. There was a significant differences in ALP, AST, ALT, and LDH activities in tobacco user groups and non- user group ($p < 0.05$). ALP, AST, ALT, and LDH levels were increased significantly in tobacco user groups with compared to non-user group (Table.3).

Plasma levels of creatinine, urea, and urea nitrogen in non- user groups and tobacco user groups (smokers, SLT users as well as smokers & SLT users) shown in Table. 3. A significant ($p < 0.05$) increases in plasma creatinine, urea and urea nitrogen levels in the tobacco user groups

(smokers, SLT users as well as smokers & SLT users) than non-user group. In tobacco user groups creatinine levels [smokers (1.89 ± 1.10), SLT users (3.17 ± 1.09), smokers and SLT users (4.57 ± 2.05)] and non-users creatinine levels (0.80 ± 0.04) respectively. The mean value of urea in tobacco user groups [smokers (29.70 ± 9.03), SLT users (34.76±10.48), smokers and SLT users (38.83 ± 12.55)] and non-users urea levels (28.05 ± 8.21) respectively.

Table 3: Biochemical parameters, [hepatic (liver) enzymes (ALP, AST, ALT & LDH) and renal (kidney) parameters (creatinine, urea & urea N2)] in non-users, smokers, SLT users and smokers and SLT users.

Parameters	Non users	Smokers	SLT users	Smokers and SLT users	P value	$F_{(3,299)}$ value
ALP (K.A)	8.41 ± 3.32a	25.10 ± 9.59b	33.04 ± 14.58c	37.99 ± 17.29d	0.0001	173.34
AST (U/mL)	29.70 ± 12.03a	34.60 ± 15.51b	37.07 ± 17.55c	39.60 ± 19.55d	0.0001	434.55
ALT (U/mL)	20.30 ± 7.32a	26.53 ± 8.99b	34.47 ± 9.66c	44.88 ± 13.72d	0.0001	188.27
LDH (U/L)	352.31 ±18.33a	473.97 ± 16.44b	527.32 ±15.88c	549.58 ± 17.31d	0.0001	2016.40
CREATININE (mg %)	0.80 ± 0.04a	1.89 ± 1.10b	3.17 ± 1.09c	4.57 ± 2.05d	0.0001	226.57
UREA (mg/dL)	28.05 ± 8.21a	29.70 ± 9.03b	34.76±10.48c	38.83 ± 12.55d	0.0001	51.63
UREA N_2 (mg/dL)	13.04 ± 3.58a	14.81 ± 4.50b	16.17 ± 6.27c	18.07 ± 9.31d	0.0001	68.88

Data are expressed in Mean ± SD, N=75/each group; $p < 0.05$ indicates a significant difference between the compared groups. Different letters indicates significant values among groups.

5.3. Estimation of lipid profiles (TC, TG, HDL, LDL & VLDL)

The present study comprises of comparison of lipid profile of 75 healthy male smokers, 75 male SLT users and 75 male SLT and smokers with 75 healthy male non-users. TC, TG, LDL-C, VLDL-C, non-HDL-C, apoB, TC/HDL-C, TG/HDL-C, LDL-C/HDL-C, non-HDL-C/HDL-C, were significantly higher in tobacco user groups [(smokers, SLT users as well as smokers & SLT

users)], whereas HDL-C was significantly lower in tobacco user groups [(smokers, SLT users as well as smokers & SLT users)] compared with non-users ($p < 0.05$) (Table. 4).

Table 2: Hematological parameters, like Haemoglobin , Erythrocytic Count, Leucocytic Count, Neutrophil Count, Lymphocyte Count, Monocyte Count, Eosinophil Count, Platelet Count , ESR, PCV, Mean corpuscular volume, MCH, MCHC, TOC M, Haematocrit findings in non-users, smokers, SLT users and smokers and SLT users.

Parameters	Non users	Smokers	SLT users	Smokers and SLT users	P value	$F_{(3,299)}$ value
Haemoglobin (gm %)	13.25 ± 2.38a	10.45 ± 2.14b	8.76 ± 1.96c	6.45 ± 1.54d	0.0001	149.53
Erythrocytic Count (millions/cu.mm)	4.90± 1.09a	4.25 ± 1.05b	3.89 ± 1.06c	3.17 ± 1.08d	0.0001	34.11
Leucocytic Count (mm^3)	7457.12 ± 1476.58a	7988.17 ± 1654.31b	8457.36 ± 1718.47c	8976.38 ± 1898.68d	0.0001	11.01
Neutrophil Count (%)	64.35 ± 9.48a	71.35 ± 9.87b	84.87 ± 10.24c	88.45 ± 10.87d	0.0001	93.76
Lymphocyte Count (%)	40.18 ± 4.98a	31.87 ± 3.98b	24.68 ± 2.84c	18.35 ± 2.41d	0.0001	486.30
Monocyte Count (%)	0.17 ± 0.08a	0.12 ± 0.09b	0.10 ± 0.04c	0.07 ± 0.03d	0.0001	31.17
Eosinophil Count (%)	0.34 ± 0.17a	0.28 ± 0.13b	0.21 ± 0.15c	0.14 ± 0.11d	0.0001	27.95
Platelet Count (lakhs/mm^3)	0.47 ± 0.18a	0.54 ± 0.12b	0.61 ± 0.35c	0.68 ± 0.27d	0.0001	10.11
ESR (mm)	1.18 ± 0.47a	1.28 ± 0.35b	1.34 ± 0.29c	1.42 ± 0.24d	0.0001	6.32
PCV (%)	42.89 ± 4.25a	34.74 ± 3.45b	28.65 ± 2.89c	21.87 ± 2.18d	0.0001	557.08
Mean corpuscular volume (fL)	83.78 ± 8.95a	74.56 ± 7.81b	66.54± 6.97c	59.87 ± 5.97d	0.0001	141.86
MCH (pg)	25.94 ± 5.41a	27.85 ± 5.7 8b	30.17 ± 6.11c	32.54 ± 4.87d	0.0001	19.82
MCHC (%)	28.98 ± 4.87a	32.47 ± 3.74b	37.48 ± 3.78c	41.45 ± 4.17d	0.0001	130.23
TOC M	740.78 ± 71.85a	785.48 ± 78.59b	865.02± 82.35c	917.85 ± 91.48d	0.0001	71.18
Haematocrit (%)	51.22± 5.89a	45.77 ± 4.52b	38.28 ± 3.98c	30.41 ± 3.41d	0.0001	297.90

Data are expressed in Mean ± SD, N=75/each group; $p < 0.05$ indicates a significant difference between the compared groups. Different letters indicates significant values among groups.

Table 4: Plasma lipid profile in non-users, smokers, SLT users and smokers and SLT users.

Parameters	Non users	smokers	SLT users	Smoker and SLT users	P value	$F_{(3,299)}$ value
Triglycerides (mg/dL)	116.92 ± 13.83a	181.08 ± 11.51b	351.59 ± 14.09c	460.55 ± 14.55d	0.0001	477.89
HDL (mg/dL)	84.70 ± 5.96a	47.27 ± 3.86b	35.19 ± 3.44c	26.51 ± 2.22d	0.0001	2936.20
LDL (mg/dL)	75.22 ± 7.32a	180.59 ± 18.24b	192.76 ± 19.05c	213.10 ± 21.66d	0.0001	934.64
Total cholesterol (mg/dL)	183.34 ± 18.01a	265.44 ± 26.95b	296.44 ± 29.17c	333.30 ± 33.90d	0.0001	401.09
VLDC (mg/dL)	23.41 ± 8.77a	35.96 ± 9.38b	68.98 ± 11.55c	90.76 ± 13.61d	0.0001	586.20
LDL/HDL (mg/dL)	0.89 ± 0.31a	3.88 ± 1.07b	5.50 ± 2.11c	8.44 ± 3.21d	0.0001	186.37
HDL/LDL (mg/dL)	1.14 ± 0.12a	0.27 ± 0.11b	0.19 ± 0.13c	0.13 ± 0.09d	0.0001	1315.10
TC/HDL (mg/dL)	2.17 ± 0.42a	5.65 ± 2.08b	10.34 ± 4.77c	13.04 ± 6.28d	0.0001	105.30
TG/HDL (mg/dL)	1.38 ± 0.54a	3.85 ± 1.06b	9.80 ± 4.22c	17.96 ± 7.40d	0.0001	220.65
HDL/TG (mg/dL)	0.75 ± 0.32a	0.26 ± 0.06b	0.10 ± 0.02c	0.06 ± 0.03d	0.0001	280.96
NONHDL (mg/dL)	98.63 ± 9.58a	216.56 ± 21.48b	263.03 ± 26.26c	303.86 ± 30.94d	0.0001	1073.90
NONHDL/HDL (mg/dL)	1.17 ± 0.62a	4.65 ± 1.08b	7.45 ± 2.16c	12.04 ± 5.28d	0.0001	185.68
HDL/TC (mg/dL)	0.46 ± 0.18a	0.18 ± 0.06b	2.40 ± 1.28c	0.08 ± 0.02d	0.0001	213.57
APOB (mg/dL)	68.73 ± 6.47a	174.77 ± 17.22b	231.85 ± 23.91c	278.91 ± 27.83d	0.0001	1459.50
LDL/APOB (mg/dL)	1.10 ± 0.41a	1.03 ± 0.06b	0.82 ± 0.50c	0.76 ± 0.30d	0.0001	15.61

Data are expressed in Mean ± SD, N=75/each group; $p < 0.05$ indicates a significant difference between the compared groups. Different letters indicates significant values among groups.

5.4. Determination of oxidative stress (lipid peroxidation) levels

Evaluation of extent of oxidative stress levels in the plasma .Assessment of generation of lipid peroxidation radicals (nmol MDA/mg protein), of non users, chewers, and smokers combinations (smokers and SLT users) in tobacco consuming mizo population, respectively. Data are presented as mean ± standard deviation (n = 75 /group). Statistical comparison was performed using one-way ANOVA followed by Tukey's post-hoc tests for all pair-wise multiple comparisons. Bar with different letter indicate that case groups are significantly different at $p < 0.05$. Non tobacco users group (individuals do not consume any form of tobacco or who have not used tobacco products for at least past one year); chewers (SLT users) group (individuals chewing tobacco products (Zarda, gutkha, sadha and khuva) habitually, at least > 20 times per week for the last 6 months); smokers group (frequency of smoking > 20 cigarettes per day); combinations (smokers and SLT users group) (individuals consume smokeless tobacco products as well as smoking), respectively. When lipid peroxidation (nmol/mg of protein, MDA) concentration was compared between tobacco user groups and non-users, significant increase was observed in tobacco user groups (smokers, chewers and combinations). The mean ± S.D values in tobacco user groups [(chewers, (8.92 ± 2.73), smokers, (4.76 ± 1.83), and combination (13.01 ± 4.17)] with compared to non-users (2.71 ± 1.78) respectively (Fig.3).

5.5. Determination of antioxidant enzyme levels in plasma

5.5.1. Determination of superoxide dismutase (U/mg of protein, SOD) levels in plasma

Evaluation of superoxide dismutase (SOD, U/mg protein) enzyme levels in the plasma of non users, chewers, smokers and combinations (smokers and SLT users) in tobacco consuming mizo population, respectively. Data are presented as mean ± standard deviation (n = 75 /group). Statistical comparison was performed using one-way ANOVA followed by Tukey's post-hoc tests for all pair-wise multiple comparisons. Bar with different letter indicate that case groups are

significantly different at $p < 0.05$. Non tobacco users group (individuals do not consume any form)

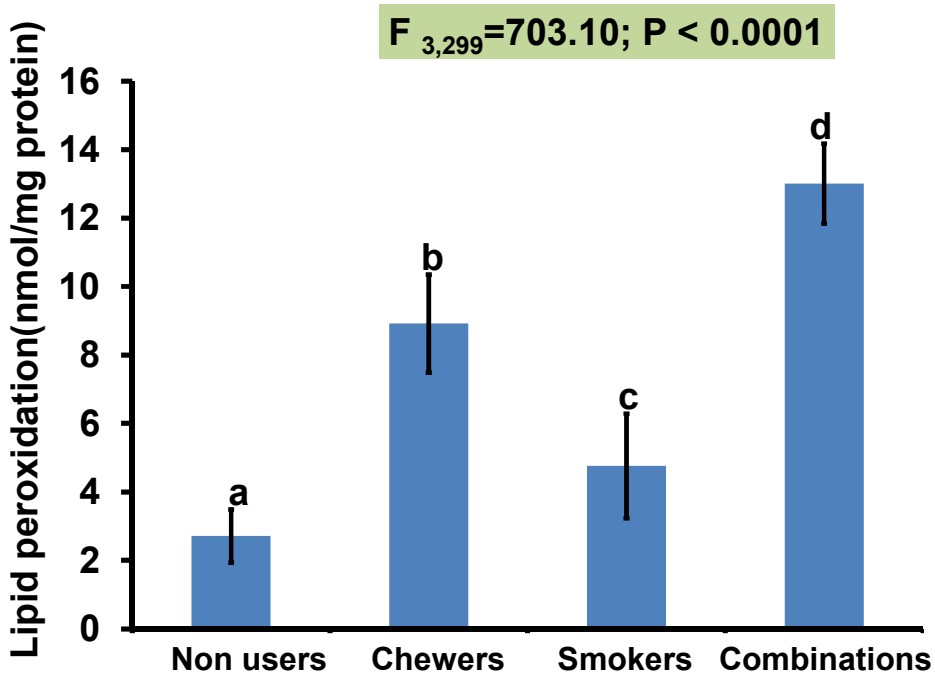


Figure 3. Lipid peroxidation (nmol/mg of protein, MDA) concentration in plasma recovered from healthy non users, chewers, smokers and combinations. Data are illustrated as Mean ± S.D, with comparisons among groups performed using the one way ANOVA followed by Tukey's post-hoc tests for all pair-wise multiple comparisons. Bar with different letter indicate that case groups are significantly different at $p < 0.05$.

of tobacco or who have not used tobacco products for at least past one year); chewers (SLT users) group (individuals chewing tobacco products (Zarda, gutkha, sadha and khuva) habitually, at least > 20 times per week for the last 6 months); smokers group (frequency of smoking > 20 cigarettes per day); combinations (smokers and SLT users group) (individuals consume smokeless tobacco products as well as smoking), respectively. SOD levels were decreased

significantly in tobacco user groups chewers (16.09 ± 2.36), smokers (21.02 ± 2.42) and combinations (9.96 ± 1.37) with respect to non-user group (25.10 ± 3.59) respectively (Fig.4).

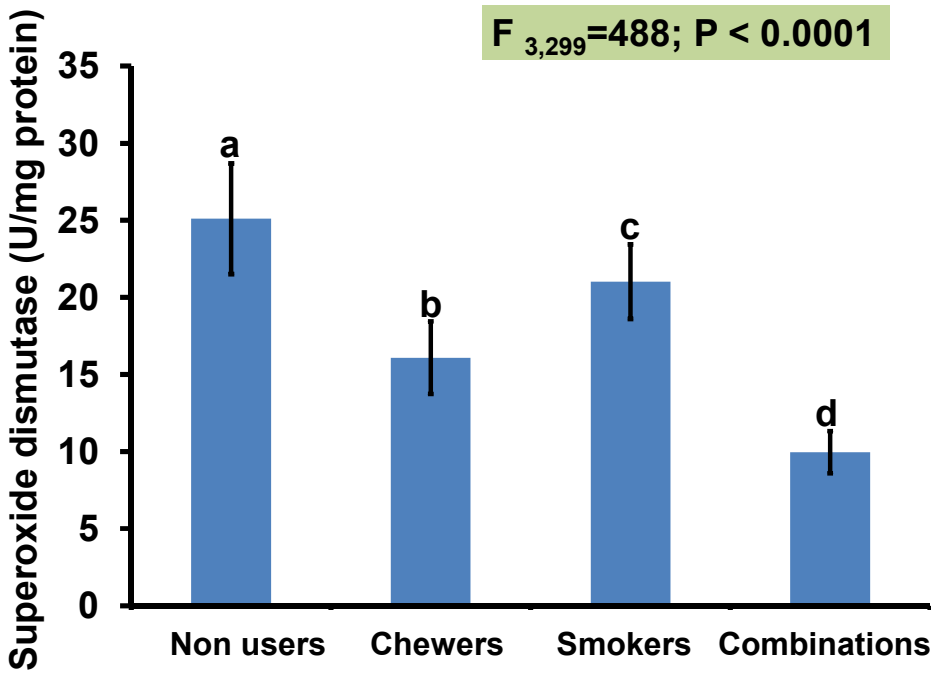


Figure 4. Superoxide dismutase (U/mg of protein, SOD) concentration in plasma recovered from healthy non users, chewers, smokers and combinations. Data are illustrated as Mean ± S.D, with comparisons among groups performed using the one way ANOVA followed by Tukey's post-hoc tests for all pair-wise multiple comparisons. Bar with different letter indicate that case groups are significantly different at $p < 0.05$.

5.5.2. Determination of catalase (µmol/min/mg of protein, CAT) levels in plasma

The mean levels of plasma catalase (µmol/min/mg of protein, CAT) levels shown significant decrease in tobacco user groups with compared to non-user group (Fig.5). The mean ± S.D values in tobacco user groups, 4.44 ± 2.06 (chewers), 5.82 ± 2.17 (smokers) and 3.11 ± 1.11 (combinations) respectively but the non-user group showed mean ± S.D value was 9.15 ± 3.23.

5.5.3. Evaluation of glutathione-s-transferase (U/mg of protein, GST) levels in plasma

A lower value of Glutathione-s-transferase (U/mg of protein, GST) level was observed in the tobacco consuming group's chewers (2.52 ± 1.14), smokers (3.75 ± 1.10) and combinations (1.63 ± 1.07) as compared to the non-users group (4.69 ± 1.06) for plasma respectively (Fig.6).

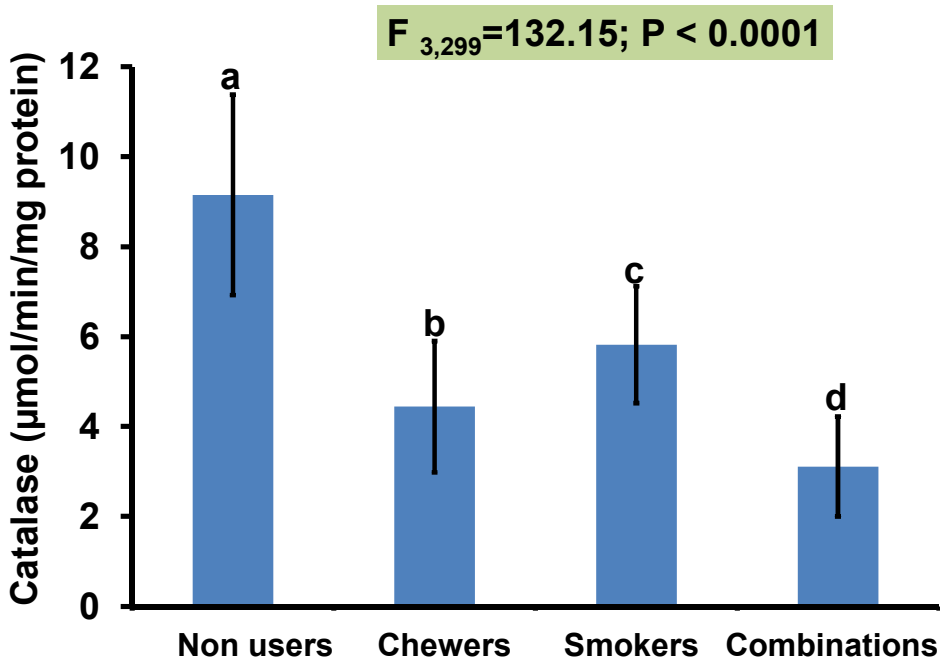


Figure 5. Catalase (µmol/min/mg of protein, CAT) concentration in plasma recovered from healthy non users, chewers, smokers and combinations. Data are illustrated as Mean ± S.D, with comparisons among groups performed using the one way ANOVA followed by Tukey's post-hoc tests for all pair-wise multiple comparisons. Bar with different letter indicate that case groups are significantly different at $p < 0.05$.

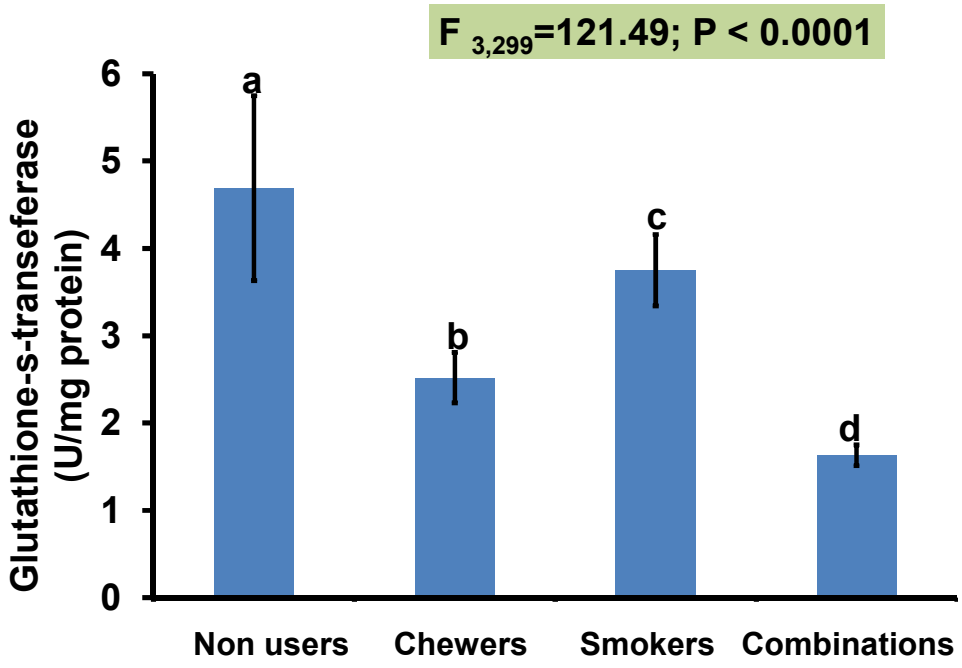


Figure 6. Glutathione-s-transferase (U/mg of protein, GST) concentration in plasma recovered from healthy non users, chewers, smokers and combinations. Data are illustrated as Mean ± S.D, with comparisons among groups performed using the one way ANOVA followed by Tukey's post-hoc tests for all pair-wise multiple comparisons. Bar with different letter indicate that case groups are significantly different at $p < 0.05$.

5.5.4. Evaluation of glutathione reduced (nmol/mg of protein, GSH) levels in plasma

A lower value of Glutathione reduced (nmol/mg of protein, GSH) level was observed in the tobacco consuming groups chewers (340.46 ± 12.74), smokers (3.75 ± 1.1454.56 ± 15.58) and combinations (248.20 ± 10.94) as compared to the non-users group (544.81 ± 18.57) for plasma respectively (Fig.7).

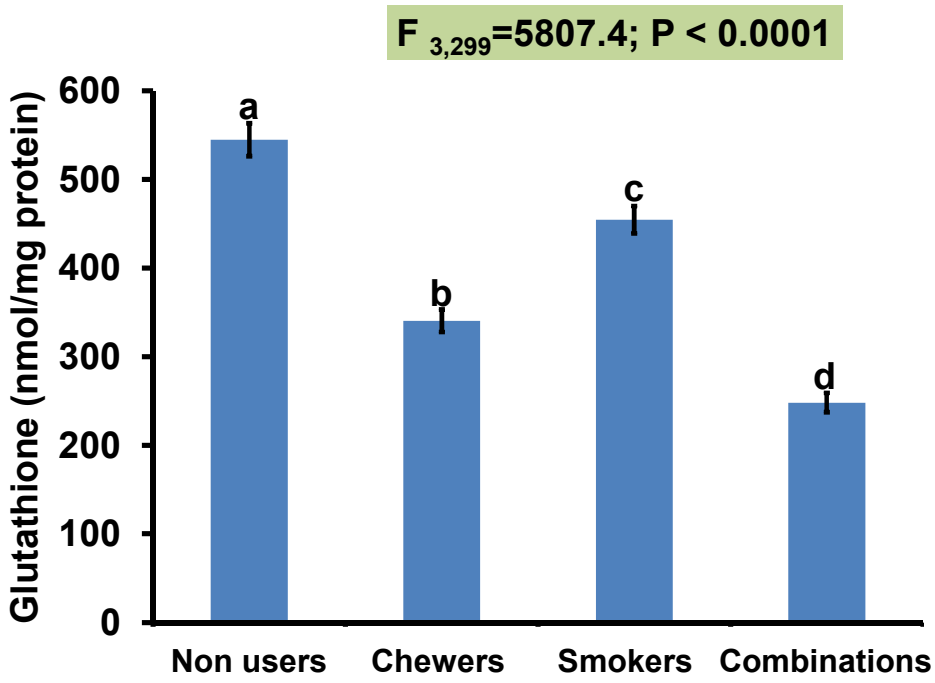


Figure 7. Glutathione reduced (nmol/mg of protein, GSH) concentration in plasma recovered from healthy non users, chewers, smokers and combinations. Data are illustrated as Mean ± S.D, with comparisons among groups performed using the one way ANOVA followed by Tukey's post-hoc tests for all pair-wise multiple comparisons. Bar with different letter indicate that case groups are significantly different at $p < 0.05$.

5.6. Determination of hormone profiles [(Testosterone, Estradiol, Leutinizing hormone (LH) and Follicle stimulating hormone (FSH)] levels in plasma

Reproductive hormone levels for non-users [Testosterone (17.45 ± 0.60); Estradiol (44.66 ± 2.82); LH (3.07 ± 0.17); FSH (3.93 ± 0.10)] and tobacco user, smokers [Testosterone (4.30 ± 0.27); Estradiol (45.76 ± 0.97); LH (4.05 ± 0.17); FSH (4.11 ± 0.14)]; SLT users [Testosterone (6.30 ± 0.13); Estradiol (70.97 ± 2.51); LH (7.37 ± 0.26); FSH (10.33 ± 0.47)]; smokers and SLT users [Testosterone (2.91 ± 0.19); Estradiol (83.36 ± 1.46); LH (9.34 ± 0.24); FSH (11.59 ± 0.55)]. Significant difference was observed in the plasma levels of testosterone, estradiol, FSH,

LH ($P < 0.0001$) in tobacco user groups, smokers, SLT users, smokers & SLT users and non-user group (Table. 5).

Table 5: Reproductive hormone profiles [(Testosterone, Estradiol, Leutinizing hormone (LH) and Follicle stimulating hormone (FSH)] concentrations in non-users, smokers, SLT users and smokers and SLT users.

Parameters	Non users	Smokers	SLT users	Smokers and SLT users	P value	$F_{(3,299)}$ value
Testosterone (ng/mL)	17.45 ± 0.60a	6.30 ± 0.13b	4.30 ± 0.27c	2.91 ± 0.19d	0.0001	4033.9
Estradiol (pg/mL)	44.66 ± 2.82a	45.76 ± 0.97b	70.97 ± 2.51c	83.36 ± 1.46d	0.0001	194.08
LH (mIU/mL)	3.07 ± 0.17a	4.05 ± 0.17b	7.37 ± 0.26c	9.34 ± 0.24d	0.0001	543.26
FSH (mIU/mL)	3.93 ± 0.10a	4.11 ± 0.14b	10.33 ± 0.47c	11.59 ± 0.55d	0.0001	735.95

Hormone profiles [(Testosterone, Estradiol, Leutinizing hormone (LH) and Follicle stimulating hormone (FSH)] concentrations in plasma recovered from healthy non users, chewers, smokers and combinations. Data are illustrated as Mean ± S.D, with comparisons among groups performed using the one way ANOVA followed by Tukey's post-hoc tests for all pair-wise multiple comparisons. Different letter indicate that case groups are significantly different at $p < 0.05$.

5.7. Estimation of cotinine levels in plasma

Estimation of cotinine concentration (ng/ml) in mizo population in the plasma of non users, chewers, smokers and smokers and SLT user groups, respectively (Fig.8). Non tobacco users group (individuals do not consume any form of tobacco or who have not used tobacco products for at least past one year); chewers (SLT users) group (individuals chewing tobacco products (Zarda, gutkha, sadha and khuva) habitually, at least > 20 times per week for the last 6 months); smokers group (frequency of smoking > 20 cigarettes per day); smokers and SLT users group (individuals consume smokeless tobacco products as well as smoking),

respectively. Tobacco consumer groups, chewers (765.15 ± 22.68), smokers (554.36 ± 28.16) and combinations (867.26 ± 21.35) showed significant levels of cotinine concentration as compared with non-users (24.07 ± 3.98).

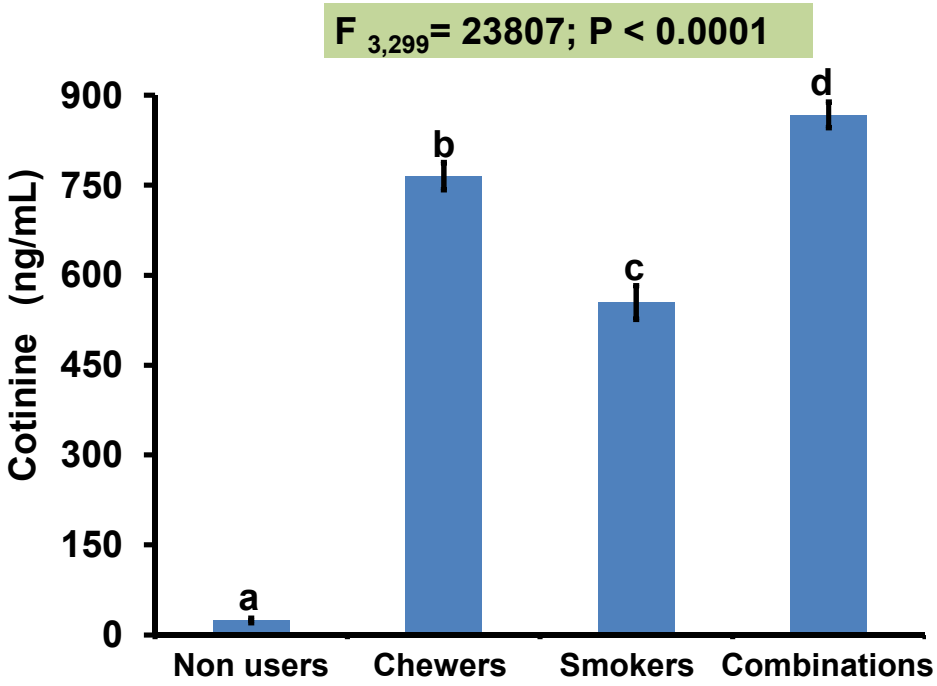


Figure 8. Data are presented as mean ± standard deviation (n = 75 /group). Statistical comparison was performed using one-way ANOVA followed by Tukey's post-hoc tests for all pair-wise multiple comparisons. Bar with different letter indicate that case groups are significantly different at $p < 0.05$.

5.8. Immunoblotting of pro apoptotic markers Bax and Bcl-2

Higher Bax and lower Bcl-2 levels were observed in tobacco user groups, smokers, chewers and combinations (smokers and chewers) group. Densitometric analysis of Bax and Bcl-2 from the smokers, chewers, combinations and non-user group were represented in (Fig. 9A,B) and (Fig. 10A,B). It showed significant higher level of expression for Bax in the smokers, chewers and combinations groups ($p < 0.05$) and Bcl-2 in the non-user group ($p < 0.05$).

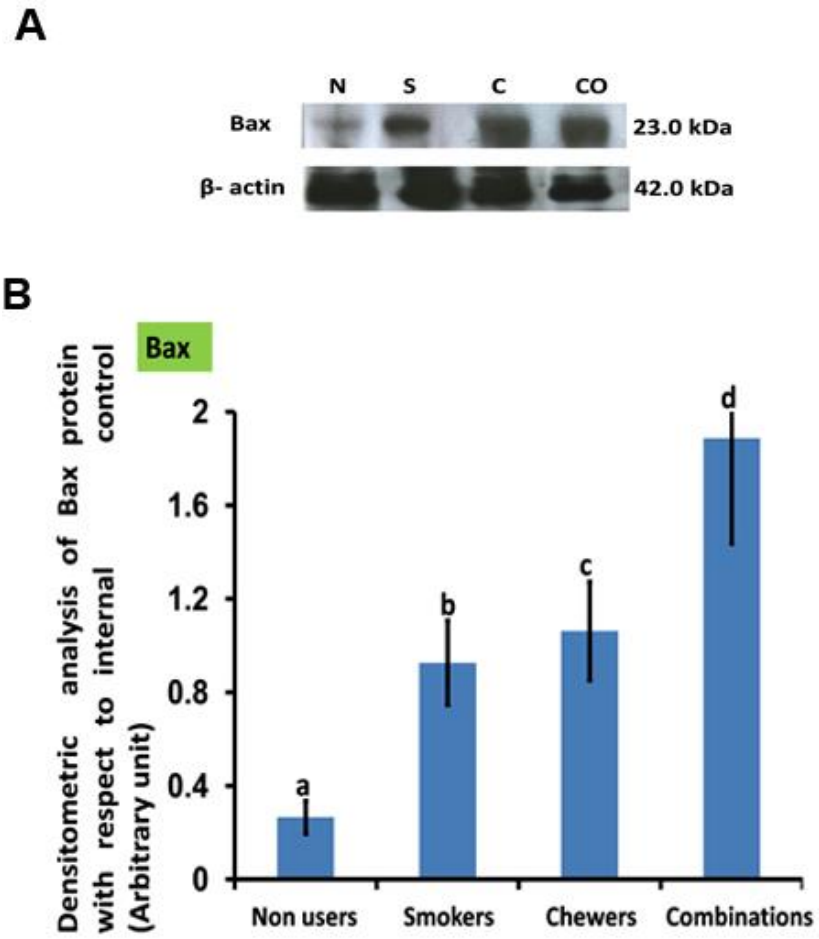


Figure 9. Induction of apoptotic marker Bax.**(A)** Representative Immunoblots of N (non users); S (smokers); C (chewers); and CO (combinations).**(B)** Quantitative analysis of densitometric values obtained from Immunoblot analysis showing increased expression of Bax compared to non-user group (N = 5/each group). Data are illustrated as Mean ± S.D, with comparisons among groups performed using the one way ANOVA followed by Tukey's post-hoc tests for all pair-wise multiple comparisons. Bar with different letter indicate that case groups are significantly different at $p < 0.05$.

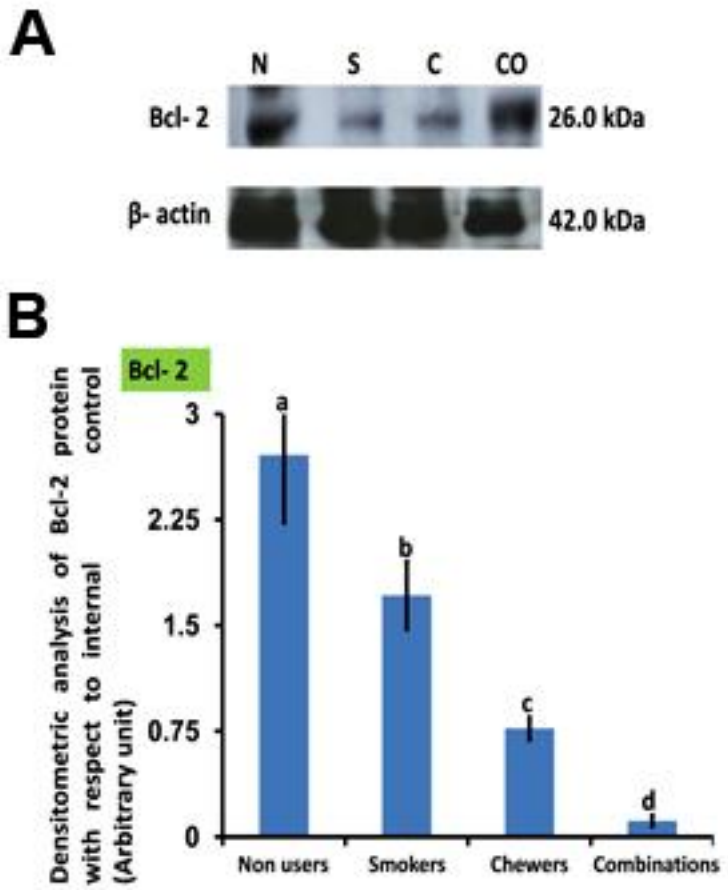


Figure 10. Induction of apoptotic marker Bcl-2.**(A)** Representative Immunoblots of N (non users); S (smokers); C (chewers); and CO (combinations).**(B)** Quantitative analysis of densitometric values obtained from Immunoblots analysis showing decreased expression of Bcl-2, compared to non-user group (N = 5/each group). Data are illustrated as Mean ± S.D, with comparisons among groups performed using the one way ANOVA followed by Tukey's post-hoc tests for all pair-wise multiple comparisons. Bar with different letter indicate that case groups are significantly different at $p < 0.05$.

5.9. Immunoblotting of Pro-inflammatory cytokines like TNF-α, Cox-2

TNF-α, Cox-2 levels were higher in tobacco user groups, smokers, chewers and combinations (smokers and chewers) group. Tobacco user groups showed significantly higher ($p < 0.05$) level of expression for pro-inflammatory cytokines such as, TNF- α (Fig. 11A,B) and Cox-2 (Fig. 12A,B) expression in comparison to the non-user group (N= 5/each group).

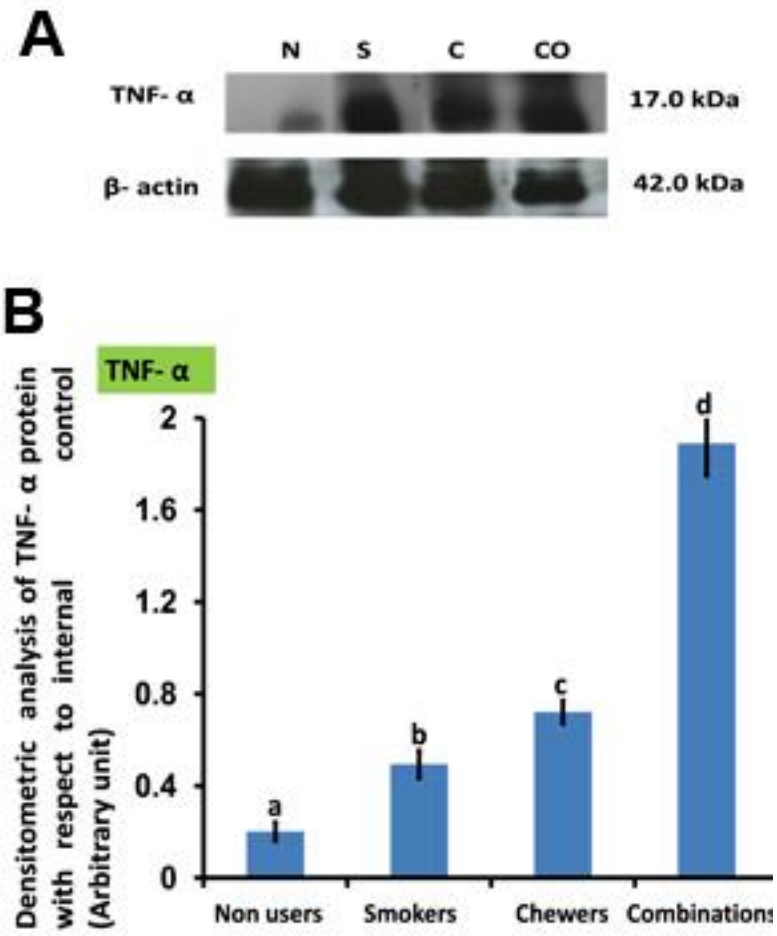


Figure 11. Tobacco Induced enhancement of pro-inflammatory cytokine likes TNF-α. **(A)** Representative Immunoblots of N (non users); S (smokers); C (chewers); and CO (combinations).**(B)** Quantitative analysis of densitometry values obtained from Immunoblots analysis showing increased expression of TNF-α, compared to non-user group (N = 5/each

group). Data are illustrated as Mean ± S.D, with comparisons among groups performed using the one way ANOVA followed by Tukey's post-hoc tests for all pair-wise multiple comparisons. Bar with different letter indicate that case groups are significantly different at $p < 0.05$.

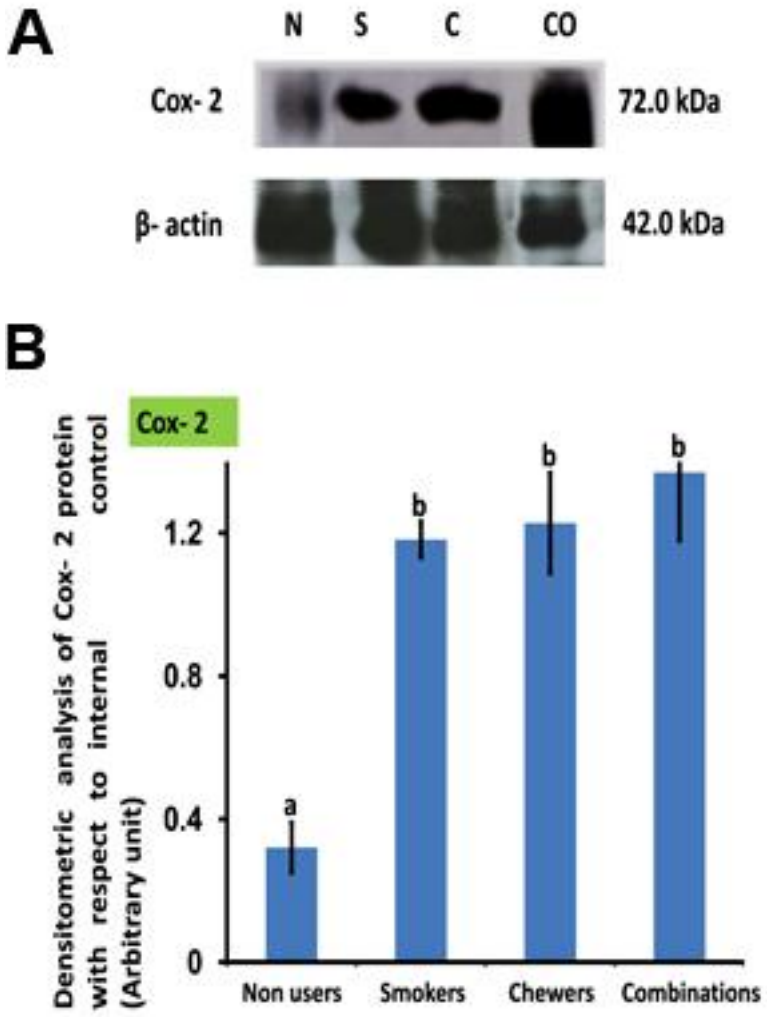


Figure 12. Tobacco Induced enhancement of pro-inflammatory cytokine like Cox-2. **(A)** Representative Immunoblots of N (non users); S (smokers); C (chewers); and CO (combinations). **(B)** Quantitative analysis of densitometry values obtained from Immunoblots analysis showing increased expression of Cox-2, compared to non-user group (N = 5/each group). Data are illustrated as Mean ± S.D, with comparisons among groups performed using the

one way ANOVA followed by Tukey's post-hoc tests for all pair-wise multiple comparisons. Bar with different letter indicate that case groups are significantly different at $p < 0.05$.

5.10. Immunoblotting of inflammatory cytokine like HSP-70

Higher HSP-70 levels were observed in tobacco user groups, smokers, chewers and combinations (smokers and chewers) group. Densitometric analysis of HSP-70 from the smokers, chewers, combinations and non-user group were represented in (Fig. 13A, B). It showed significant higher level of expression for HSP-70 in the smokers, chewers and combinations groups ($p < 0.05$) in comparison to the non-user group (N= 5/each group).

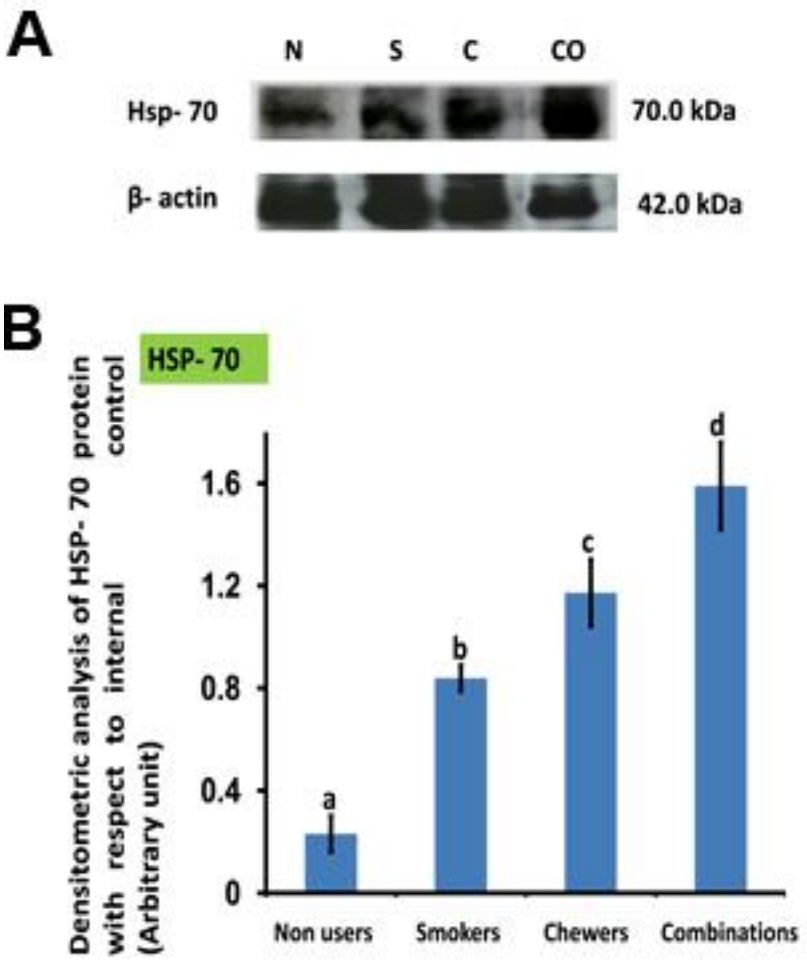


Figure 13. Tobacco Induced enhancement of inflammatory cytokine like HSP-70. **(A)** Representative Immunoblots of N (non users); S (smokers); C (chewers); and CO

(combinations).**(B)** Quantitative analysis of densitometry values obtained from Immunoblots analysis showing increased expression of HSP-70, compared to non-user group (N = 5/each group). Data are illustrated as Mean ± S.D, with comparisons among groups performed using the one way ANOVA followed by Tukey's post-hoc tests for all pair-wise multiple comparisons. Bar with different letter indicate that case groups are significantly different at $p < 0.05$.

5.11. Genotyping of GSTT1, GSTM1and GSTP1

Non significant differences in genotype distribution were observed between tobacco consumers (smokers, chewers and combinations) and control samples (Table. 6). The frequencies of GSTM1 [Non-users (8.41%); Smokers (15%); Chewers (21.47%) & (67.50%)], and GSTT1 [Non-users (12.50%); Smokers (47.50%); Chewers (53.42%) & (67.21%)] null genotypes were non-significant from those observed in non-users (Fig. 14). Non-significant differences in genotype or allele frequencies of the GSTP1 IIe105Val and GSTP1 Ala114Val polymorphisms were also observed (Fig. 15). Presence of heterozygous allele A/G in rs1695 was [Non-users (20%); Smokers (32.5%); Chewers (45.27%) & (57.54%)] and homozygous allele G/G in rs1995 was [Non-users (5%); Smokers (27.5%); Chewers (35.78%) & (27.15%)]. In case of rs1138272, C/T heterozygous was [Non-users (15%); Smokers (25%); Chewers (45%) & (56.21%)] and T/T homozygous allele was [Non-users (0%); Smokers (5%); Chewers (3%) & (5%)].

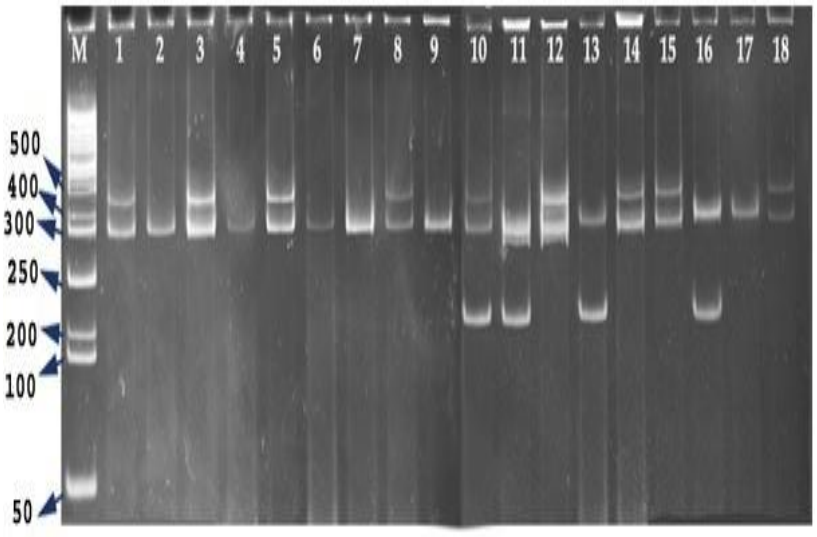


Figure.14. Multiplex PCR amplified products of GSTT1 (459 bp), GSTM1 (219 bp) and internal control gene albumin (349 bp). M – Low range ruler plus; 1 – 5 smokers; 6-10 chewers; 11 –14 combinations; 15-18 non-users Samples.

Table 6: Genotype distribution of GST gene polymorphisms in non-users, smokers, chewers and combinations

Genotypes	Non-users	Smokers	Chewers	Combination	*p* Value
GSTM1 (+)	17 (55%)	12 (22.5%)	11(12.41%)	10 (11.47%)	0.973
(-)	3(8.41 %)	8 (15%)	9 (21.47%)	10 (67.5%)	
GSTT1 (+)	18 (67.5%)	13 (32.5%)	14 (21.42%)	12 (13.41%)	0.60
(-)	10 (12.5%)	14 (47.5%)	17 (53.42%)	18 (67.21%)	
GSTP1105(rs1695) A/A	18 (75%)	12 (40%)	11 (31.04%)	9 (24.78%)	0.057
A/G	10 (20%)	13 (32.5%)	16 (45.27%)	18 (57.54%)	
G/G	4 (5%)	14 (27.5%)	15 (35.78%)	14 (27.15%)	
GSTP1114(rs1138272) C/C	18 (65%)	14 (25%)	12 (17.58)	12(17.48%)	0.261
C/T	12 (15%)	13 (25%)	16 (45%)	17 (56.21%)	
T/T	0 (0%)	16 (5%)	14 (3%)	16 (5%)	

$p < 0.05$ indicates significant association of the genotype with tobacco user groups.

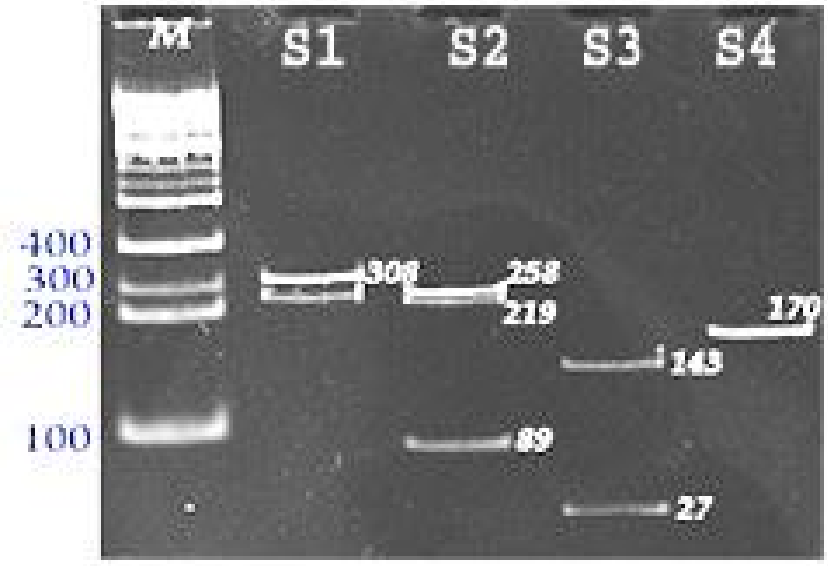


Figure 15. PCR -RFLP of GSTP1.1 (rs1695) and GSTP1.2 (rs1138272) regions. S1 - BsmAI RFLP (A - Digested fragment); S2- BsmAI RFLP (G - Digested fragment) S3- AciI RFLP (C - Digested fragment); S4- AciI RFLP (T - Intact fragment).

CHAPTER-6

DISCUSSION

6.1. Hematology parameters

Tobacco have been associated with a high risk factor for atherosclerosis, polycythemia vera, chronic obstructive pulmonary disease, and cardiovascular diseases, and also higher risk of hypertension, inflammation, stroke, coagulopathies, and respiratory diseases [Abel *et al.,* 2005; Inal *et al*., 2014; Biswas *et al.,* 2015]. Since tobacco causes number of health problems besides, it affects on various organs as lungs, pancreas, kidney, and liver shown in various studies. RBC number decreases significantly in tobacco users. Previous studies showed that changes in the hematological parameters associated with number cigarettes smoked per day **(**Zafar *et al*., 2003; Biswas *et al.,* 2015**).** In our study, we found that significant decrease in hemoglobin, erythrocyte count, lymphocyte count, Monocyte count, Eosinophil count, packed cell volume (PCV), mean corpuscular volume (MCV) levels in tobacco user groups (smokers, SLT users as well as smokers & SLT users) compared to the non-user group. This might be possible, known fact that the function of erythrocytes inhibited by nicotine present in tobacco products (Hibbeln *et al.,* 2003). The low erythrocyte count may lead to the number of physiological disorder and may also affect the efficiency of various enzymes that may play role in the metabolism of drugs. Leukocyte counts, Neutrophil Count, ESR, MCH, MCHC and TOC are increased significantly in tobacco consumers showed in our study. Our results are similar to the previous study, significant increase in leucocytes with number of cigarettes per day (Helman and Rubenstein, 1975; whitehead *et al*., 1995; Zafar *et al*., 2003; Biswas *et al*., 2015). Lung emphysema damage alveolar wall due to increase in leucocytes (Terashima *et al*., 1999). The increase in leucocytes which inturn decrease the Eosinophils levels is associated with tobacco quantity (Winkle, 1981). According to Watanabe, 2011, the increase in WBC count in turn increase tumor necrosis factor (TNF) system activities in tobacco users compared with non-users. Leukocyte counts, Neutrophil Count, ESR, MCH, MCHC and TOC count increases might be the reason of catecholamine's

secretion is increased in WBC, induced by nicotine present in tobacco. Besides, higher WBC count might be resultant inflammation in the respiratory system affected by nicotine. Also, it has been suggested that inflammatory stimulation of the bronchial tract induces an increase in inflammatory markers in the blood circulation. Greater amount of leukocyte counts, Neutrophil Count, ESR, MCH, MCHC and TOC may suggest greater risk for developing cardiovascular diseases.

6.2. Biochemical profiles [hepatic (ALP, AST, ALT & LDH) and renal (Urea & Creatinine) functional enzymes].

AST, ALT, LDH and ALP are essential enzymes mainly present in the liver and bones also present in the kidneys and the leukocytes (Chan-Yeung *et al.,* 1981; Gordon, 1993). The results showed a significant rise in plasma AST, ALT, LDH and ALP of tobacco user groups [(smokers, SLT users as well as smokers & SLT users)] compared with non-users. Our results are very similar to the previous studies, tobacco components are linked with AST, ALT, LDH and ALP enymes. The inflammatory markers, C- reactive protein and leucocyte count explained the relationship between tobacco components and AST, ALT, LDH and ALP (Whitehead *et al*., 1996; Wannamethee and Shaper, 2010). AST, ALT, LDH and ALP levels elevated due to reactive oxygen species generated by tobacco. This might also be a reason in elevated levels of AST, ALT, LDH and ALP enzymes in plasma, resultant of hepatomegaly and liver enzyme abnormality causes glycogen storage in liver may leads to diabetes (Chatila and West, 1996; Abdrabo and Elameen, 2013). Tobacco contains number of chemicals with prospective of liver toxicity including nicotine. This may possible the production of chemicals, nitrous oxide leads to nitrosative stress that causes protein alteration (Padmavathi *et al.*, 2009; Chakraborty and Selvaraj, 2000; Abdul-Razaq and Ahmed, 2013). Elevated levels of urea, creatinine and urea nitrogen were extensively linked with post renal obstruction and pre-renal uremia. Tobacco users were linked with

the risk of kidney failure compared with non-users (Haroun *et al.,* 2003; Hallan and Orth, 2011; Hall *et al.,* 2016).The findings of our study shows elevated levels of urea, creatinine and urea nitrogen in tobacco consuming groups compare with non-user groups. Our results similar to previous studies (Bleyer *et al.,* 2000; Noborisaka *et al.,* 2012; El Sayed *et al.,* 2013; Desai *et al.,* 2016; Ahmed *et al.,* 2016; Hall *et al.,* 2016; Murtadha, 2017). This might be due to tobacco and its components increases inflammation, renovascular resistance that lead to failure in glomerular filtration rate (GFR), which inturn decrease distal tubular flow rate. It leads to increase in urea reabsorption (Lindenfeld and Schrier, 2011). Various mechanisms may cause inflammation, renal dysfunction, vasoconstriction and vascular damage include oxidative stress, endothelial dysfunction, and atherogenesis (Papagianni *et al*., 2003; Perticone *et al.,* 2004; Cottone *et al.,* 2006; Hall *et al.,* 2016; Roehm *et al.,* 2017). Catecholamines, arginine, vasopressin and endothelin-1 are vasoconstrictors, increased in tobacco users due to nicotine, which inturn damages endothelial cells and smooth muscles (Pittilo *et al.,* 1990; Gambaro *et al.,* 1998; Ritz *et al.,* 1998).

6.3. Estimation of lipid profiles (TC, TG, HDL, LDL & VLDL)

Tobacco users have a high risk of coronary heart disease, atherosclerosis, IR and CVD risks than non-users due to altered blood coagulation, impaired integrity of the arterial walls, changes in the blood lipid and lipoprotein concentration explained by various potential mechanisms.This study results are similar to that of previous studies **(**Wynder *et al.,* 1989; Rustogi *et al*., 1989; Devaranavadgi *et al.,* 2012; Joshi *et al.,* 2013; Biswas *et al.,* 2015). In our study, all lipid profiles (total cholesterol, triglycerides, LDL, VLDL) were increased in tobacco users except HDL cholesterol was decreased in tobacco users. In this study, we also measured various lipid ratios, for determination of risk of coronary heart disease, atherosclerosis, IR and CVD diseases (Walldius *et al*., 2001; van der Steeg *et al*., 2007; McQueen *et al.,* 2008; Kimm *et al.,* 2010; Taskinen *et al*., 2010). Higher levels of cholesterol, triglycerides, LDL-C and VLDL-C and lower levels of HDL-C

were associated with CHD, coronary artery disease and ischaemic stroke (Carlson and Böttiger, 1972; Chary and Sharma, 2004). Reduced activity of lipoprotein lipase might be a reason to increase in the levels of cholesterol, triglycerides, LDL-C and VLDL-C is linked with tobacco consumers. Nicotine may be increase myocardial oxygen levels by using free fatty acids which might be a reason to the lower levels of HDL-C in tobacco users with compared to non-users. Other reason to increase in lipid profiles in tobacco users through catecholamine and adenyl cyclase axis induced tissue lipolysis. Various mechanisms leading to lipid alteration by smoking are: (a) nicotine stimulates sympathetic adrenal system leading to increase secretion of catecholamines resulting in increased lipolysis and increased concentration of plasma free fatty acids (FFA) which further result in increased secretion of hepatic FFAs and hepatic triglycerides along with VLDL in the blood stream (Mpabulungi and Muula, 2004; Hassan, 2013) (b) Fall in oestrogen levels occurs due to smoking which further leads to decreased HDL (c) Presence of hyperinsulinaemia in smokers leads to increased cholesterol, LDL and TG due to decreased activity of lipoprotein lipase (Bhatt, 2003; Hassan, 2013; Shenoi *et al*., 2015).

6.4. Oxidative stress and antioxidant enzymes

Tobacco is available in various forms in India, cigarette, Bidi, gutkha, khaini, zarda, Tuibur and Meizial (especial in northeastern states of India) etc. These tobacco products composed with various chemicals, tobacco-specific nitrosamines (TSNAs), polycyclic aromatic hydrocarbons, nitrate, nitrite, nicotine, acrolein, chemicals such as crotonaldehyde, substantial amounts of formaldehyde, acetaldehyde, etc have deleterious effects on human health (Stepanov *et al*., 2006, 2010). The imbalance between pro-oxidant and anti-oxidant states is associated with the levels of ROS (reactive oxygen species). Oxidative stress status leads to cause various diseases, coronary heart disease, chronic obstructive pulmonary disease, cataract, heart attack, emphysema, chronic kidney diseases, and various cancers etc. (Mcghee *et al*., 2006). Tobacco

user groups, chewers (8.92 ± 2.73), smokers (4.76 ± 1.83) and combinations (13.01 ± 4.17) showed significant increase ($p < 0.05$) in MDA levels which amplified the levels of TBARS as compared to non-user groups (2.71 ± 1.78). Our results were similar to previous studies, the MDA levels were increased in chewers in government employees of Kolkata, India (Biswa et al., 2015). Our results were also supported to previous reports on smoking effect on MDA levels (Yanbaeva *et al*., 2007; Emerging Risk Factors Collaboration, 2010). The possible reason to increase in MDA levels (lipid peroxidation) is the tobacco components, nicotine and TSNA may cause production of ROS, which is proportional to the oxidative stress (Kwiatkowska *et al*., 1999). The lipid peroxidation in tobacco users, is very common in cancer patients (Arivazhagan *et al.,* 1997; Nagini *et al*., 1998; Samir and Kholy, 1999; Abou-Seif *et al.,* 2000; Nishino *et al*., 2006). Various types of antioxidant systems are present in cells. These antioxidants can reduce or remove reactive oxygen species (ROS) from damaged cells. According to (Ho *et al.,* 2010), glutathione peroxidase, superoxide dismutase, glutathione-s-transferase, reduced glutathione and catalase are most important antioxidant enzymes in living cells. SOD converts Superoxide radicals into hydrogen peroxide and molecular oxygen. SOD levels were significantly lower in tobacco consumers, the present study showed the SOD levels were drastically decreased in smokers, chewers and combinations as compared to non-users group. Our study was parallel to earlier studies, which showed significant decrease of SOD in smoker's erythrocytes (Zhou *et al.,* 1997; Fouad, 2005; Sirisha and Manohar, 2013). Our results are very parallel to previous studies, the sod levels were decreased in the GCF and saliva of smokers associated with chronic periodontitis (Agnihotri *et al*., 2009). The changes in antioxidant enzyme levels, due to tobacco components are not yet known clearly (Kanehira *et al.,* 2006). Oxidative stress is more in tobacco consumers or smokers due to high lipid peroxidation. Its products can easily diffuse from inflammatory sites, which in turn cause huge production of reactive oxygen species

(Dekhuijzen *et al*., 1996; Kwiatkowska *et al.,* 1999; Kostikas *et al*., 2003; Rosamund *et al.,* 2015). According to (McLellan and Wolf, 1999), tri-peptide glutathione plays a very crucial role in defending cells from oxidative stress. Tobacco users, smokers (454.56 ± 15.58), chewers (340.46 ± 12.74) and combination (248.20 ± 10.94) groups showed significant lower levels of glutathione (GSH) as compared to non-users (544.81 ± 18.57) respectively. Our study result was parallel with Biswas *et al*., 2015, reported that low levels of glutathione in chewers. The reduction of GSH content in smokers, chewers and combinations may cause to be the direct or indirect effect of nicotine and TSNA induced ROS or direct effect of nicotine to GSH content of PBMC and RBC membrane. Catalase converts hydrogen peroxide into water and oxygen. It is widely present in tissues and RBC cells. It suggested that catalase can protect the cells from oxidative damage. Catalase was decreased significantly in smokers, chewers and smokers compared with non-users. Betsuyaku *et al.,* 2013, also reported that catalase activity was reduced at m-RNA and protein levels in smokers associated with mild COPD, parallel to our study results. Evolutionary study on salivary catalase, showed significant ($p< 0.01$) decreases in smokers as compared to non-smokers (Ahmadi Motamayel *et al*., 2017). Our results were similar to previous studies (LaLonde *et al*., 1997) reported that catalase activity was decreased in smokers. The decrease in catalase activity in tobacco consumers associated with oxidative stress, due to toxic components of tobacco increases lipid peroxidation which in turn increase reactive oxygen species. Glutathione-s-transferase (GST) is a key enzyme in detoxification of xenobiotics and drugs. Our study showed, GST was significantly lower in smokers, chewers, and combinations compared with non-users. Our study results were similar to previous studies reported that smokers have low levels of glutathione-s-transferase (Harju *et al.,* 2007). The levels of GST were decreased based on the oxidative stress, generated by reactive oxygen species causes lipid peroxidation (Kwiatkowska *et al*., 1999). The levels of MDA, associated with GST

and changed its structure, leads to oxidative stress. The imbalance of antioxidant defense system is due to cancer development and leads to abnormalities in antioxidant metabolism.

6.5. Reproductive hormones (Testosterone, Estradiol, LH and FSH)

The present study included the relationship between tobacco consumers and reproductive hormones levels (Testosterone, Estradiol, LH and FSH). Previous research showed, conflicting results about the association between tobacco and reproductive hormone levels in men (Vine, 1996). Our findings were parallel to certain studies Shen *et al*., 2016 included that significant decrease in testosterone levels in smokers, chewers and combinations. Certain studies showed diminished levels of testosterone (Andersson *et al*., 2007; Travison *et al.,* 2007; Pan *et al*., 2011). HPT (hypothalamic-pituitary-testicular axis) regulates pituitary gland, to produce testosterone, LH and FSH. Testes Leydig cells interact with LH to produce testosterone, which in turn reduce the LH hormones (Baker *et al.,* 1975). This migh be a possible reason to reduce in testosterone levels in smokers, chewers as well as combination groups, due to degeneration of Leydig cells in their testes (Yardimci *et al.,* 1997). The feedback function of the HPT axis is forbidden due to rises in LH and FSH levels, and the LH/ fT ratio (Pan *et al*., 2011). LH, FSH and Estradiol were increased significantly in our study. Our results were similar to some previous studies (Agarwal *et al*., 1985; Pan *et al*., 2011; Shen *et al*., 2016). The true mechanism is not yet clear but a possible reason is that decreased testosterone levels associated with higher levels of LH, Estradiol and FSH. DEHP associated with Leydig cell impairment and cause cell hyperplasia leads to low testosterone, high LH, FSH and Estradiol concentrations (Akingbemi *et al.,* 2004). EDC may affect at multiple sites in HPT axis which in turn affects pituitary gland in later stages due to changes in testicular structure leads to low testosterone, high LH, FSH and Estradiol concentrations. This type of results might be possible in hypogonadism persons (Carnegie,

2004). Low testosterone, high LH, FSH and Estradiol concentrations might be possible due to abnormalities in testicular cytoarchitecture.

6.6. Cotinine

The current study explained the relationship between cotinine concentration and tobacco users in Mizoram, India. In our study cotinine levels in smokers, chewers and combinations were significantly high smokers (554.36 ± 28.16), chewers (765.15 ± 22.68) and combinations (867.26 ± 21.35) with compared to non-users (24.07 ± 3.98) as found in previous studies (Williams *et al.,* 2005; Rostron *et al*., 2015; Huque *et al*., 2016). Olincy *et al*., 1997, reported that high levels of cotinine in schizophrenic smoker's urine. We found that frequency, different tobacco use pattern and duration of smoking chewing significantly associated with cotinine concentration. Same results found in our previous studies (Mushtaq *et al.,* 2012; Huque *et al*., 2016). High cotinine concentration in tobacco users in Mizoram, India, suggested that urgent need of awareness and tobacco control programmes.

6.7. Immunoblotting of Pro-apoptotic, Pro-inflammatory cytokines like Bax, Bcl-2, TNF-α, Cox-2 and HSP70

Tobacco and its components were responsible for various cellular activities, including perturbing the pro- and anti-apoptotic pathways. We found increased expression of Bax, a pro-apoptotic protein in tobacco user groups compared to the non-users. However, non-users showed increased level of expression of anti-apoptotic protein, Bcl-2. Our findings were similar to previous studies, found that Bax expression was higher in chewers and Bcl-2 was low in chewers group as compared to non-chewers (Biswas *et al*., 2015). Mitra *et al*., 2015, reported that cannabis smoke addicted group showed the highest expression of both p-Akt and Bax proteins. The tobacco could lead to interruption of cell cycle progression through the p53/p21 signalling axis and induced cell cycle arrest leading to subsequent apoptosis through Bcl-2 and Bax

disequilibrium. We found higher TNF-α ($p < 0.05$) expression in the user group. Biswas *et al*., 2015, showed higher expression of TNF-α in government employees in Kolkata. Kushner and coworkers report that the concentration of TNF-α in the pulmonary microenvironment is greater in healthy smokers compared to healthy nonsmokers (Kushner *et al*., 1996). However, in a study using induced sputum sample, a non-invasive method of studying airway secretion, showed that the concentration of TNF-α is greater in smokers with COPD compared to healthy smokers and nonsmokers (Keatings *et al.,* 1996). Demirjian *et al.,* 2006, high levels of TNF-α expression in macrophage mediated through the erk1/2 pathway. The acute effect of smoke exposure on mice was studied by Churg and coworkers. A single acute smoke exposure from four cigarettes caused an increase in the gene expression of TNF-α from whole lung extract 2 h after smoke treatment (Churg *et al.,* 2002). IL-6 is critical as an anti-inflammatory mediator by inhibition of other cytokines such as IL-1, IL-10 and TNF-α (Hirano, 1998). In our study, we found significant increase in COX-2 in smokers, chewers and combinations. We seen parallel findings in Biswas *et al*., 2015 and the elevated levels of COX-2 found in the oral mucosa of smokers (Moraitis *et al.,* 2005). In inflammatory processes, the inducible isoform of cyclooxygenase (COX-2) is expressed in many cells, including fibroblasts and macrophages, accounting for the release of large quantities of pro-inflammatory prostaglandins at the site of inflammation (Wu, 1995). The NOS and COX systems are often present together, share a number of similarities and mediate fundamental roles in pathophysiological conditions (Bassuk *et al*., 2004). Increasing evidences suggest that there is considerabl‘cross-talk’ between COX and NOS. In vivo studies revealed that the regulation of COX by NO is a powerful mechanism that is used to magnify the course of the inflammatory response. We found significant increase in HSP-70 levels in smokers,chewers and combinations as compared to non-users. This findings are similar to previous study reported

that increased levels of HSP70 in current smokers than non-smokers associated with COPD (Dong *et al.,* 2013).

6.8. Genotyping of GSTT1, GSTM1and GSTP1

The frequency of null genotype of GSTM1 (55%) observed in our study was similar to that reported in North Indian controls (Mishra *et al*., 2004). However, this was similar previous studies found in other Asian populations (35–63% in Japanese, Chinese and Koreans), Caucasians (48–57%) and Pacific-Rims (approx. 75%). Likewise, the GSTT1 null genotype frequency was found to be 67.5% in the controls which was similar to that seen in Caucasians and African- Americans but higher than reported in other Asian (approx. 42–58% in Chinese, Japanese and Korean) and Pacific Rim (33%) populations. Similarly the frequency of variant alleles of GSTP1 was almost equal to that observed with Caucasians but more than that reported with Japanese and African-Americans and less than Hispanics and Pacific-Rim populations (Geisler and Olshan, 2001).

CHAPTER-7

SUMMARY

- A significant increase in the levels of leukocyte count, Neutrophil count, platelet count, erythrocyte sedimentation rate (ESR), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) in tobacco user groups (smokers, SLT users as well as smokers & SLT users) compared to the non-user group ($p < 0.05$).
- Hemoglobin, erythrocyte count, lymphocyte count, Monocyte count, Eosinophil count, packed cell volume (PCV), mean corpuscular volume (MCV) levels were decreased significantly in tobacco user groups (smokers, SLT users as well as smokers & SLT users) compared to the non-user group ($p < 0.05$).
- ALP, AST, ALT, and LDH levels increased as daily tobacco amount increased. There was a significant differences in ALP, AST, ALT, and LDH activities in tobacco user groups and non- user group ($p < 0.05$). ALP, AST, ALT, and LDH levels were increased significantly in tobacco user groups with compared to non-user group.
- A significant ($p < 0.05$) increases in plasma creatinine, urea and urea nitrogen levels in the tobacco user groups (smokers, SLT users as well as smokers & SLT users) than non-user group.
- TC, TG, LDL-C, VLDL-C, non-HDL-C, apoB, TC/HDL-C, TG/HDL-C, LDL-C/HDL-C, non-HDL-C/HDL-C, were significantly higher in tobacco user groups [(smokers, SLT users as well as smokers & SLT users)], whereas HDL-C was significantly lower in tobacco user groups [(smokers, SLT users as well as smokers & SLT users)] compared with non-users ($p < 0.05$).
- When lipid peroxidation (nmol/mg of protein, MDA) concentration was compared between tobacco user groups and non-users, significant increase was observed in tobacco user groups (smokers, chewers and combinations). The mean ± S.D values in tobacco

user groups [(chewers, (8.92 ± 2.73), smokers, (4.76 ± 1.83), and combination (13.01 ± 4.17)] with compared to non-users (2.71 ± 1.78) respectively.

- SOD levels were decreased significantly in tobacco user groups chewers (16.09 ± 2.36), smokers (21.02 ± 2.42) and combinations (9.96 ± 1.37) with respect to non-user group (25.10 ± 3.59) respectively.
- The mean levels of plasma catalase (μmol/min/mg of protein, CAT) levels shown significant decrease in tobacco user groups with compared to non-user group.
- A lower value of Glutathione-s-transferase (U/mg of protein, GST) level was observed in the tobacco consuming group's chewers (2.52 ± 1.14), smokers (3.75 ± 1.10) and combinations (1.63 ± 1.07) as compared to the non-users group (4.69 ± 1.06) in plasma respectively.
- A lower value of Glutathione reduced (nmol/mg of protein, GSH) level was observed in the tobacco consuming groups chewers (340.46 ± 12.74), smokers (3.75 ± 1.1454.56 ± 15.58) and combinations (248.20 ± 10.94) as compared to the non-users group (544.81 ± 18.57) in plasma respectively.
- Significant difference was observed in the plasma levels of testosterone, estradiol, FSH, LH ($p < 0.0001$) in tobacco user groups, smokers, SLT users, smokers & SLT users and non-user group.
- Tobacco consumer groups, chewers (765.15 ± 22.68), smokers (554.36 ± 28.16) and combinations (867.26 ± 21.35) showed significant levels of cotinine concentration as compared with non-users (24.07 ± 3.98).
- It showed significant higher level of expression for Bax in the smokers, chewers and combinations groups ($p < 0.05$) and Bcl-2 in the non-user group ($p < 0.05$).

- Tobacco user groups showed significantly higher ($p < 0.05$) level of expression for pro-inflammatory cytokines such as, TNF- α and Cox-2 expression in comparison to the non-user group.
- It showed significant higher level of expression for HSP-70 in the smokers, chewers and combinations groups ($p < 0.05$) in comparison to the non-user group.
- Non significant differences in genotype distribution were observed between tobacco consumers (smokers, chewers and combinations) and control samples.
- Tobacco is associated with various diseases such as atherosclerosis, polycythemia vera, chronic obstructive pulmonary disease, higher risk of hypertension, inflammation, stroke, coagulopathies, and respiratory diseases, post renal obstruction and pre-renal uremia, CHD, coronary artery disease and ischaemic stroke, hyperinsulinaemia, IR and cardiovascular diseases etc.
- Tobacco and its components have hostile effects on hematology parameters, liver function enzymes, kidney parameters and also on lipid profiles.
- It is suggested that while measured the hematology parameters, liver function enzymes, kidney parameters and lipid profiles, social habits like tobacco using should be considered as a significant factor. Even though public awareness about harmful effects and cessation of tobacco has been raised considerably, still every effort should be made on this issue.
- The current study appraises relationship between anti-oxidant enzyme levels, oxidative stress, hormones, cotinine levels and tobacco habit. The imbalance of antioxidant defense system, hormone profiles and increased levels of cotinine concentration in tobacco users associated with various diseases such as cardiovascular diseases, stroke, heart diseases, type-2 diabetes, COPD etc.

- Therefore, from the studied group, it may be conferred that, tobacco remains a threat locally in the oral cavity and following its absorption; it modulates the metabolic pattern in a robust way, escalate the risk of systemic inflammation and promotes the risk of coronary artery disease, cardiovascular disease and dyslipidemia.This is a comprehensive evidence of systemic toxicity, apoptosis, metabolic disorder and gene polymorphism modulation after smoking and smokeless tobacco chewing.

CHAPTER-8

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VIII. APPENDIX

1. List of acronyms

ABBREVIATED	FULL FORM
°	Degree
%	Percent
µl	Micro litre
Mg	Mili gram
µg	Micro gram
ng/ ml	Nano-grams per milli Liter
Kg	Kilo gram
C	Celsius
DNA	Deoxyribonucleic acid
dNTPs	Deoxinucleotide Triphosphate
EDTA	Ethylene-diamine tetra-acetic acid
Kb	Kilo base pair
PCR	Polymerized Chain Reaction
rpm	Rotation per minute
TAE	Trisbase acetic acid EDTA
SDS	Sodium Dodecyl Sulfate
h	Hour
M	Molar
Min	Minutes
bp	Base pair
RFLP	Restriction fragment length polymerphism
CAT	Catalase
GSH	Reduced glutathione
SOD	Superoxide dismutase
GST	Glutathione-s-transferase
MDA	Malondialdehyde
Hb	Hemoglobin
RBC	Red blood cells
WBC	White blood cells
GSTM1	Glutathione-s-transferase mu1
GSTT1	Glutathione-s-transferase theta1
GSTP1	Glutathione-s-transferase para1

pg/ml	Picogram per milli litre
MCV	Mean cell volume
PCV	Packed cell volume
EtBr	Ethidium bromide

2. List of Conference/Seminar/Workshop attended and participated

S.No.	Conference/Seminar/Workshop attended and participated	Date
1	The work shop on "*Mechanism of adaptation in the Temporal Environment"*, May 23, 2017. Organized at: Department of Zoology, Mizoram University, Aizawl, Mizoram.	23-25, May 2017
2	*National Seminar on "Biodiversity, Conservation and utilization of Natural Resources With Reference to NortheastIndia (BCUNRNEI)" 30-31th March 2017, Mizoram University.*	30-31, March, 2017
3	*Workshop/ Training on "Cancer Epidimiology" in Mizoram University held during 29-30th NOV. 2016.*	29-30, NOV. 2016

3. List of published papers

S.No.	Paper
1	Testicular toxicity and sperm quality following copper exposure in Wistar albino rats: ameliorative potentials of L-carnitine. Environ.Sci.Pollut.Res. 2017, 1-26.