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Department of Biochemistry

Investigation of Toxicity of Cigarette Butts Collected in Addis Ababa to Swiss Albino Mice

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Approved by the Examination Board

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Abstract

Background: Cigarette butts are the most common form of litter in the world; approximately 5.6 trillion cigarettes are smoked yearly worldwide. Over 7000 chemicals may be introduced to the environment in cigarette butts and smoke constituents. These include chemicals such as nicotine, carbon monoxide, hydrogen cyanide, polycyclic aromatic hydrocarbons, heavy metal ions, nitrosamines, ammonia, acetaldehyde, formaldehyde, benzene, phenol and acetone, over 70 of which are carcinogenic to humans. Environmental cigarette butts are toxic to microorganisms, fish, other marine organisms and birds, but no studies have been done on their toxicity to mammals.

Methods: In this study the toxicity of cigarette butt leachates to Swiss albino mice was investigated, and the effects of cigarette butts with and without associated tobacco were compared. Cigarette butt leachates were made by soaking different numbers of cigarette butts in 250 mL of tap water overnight, and leachates were given to mice to drink. The weights of mice as well as amounts of food and fluids consumed were followed over time. Necropsies were performed and tissues were weighed and evaluated by histological staining with eosin and hematoxylin. Blood glucose and liver function tests (ALT, AST and ALP) were also measured.

Results: Mice given high-concentration cigarette butt leachates during their growth from 6 weeks to 16 weeks preferred water over any of the leachates, whereas mice given lower butt concentrations of leachates representative of the relative frequencies of butt types in the environment drank butt leachates as well as water. Food intake was not affected by cigarette butt leachates. Mean fasting blood glucose and activities of serum liver enzymes were not different between mice given leachates or water to drink. The weights of the mice, as well as tissues (liver, lung, spleen, heart, kidney, epididymal fat pads) were significantly lower in mice that drank leachates with associated tobacco than in mice that drank water alone or leachates made from butts without associated tobacco. This suggests that a component(s) of tobacco is/ are responsible for reducing weight gain during mouse growth. Cigarette butt leachates had no effect on histopathology of liver, heart and kidney, whereas lung tissue of the mice that drank leachates made with tobacco-associated butts showed increased air space volumes and alveolar fibrosis, compatible with pulmonary emphysema.

Conclusions: Cigarette butts are toxic to laboratory mice, causing reduced weight gain during growth, reduced mass and size of tissues and organs, and pulmonary emphysematous changes. An average cigarette butt thrown into the environment contains 1.5 mg of nicotine, enough to potentially kill 15 mice. The implications of these studies to environmental animals in Ethiopia and elsewhere are discussed.

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Chapter 1

Introduction

1.1. Epidemiology of tobacco smoking in Ethiopia

Tobacco is the only consumer product that kills many of its users when used exactly as intended by manufacturers. WHO has estimated that tobacco use is currently responsible for the deaths of about six million people across the world each year, with many of these deaths occurring prematurely. On average, a smoker can expect to live for 10 years less than a non-smoker. A total of about 600,000 people are also estimated to die yearly from the effects of second-hand smoke (passive smoking) and many of these are children and women.

Although often associated with ill-health, disability and death from non-communicable diseases (NCDs), tobacco smoking is also associated with an increased risk of death from communicable diseases.Over 50 % of smokers die of smoking-related diseasesand 75% of the ones who do not die from smoking-related diseases suffer from chronic illness and debilitation, for example from the effects of chronic obstructive pulmonary disease. Tobacco use is also the single greatest preventable cause of NCDs. Tobacco use kills more than 15,000 people a day and accounts for one in six of all NCD deaths. Although fewer people are using tobacco in some countries, particularly in high income countries where tobacco control laws are being enforced, in some low-income countries, including Ethiopia, smoking prevalence is on the rise. By 2020, WHO estimates that tobacco will cause 7.5 million deaths annually, or about one in ten of all deaths. An estimated 100 million people died from tobacco-related diseases during the 20th century. Unless steps are taken to avoid it, a billion lives will be lost in this 21stcentury to tobacco use (World Health Organization, 2015).

There are relatively few studies on smoking practices and epidemiology in Ethiopia and it is unclear just how common smoking is, in particular because its prevalence differs significantly in different areas of the country. For example, in 2008 the World Health Organization (WHO) estimated that about 7.6 % of adult males and 0.9% of adult females smoke in Ethiopia (World Health Organization, 2008), although a subsequent WHO report for 2011 did not include Ethiopia due to there being insufficient data (World Health Organization, 2011). A more recently published survey of tobacco smoking prevalence in 187 countries showed that, although the total number of smokers globally has increased due to population increase, global smoking prevalence has fallen from about 41% in 1980 to about 31% in 2012. In this survey, Ethiopia ranked in the bottom five countries with the lowest smoking prevalence, with a smoking prevalence below 10% (Ng *et al.*, 2014). The WHO estimated that 2% of all deaths in Ethiopia were attributable to tobacco use in 2004, including 3% of deaths due to non-communicable diseases (NCDs). This compares with countries with high smoking prevalence, such as the USA, where over 20% of all deaths are due to smoking. In Ethiopia, tobacco use accounted for 25% of deaths due to lung, trachea and bronchus cancer, and 8% of deaths due to non-malignant respiratory diseases, including 15 % of deaths due to COPD (World Health Organization, 2011).

However, in one study of a rural town in Eastern Ethiopia, about 28% of adults interviewed said that they smoked cigarettes daily; this prevalence is higher than that in many countries (Reda *et al*, 2013). In addition, tobacco use is increasing in many developing countries, including sub-Saharan African countries. In Ethiopia, the only tobacco company, National Tobacco Enterprise (NTE), recently increased its production of cigarettes from 4 billion to 6 billion annually, stating that the demand for cigarettes is increasing in Ethiopia (Ashall, 2015).

Because tobacco use practices in Ethiopia vary around the country, and since the data available is relatively minimal, published data on national tobacco use practices are necessarily only estimates. Another survey reported an adult smoking prevalence of 15.8% in the Gilgel Gibe Field Research Center (Alemseged *et al.*, 2013). Most studies on smoking in Ethiopia have been conducted in urban populations or specific groups such as students (Misganaw *et al.*, 2014) while rural areas and towns, where the majority (84.0%) of the population lives (Fisher *et al,* 2008), are relatively neglected in tobacco research (Slama, 2008), although one study, for example, in the Oromia region of Kersa rural town showed 28% of residents were current smokers. Of these, 68% of smokers expressed an interest to quit while 37% had tried to quit previously but without success. This study also showed a high exposure to second-hand smoke (52%) in homes and a third of this indoor smoking took place daily (Reda et *al*., 2013).

A recent study reported that the prevalence of cigarette smoking in Ethiopia is increasing among adolescents and that the proportion of female smokers is increasing; in this study, the prevalence of current smokers in adolescents was 17.2% and the prevalence of ever-smokers among adolescents was 28.6% in 2014 (Dereje *et al.,* 2014).

To date, little is known about both the prevalence of smoking or passive smoking and their relationship with other substances of abuse such as khat and alcohol in Ethiopia; although a study conducted in Addis Ababa showed that daily smoking and regular khat chewing were significantly associated with each other (Tesfaye *et al.*, 2008).

Lung cancer is relatively uncommon in Ethiopia, but if the increase in smoking prevalence continues, it will likely to become a worsening health problem. It was estimated that there were almost 3000 lung cancer deaths per year in Ethiopia (Winkler *et al*, 2011). There is a 20-year lag between smoking increases and corresponding increases in lung cancer prevalence, so an increased smoking prevalence in the next 5 years will reveal itself as a lung cancer epidemic in 20 years, time.

1.2. Tobacco-related diseases

The three major groups of tobacco-related diseases are cardiovascular diseases, lung cancer and other cancers, and obstructive respiratory diseases. However, smoking also contributes to many other diseases.

Smoking is the major cause of cardiovascular diseases. Cigarette smoking is the most powerful risk factor predisposing to atherosclerotic vascular disease (McBride, 1992).Smoking increases the risk and progression of atherosclerosis, with resultant cardiovascular disease. Smokers are two to four times more likely than non-smokers to have coronary artery disease and have a stroke. This affects most arterial vessels, particularly the coronary arteries (causing heart attacks), arteries to the brain (causing strokes), arteries to the kidneys and other organs, and arteries to the legs (causing peripheral vascular disease) and intestines (causing mesenteric artery ischemia).Abdominal aortic aneurysms occur mainly in smokers.

Smoking is the main cause of chronic obstructive pulmonary disease (COPD) in adults. COPD refers to two major smoking-related diseases: chronic bronchitis ("smoker's cough") and emphysema (loss of lung tissue). Asthma, another form of COPD, is also significantly increased in children who passively smoke.The number of pack-years (number of packs of 20 cigarettes smoked by a person per day multiplied by the number of years that person has smoked) smoked is related to the risk and severity of COPD. Over 40 pack-years increases the risk of having COPD 8-fold compared with a non-smoker. COPD due to smoking is very debilitating and progressive, leading to severe shortness of breath even with mild activity. Over 85% of deaths due to COPD are caused by smoking. Smokers are 12 times more likely to die from COPD than non- smokers.

Smoking is also associated with all four major types of lung cancer: adenocarcinoma, squamous cell carcinoma, large cell carcinoma and small cell carcinoma of the lung. Over 75% of all four types of lung cancer are caused by smoking, and over 90% of squamous cancers and small cell cancers are due to cigarette carcinogens. Adenocarcinoma has now become the most common type of lung cancer in smokers; previously it was relatively less common. The basis for this shift is unclear, but there is evidence it may be due to the use of cigarette filters. Filters were introduced in the 1950s and the increased relative prevalence of lung adenocarcinoma coincides with the use of filters. One theory is that smokers take deeper puffs of cigarettes with filters, and this allows carcinogens in smoke to gain access to the deeper alveolar tissue of lungs, where adenomas are found. With lighter puffs, airways are more likely to be affected by tobacco carcinogens, causing squamous and small cell cancers. Increased adenocarcinoma prevalence may also reflect changes in the proportions of different carcinogens in cigarette smoke that have occurred over time (Vander *et al*., 1992).

Cigarette smoking also is associated causally with other cancers, including bladder cancer, cervical cancer, esophageal cancer, kidney cancer, laryngeal cancer, leukemia, lung cancer, oral cancer, pancreatic cancer and stomach cancer (Pollayand Dewhirst 2001).

Cigarette smoking during childhood and adolescence produces significant health problems among young people, including cough and phlegm production, an increased number and severity of respiratory illnesses, decreased physical fitness, an unfavorable lipid profile, and potential retardation in the rate of lung growth and reduced level of maximum lung function. Active smoking causes injurious biologic processes (oxidant stress, inflammation, and a protease-antiprotease imbalance) that result in airway and alveolar injury. This injury, if sustained, ultimately leads to the development of chronic obstructive pulmonary disease. (Surgeon General, 2014)

Smoking also decreases exercise tolerance and causes coronary thrombosis (Bild *et al*., 1993). Cigarettes and tobacco have been shown to affect offspring of mothers exposed to smoking. For example, intrauterine growth retardation has been observed in babies of smokers (Iranloye and Bolarinwa 2009), so also low birth weight is associated with smoking in pregnant women (Bardy *et al*.,1993), increased incidence of respiratory tract infection (Fergusson *et al*., 1981), reduced forced expiratory flow (Hanrahan *et al.,* 1992), asthma (Weitzman *et al*., 1990), prenatal and neonatal death or mortality

(Walsh,1994) and reduction in uterine blood flow in both rats and humans(Bainbridge *et al*., 2006).

Men who smoke cigarette have been shown to have immature sperm, low sperm count, sperm with multiple heads (Weisberg, 1985) and decreased sperm motility and decreased penetrability through of sperm through the female cervical canal. Cigarette smoking is known to have deleterious effects on fertility in women as epidemiologic studies have clearly indicated that women who smoke suffer a lowered fertility rate (Rosevear *et al*., 1992; Fagerström *et al*., 1996; Khaw and Barrett-Connor, 1988). These harmful effects of cigarette smoking are associated with the nicotine content of cigarettes.

Exposure to second-hand smoke causes significantly increased deaths of passive smokers due to cardiovascular disease and lung cancer. Exposure to second hand smoke increases the risk for stroke by an estimated 20–30%. Second hand smoking (passive smoking) also increases the risk of pneumonias, middle ear infections, meningitis and asthma in children who are exposed to the smoke of adults (Surgeon General US, 2014).

In addition to tobacco-related diseases in smokers, passive smokers and developing fetuses of pregnant women who smoke, tobacco presents several hazards to those who cultivate and harvest the plant. Tobacco production presents some unique hazards, most notably acute nicotine poisoning, particularly in the form of a condition known as green tobacco sickness (GTS). GTS is a unique occupational nicotine poisoning associated with tobacco farming. Nicotine from tobacco leaves leaches out into moisture or rain water on the surface of the leaves, significantly increasing risk of GTS. Moisture on tobacco leaves from dew or rain may contain as much as 9 mg of dissolved nicotine per 100 mL of dew, roughly equivalent to the nicotine content of six average cigarettes. Green tobacco sickness occurs when workers, especially young children, absorb nicotine through the skin as they come into contact with moisture on leaves of the mature tobacco plant. GTS is characterized largely by nausea, vomiting, headache, muscle weakness, and dizziness (McKnight and Spiller 2005).

Other problems associated with tobacco include the use of child laborers in tobacco farms and other areas of the tobacco industry. Millions of children work as child laborers in many countries, and are not only exposed to green tobacco sickness, but also to occupational injuries and abuse. These children lose out on an education and a healthy and happy upbringing that all children deserve and human rights of children are abused by the tobacco industry.

1.3. Co-epidemics of smoking with HIV and TB

It has become clear that smoking and its health hazards cannot be clearly separated from other diseases, including infectious diseases such as pneumonias, HIV and TB, and that these diseases are part of a co-epidemic along with smoking. There is evidence that cigarette smoking increases the risk that someone will contract HIV or TB; increases the risk of a person with HIV or TB becoming ill from the diseases; increases the risk that a person will die from HIV/TB; increases the incidence and severity of side-effects of drugs used to treat HIV/TB; and increases the risk that drugs used to treat HIV/TB will not work as well and will be less effective (Rajendiran *et al*.,2013).

The association between tobacco smoking and tuberculosis was suggested many years ago(Webb, 1918). Evidence of the impact of tobacco smoking on TB infection has been confounded by it is almost universally association with poverty, overcrowding and alcohol usage. Both tobacco smoking and HIV infection may also be associated through their common associations with poverty and high-risk behavior. Tobacco smoking appears to be an independent and important risk factor for contracting HIV (Boulos *et al*., 1990, Halsey *et al*.,1992). Other studies have demonstrated higher viral loads in HIV patients who smoke (Wojna *et al*., 2007) and rate of progression of HIV infection to AIDS in smokers (Feldman *et al*., 2006), but this association has not been observed in all studies. Smoking further raises the extremely high risk of contracting TB in HIV-positive persons (Miguez-Burbano *et al*., 2003), in addition to increased susceptibility of community-acquired bacterial pneumonia (Van Zyl-Smit *et al*., 2010).

There are many carcinogens and toxic agents in cigarette smoke that make it a leading cause of death in the US. Nicotine is the addictive agent while tobacco interrupts innate and adaptive immunity, an important problem for people who are already immune-suppressed, including TB and HIV patients. The combination of being HIV positive and a smoker accounts for the largest number of lost lives/year among HIV positive patients. Smoking cessation causes tangible health benefits for HIV patients. Smoking increases HIV-related morbidity and mortality, with infectious complications, lung diseases and increased risk of neuro-cognitive impairment. Smoking causes increased white blood cell counts and carbon monoxide that converts hemoglobin to carboxy-hemoglobin, which produces reactive oxygen species that abnormally activate cell signaling pathways(Van Zyl-Smit *et al*., 2013).

These findings emphasize the importance of providing HIV and TB patients with relevant knowledge about the impact of smoking on their disease and its treatment. Multiple strategies will likely need to be combined to reduce the impact of smoking on TB and HIV, such as counseling of patients to quit or not to start smoking, and using pharmacologic approaches to help them quit smoking. The high prevalence of smoking among this population, the multiple health risks that can result from it, and the demonstrated success of cessation efforts in these patients, should encourage clinicians and other HIV/TB service providers to makes smoking cessation for their patients and clients a high priority(Van Zyl-Smit *et al*., 2013).

1.4. Components of cigarettes

1.4.1. Tobacco

Tobacco plants belong to the nightshade family, *Solanaceae*, and therefore are related to the tomato and potato. Tobacco belongs to the genus *Nicotiana.* There are many species of tobacco plant but *N. tabacum* is the most common source of commercial tobacco. *N. rustica* contains a higher proportion of nicotine in its leaves but it is rarely used as a source of tobacco for smoking (Hecht *et al*., 1998). Tobacco is used in two major forms: smoking (combustible) tobacco and smokeless tobacco. Smoking tobacco includes manufactured cigarettes, bidis, cigars, kreteks, pipes and shishas. A manufactured cigarette consists of shredded or reconstituted tobacco processed with hundreds of additive chemicals. Most often with a filter, cigarettesare manufactured by machines, and are by far the predominant form of tobacco used worldwide.

Smokeless tobacco includes chewing tobacco, snus or moist snuff, (which is placed in the mouth from where the nicotine and other products are absorbed) and dry snuff, which is usually taken in through the nose by sniffing. Pan massala, or betel quid, used in India, consists of tobacco, areca nuts and slaked lime wrapped in a betel leaf, often with added herbs and spices. Chewing tobacco can also contain sweetening and flavoring agents. Tobacco lozenges are also a form of smokeless tobacco (Brundtland, 2002).

1.4.2.Chemical compounds in cigarettes

Chemicals in cigarettes can be grouped into non-harmful chemicals, harmful chemicals (see Table 1 for examples) that may cause cancer, or may contribute for example to cardiovascular disease and emphysema; and addictive components, the major addictive component being nicotine.

There are about 70 known carcinogens in tobacco smoke. Some of them are listed in Table 2. These include nitrosamines, polycyclic aromatic hydrocarbons, and heavy metals. Carcinogens that are found in both combustible and smokeless tobacco include aflatoxins, amino-biphenyl, benzene, cadmium, chromium, naphthylamine, nickel, polonium-210, vinyl chloride, acrylonitrile,benzo[a]anthracene,benzo[a]pyrene, 1,3-butadiene,dibenz(a,h)anthracene, formaldehyde, N-nitrosodiethylamine and N-nitrosodimethylamine(Mahood, 1999).

Benzo[a]pyrene is one of the most potent of all known carcinogens in tobacco. Most of the carcinogenic compounds identified in cigarette smoke are not present in the native tobacco leaf but are formed by pyrolysis at the high burning temperature of cigarettes. It appears that the pyrolysis of many organic materials can lead to the formation of components which are carcinogens. Cigarette paper consists essentially of cellulose. Pyrolysis of cellulose has been shown to produce benzo[a]pyrene.

Table 1.List of some toxic constituents of cigarettes

Compound	µg/Cig	Compound.	µg/Cig
Nicotine	100-3000	Scopoletin	15-30
Dihydroxybenzenes	200-400	Alkaloids,solanesol	1000
Bipyridils	10-30	Neophytadienes	200-350
Total non-volatile HC	300-400	Limonene	30-60
Naphthalene	2-4	Palmitic Acid	100-150
Naphthalenes	3-6	Stearic Acid	50-75
Phenanthrene	0.2-0.4	Oleic Acid	40-110
Anthracenes	0.05-0.1	Linoleic Acid	150-250
Fluorenes	0.6-1.0	Linolenic Acid	150-250
Pyrenes	0.3-0.5	Lactic Acid	60-80
Fluoranthenes	0.3-0.45	Indole	10-15
Carcinogen PAH	0.1-0.25	Skatole	12-16
Phenols	80-180	Quinolines	2-4

Tobacco specific nitrosamines (TSNA), which are highly carcinogenic, are only found in tobacco products(Hecht and Hoffmann 1988). There is no safe level of exposure to the most potent of the TSNAcarcinogens. Besides the TSNA, non-tobacco specific nitrosamines are also formed duringthe smoking process. Nitrosamines contain the organic functional group N-N=O, and are formed by the nitrosation (addition of an N=O group) of secondary and tertiary amines. The nitrosating agent is nitrite, derived from tobacco nitrate by the action of bacteria and tobacco enzymes during curing. In tobacco, these amines are nicotine, nornicotine, anabasine, and anatabine (alkaloids). When tobacco burns during cigarette smoking, the tobacco specific nitrosamines can transfer to smoke and decompose thermally; additional nitrosamines can form by combustion.TSNA are created during fermentation, curing and burning of the tobacco leaf.

Tobacco also contains fungal-derived mycotoxins (aflatoxins, AF type B1, a powerful human carcinogen). In 1991, Varma reported the isolation of nine species of *Aspergillus* in stored leaves of chewing tobacco (Varma *et al*., 1991). Approximately 18 of the *Aspergillus* species were found to be mycotoxigenic. All aflatoxigenic strains of *A. flavus* produced aflatoxin B1, one of the most powerful carcinogens known to man. (Pauly and Paszkiewicz 2011).

The aflatoxins by *Aspergillus*fungi forms adduct in the p53 tumor-suppressor gene that mutates in approximately half of all human cancers. Aflatoxin B1 binds to the middle and third positions of p53 codon 248, inducing G-T transversions associated with lung cancer, and binds strongly to the third base pair of codon 249, generating a G-T transversion in a liver cancer mutational hotspot.

Table 2. Examples of carcinogens found in cigarette smoke.

PAHs	**N-Nitrosamines**
Benz[a]anthracene	N-Nitrosodimethylamine
Benzo[b]fluoranthene	N-Nitrosoethylmethylamine
Benzo[j]fluoranthene	N-Nitrosodiethylamine
Benzo[k]fluoranthene	N-N itrosopyrrolidine
Benzo[a]pyrene	N-Nitrosodiethanolamine
Dibenz[a,h]anthraceneDibenzo[a,i]pyrene	N-Nitrosoarcosine
Dibenzo[a,l]pyrene	N-Nitrosonornicotine
Indeno[1,2,3-cd]pyrene	N'-Nitrosoanabasine
5-Methylchrysene	N'-Nitrosamorpholine
Aza-arenes	Aromatic Amines
	2-Toluidine
QuinolineDibenz[a,h]acridineDibenz[a,j]acridine	2-Naphthylamine
7H-Dibenzo[c,g]-carbazole	4-Aminobiphenyl

Tobacco smoking also exposes smokers and passive smokers to nickel toxicityand other carcinogenic heavy metal ions. Acute toxicity to nickel from smoking cigarette results frominhalationof the nickel in tobacco smoke. The primary route for the toxicity of nickel is though the depletion of glutathione levels and bonding to the sulfhydryl groups of proteins. (Das andBüchner 2007).

In addition, tobacco and tobacco smoke also contain up to 600 additives: these are substances that are not natural components of tobacco or tobacco smoke but are added to tobacco for various reasons. Over 7000 chemicals are introduced to the environment via cigarette particulate matter (tar) and mainstream smoke. Tobacco smoke also includes toxic volatile small molecules, including acetone, ammonia, carbon monoxide, cyanide, methane propane and butane (Chen *et al*., 2008).

Some additives are used to make cigarettes that provide high levels of 'free base' nicotine, whichincreases the addictive 'kick' of the nicotine. Ammonium compounds, which are added to tobacco, can fulfill this role by raising the alkalinity of cigarette smoke.When added to a tobacco blend, ammonia reacts with the nicotine salts and liberates free [base] nicotine, which is volatile and easily absorbed through biological cellmembranes, mucous and alveolar membranes.When used as an additive in tobacco, ammonia causes nicotine to convert more to its free base, volatile form (see Figure 1). This change results in more nicotine being delivered to the smoker (Geissand Kotzias 2007).

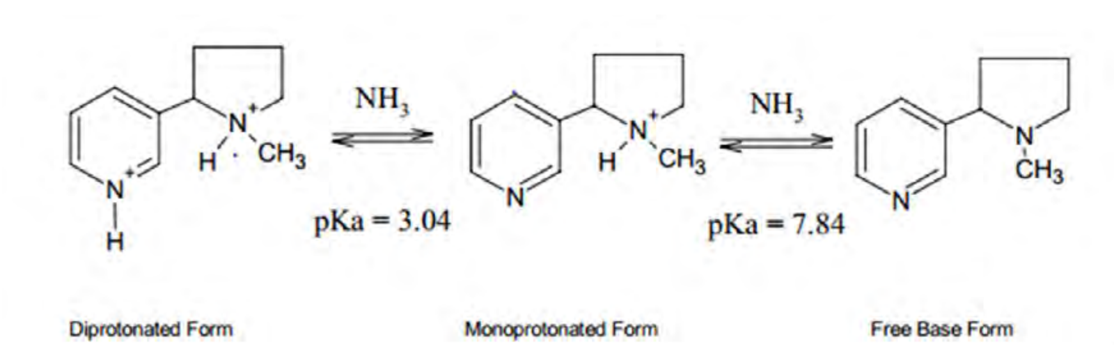


Figure 1. Alkalinization of nicotine by ammonia to its free base form

Additives are also used to mask the smell and visibility of side-stream smoke, undermining claims that smoking is antisocial without at the same time reducing the health risks of passive smoking. (Bates *et al*., 1999).

Additives are used also to enhance the taste of tobacco smoke, to make the product more desirable to consumers. Sweeteners and chocolate may help to make cigarettes more palatable to children and first time users. Eugenol and menthol numb the throat so the smoker cannot feel the smoke's aggravating effects, and also provide a “cool” flavour. Additives such as cocoa may be used to dilate the airways allowing the smoke an easier and deeper passage into the lungs exposing the body to more nicotine and higher levels of tar.

1.5. Nicotine, Nicotine Addiction and Dependence

1.5.1. Nicotine and its molecular targets

Nicotine is a naturally occurring alkaloid found in tobacco plants. In humans, when nicotine is inhaled, it rapidly enters the bloodstream, crosses the blood–brain barrier and reaches the central nervous system (CNS) where it acts as a stimulant **(**Hukkanen*et al*., 2005).

Nicotine can affect the levels of multiple neurotransmitters in addition to dopamine (Figure 2), and these can have multiple effects on central and peripheral neurologic circuits as well as neuro-endocrine systems.Activation of dopamine production is a key component in the addictiveness of nicotine.

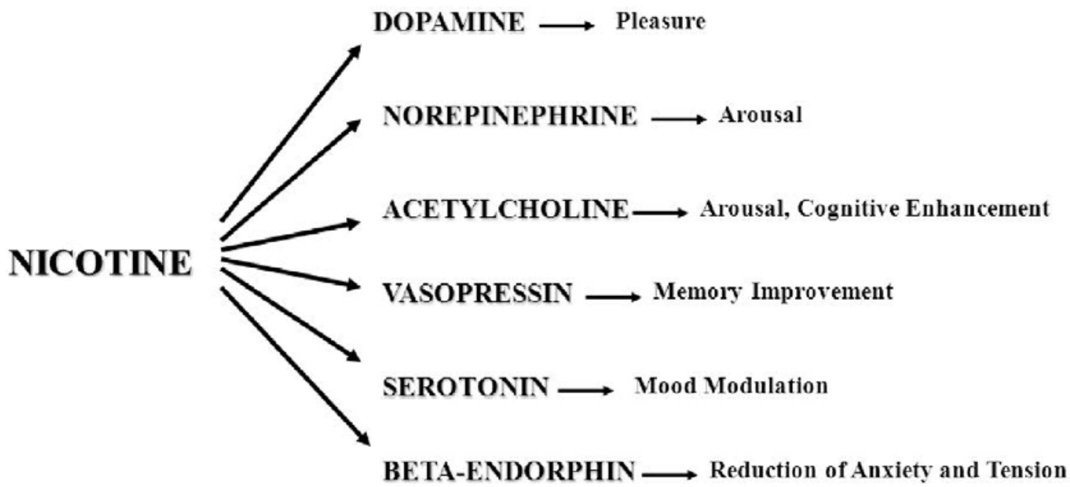


Figure 2.Some neuro-hormones and neurotransmitters activated by nicotine

1.5.2. Nicotine and its effect on nicotinic acetylcholine receptors (nAChR)

It has been documented that nicotine crosses biological membranes and the blood brain barrier easily(Cobb *et al.,* 2010). Nicotine acts in the brain through the neuronal nicotinic acetylcholine receptors (nAChRs).The nAChRs are allosterically regulated, ligand-gated ion channels consisting of five membrane spanning subunits.The predominant nAChR subtypes in mammalian brain are those containing α4 and β2 subunits (denoted as α4β2 nAChRs). The α4β2 nAChRsand α7 nAChRs mediate many behaviors related to nicotine addiction, and some of these receptors are the primary targets for currently approved smoking cessation agents.

The binding of nicotine to a nAChR mediates the release of neurotransmitters, either by promoting membrane depolarization and opening of voltage activated calcium channels (which causes exocytosis of neurotransmitters), or by the intrinsic calcium permeability of the nAChR (Benowitz, 2010; Dajas-Bailador*et.al*., 2004).

One neurotransmitter of importance to nicotine addiction is dopamine, which is critical in the acute reward pathways associated with abuse of nicotine (Nestler, 2004). Nicotine stimulates dopaminergic transmission in the ventral tegumental area (VTA) of the midbrain, which in turn activates the nucleus accumbens, an area critical for nicotine-mediated physiological effects such as addiction, pleasure, and reward (Changeux*et al.,* 2010;Nestler, 2005).Another function of nicotine is to facilitate the release of glutamate from the amygdala, which further activates the dopaminergic neurons of the VTA.

In addition, nicotine activates GABA-ergic neurons, which inhibit dopamine release from the VTA. Chronic stimulation of nAChRs during nicotine addiction will desensitize the GABA-ergic neurons, which lose their inhibitory effect on dopamine release (Dajas-Bailador *et al.,* 2004; Laviolette *et al.,* 2004;De Rover *et al*., 2004). Through time, as the GABA-ergic response decreases, the dopaminergic response is heightened; further potentiating the addictive effects associated with nicotine.Chronic exposure to nicotine can cause desensitization of nAChRs, resulting in neuro-adaptation, or tolerance, to the effects of nicotine.

During neuro-adaptation, the total number of nAChRs increases, and this is thought to be a result of increased nicotine-mediated nAChR inactivation (Wang and Sun 2005; Govind*et al.,* 2009).The resulting unresponsiveness of the receptors plays a role in the development of addiction. For example, feelings of craving and withdrawal arise when $\alpha4\beta2$nAChRs, desensitized in smokers from chronic nicotine exposure, become sensitized during periods of abstinence, such as during sleep (Govind*et al.,* 2009). Reintroduction of nicotine to these receptors decreases the feelings of withdrawal.

People initially start smoking for different reasons, but whatever the reason might be once they start to smoke they are likely to continue smoking. The primary reason why people continue to smoke is that they are nicotine-dependent. When inhaled, nicotine reaches the brain in 10 to 16 seconds (faster than if it was delivered intravenously), and has a terminal half-life of about two hours. Given this short half-life, regular cigarettes are required to maintain nicotine levels and avoid symptoms of withdrawal. (McEwen*et al*.,2008).

Distinct definitions or criteria for drug dependence or addiction have been put forth by different health organizations or authorities. Most of the time the terms "dependence" and "addiction" are used interchangeably, but dependence is generally a milder form of reliance on the drug of abuse, whereas addiction involves extreme dependence and drug-seeking behaviour. These terms are considered equivalent because they describe similar neuro-chemical and psychological processes that sustain drug use(Surgeon General, 2014).Furthermore, both terms indicate loss of control over drug-taking behavior the principal characteristic of drug addiction. In both cases the substance use is continued despite knowledge of having had a persistent or recurrent physical or psychological problemthat is likely to have been caused or exacerbated by the substance. Thus, once a person is addicted to nicotine, it is difficult to stop using it even when there are compelling reasons to do so. Drug dependence has been defined by the World Health Organization (WHO) as "a behavioral pattern in which the use of a given psychoactive drug is given a sharply higher priority over other behaviors which once had a significantly higher value.”(Edwards *et al*., 1982).

Tolerance is defined by either need for markedly increasing amountsof the substance over time to achieve the desired effect or markedly diminished effect with continued use of the same amount of the substance.

Withdrawal is manifested by either the characteristic withdrawal syndrome for the substance or the same (or closely related) substance, which is taken to relieve or avoid withdrawal symptoms. In the case of nicotine use, withdrawal symptoms include in feelings of anxiety, tremors, depression, anger and irritability.

1.5.3. Nicotine metabolism

Nicotine absorption can occur through the oral mucosa,skin, lung, urinary bladder, and gastrointestinal tract(Schevelbein*et al*., 1973). The rate of nicotine absorptionthrough biological membranes is a pH dependentprocess. The presence of both apyrrolidine and a pyridine nitrogen means that nicotine isdibasic with pKa of 7.84 and 3.04 at 25°C. The proportionof uncharged nicotine increases as the pH of the solutioncontaining nicotine increases whereas the proportion ofcharged nicotine increases as the pH of the solutioncontaining nicotine decreases.The rate of nicotineabsorption through biological membranes thus increases asthe pH of the aqueous solution increases, whereas nicotineabsorption decreases as the pH decreases. The absorption ofnicotine through the oral mucosa has been shown to be theprincipal route of absorption for smokers who do not inhaleand for smokeless tobacco users, whereas absorption through the lung alveoli is the main route of nicotine absorption in smokers who inhale(Yildiz, 2004).

Nicotine is metabolized in the liver by the cytochrome P450 enzymes CYP2A6 and CYP2B6 to form a variety of metabolites, 70 to 80% of which are converted initially, in humans, to cotinine, which is excreted in the urine (see Figure 3).

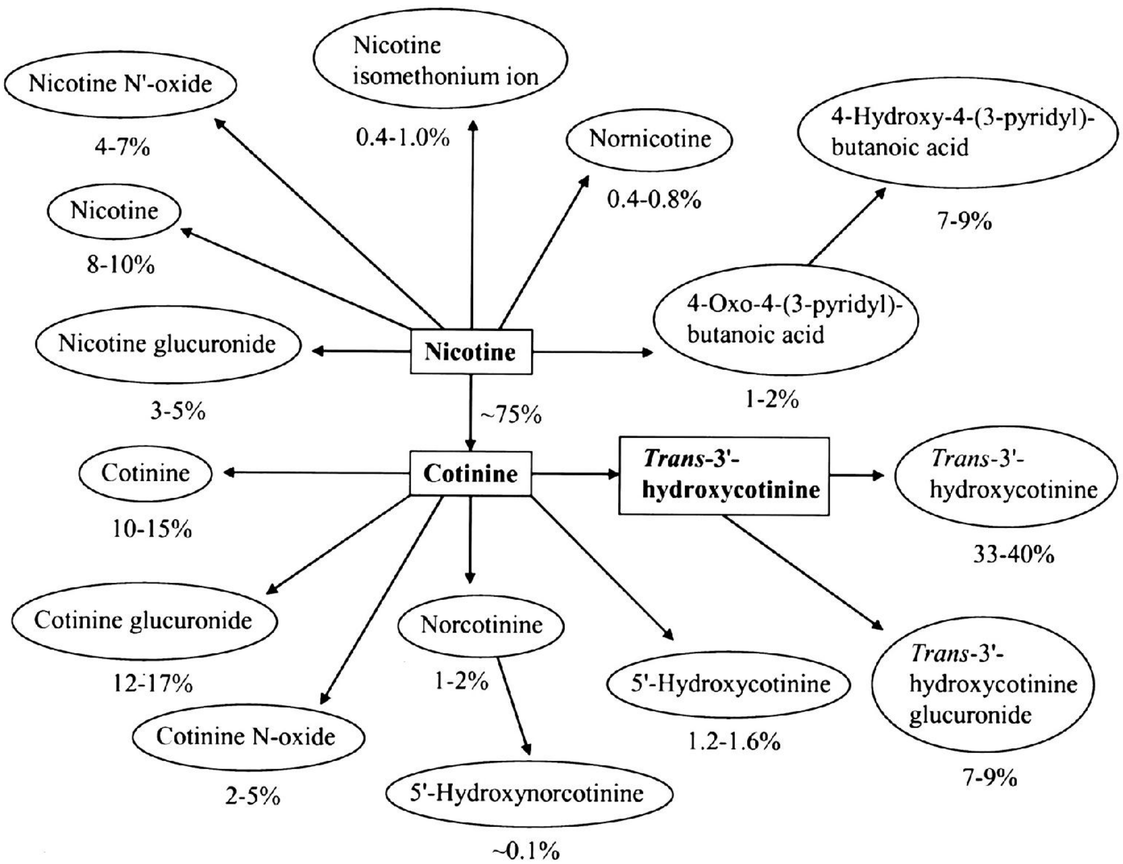


Figure 3.Various metabolites of nicotine after being metabolized in the liver by the cytochrome P450 enzymes CYP2A6 and CYP2B6

Quantitatively, the most important metabolite of nicotine in most mammalian species is the lactam derivative, cotinine. This transformation involves two steps. The first is mediated primarily by CYP2A6 to produce nicotine-Δ1′(5′)-iminium ion, which is inequilibrium with 5′-hydroxynicotine. The second step is catalyzed by a cytoplasmic aldehydeoxidase(Shigenaga*et al.,* 1988).Although on average about 70–80% of nicotine is metabolized via the cotinine pathway in humans, only 10–15% of nicotine absorbed by smokers appears in the urine as unchanged cotinine (Benowitz *et al*., 1988). Six primary metabolites of cotinine have been reported in humans: 3′-hydroxycotinine (McKennis *et al.,* 1963; Neurath *et al*., 1987), 5-hydroxycotinine (also called allohydroxy cotinine) (Neurath, 1994), cotinine N-oxide, cotinine methonium ion, cotinine glucuronide, and norcotinine.3′-Hydroxycotinine is the main nicotine metabolite detected in smokers' urine. It is alsoexcreted as a glucuronide conjugate (Benowitz *et al*., 1988). 3′-Hydroxycotinine and itsglucuronide conjugate account for 40–60% of the nicotine dose in urine (Byrd *et al*., 1992).

1.5.4. Gateway hypothesis of addictive substances

Epidemiologic studies have shown that nicotine use is a gateway to the use of marijuana and cocaine in human populations.The gateway hypothesis was developed by Denise Kandel, who observed that young people become involved in abuse of drugs in stages and sequences**.**(Kandel, 2002)a well-defined developmental sequence of drug use occurs that starts with a legal drug and proceeds to illegal drugs. Specifically, the use of tobacco or alcohol precedes the use of marijuana, which in turn precedes the use of cocaine and other illicit drugs(Huizink *et al*., 2010).

Scientists explored the learning mechanisms underlying addiction in the striatum, a critical brain area that is targeted by drugs of abuse. They found that the same molecular steps Kandel had delineated as underlying memory also play a major role in addiction**.** CREB-binding protein controls response to cocaine by acetylating histones at the fosB promoter in the mouse striatum: activation of CREB and the transcription of downstream target genes such as FosBand its isoform ΔFosB. The accumulation of ΔFosB in the striatum is a crucial step in establishing addiction to most drugs of abuse and has been used as a molecular marker for these processes and structural changes occur in the chromatin of FosBin response also to cocaine. (Hyman *et al*.,2006).

Nicotine acts as a gateway drug and exerts a priming effect on cocaine in the sequence of drug use through global histone acetylation in the striatum, specifically inhibiting deacetylation of histones associated with FosB expression; this increases, epigenetically, histone acetylation and activation of FosB expression, which leads to altered control of multiple genes associated with an addictive phenotype (Levine *et al*., 2011).

Of importance to Ethiopia in particular is the possibility that chewing khat might be a gateway drug to cigarette smoking. Cigarette smoking is associated with khat chewing (Tesfaye*et al*., 2008). A study of Yemeni diaspora in the UK indicated that khat chewing was associated with an increased initiation of subsequent cigarette smoking, suggestive that khat may be a gateway drug for initiating cigarette smoking. This would be very important in Ethiopia, where khat chewing is common, and so might exacerbate the tobacco epidemic in Ethiopia (Kassim *et al*., 2014).

1.5.5. Toxicity of nicotine, other than addiction

Although nicotine is not considered to be the direct major cause of most tobacco-related diseases, it is the main addictive component of tobacco and therefore is an indirect cause of most tobacco-related diseases; without nicotine, there would be far fewer smokers and hence a much reduced prevalence of tobacco-related diseases. However, evidence is accumulating that nicotine is not simply addictive, but has many other pathological activities.

Several studies find that nicotine can acutely increases activity in the prefrontal cortex and visual systems. There is release of a variety of neurotransmitters important in drug-induced reward. Nicotine also causes an increased oxidative stress and neuronal apoptosis, DNA damage, reactive oxygen species and lipid peroxide increase. nAChRs were originally thought to be limited to neuronal cells, however, studies have identified functional nAChRs in tissues outside the nervous system. Actions on nicotinic receptors produce a wide variety of acute and long-term effects on organ systems, cell multiplication and apoptosis, throughout the body.

Nicotine on direct application in humans causes irritation and burning sensation in the mouth and throat, increased salivation, nausea, abdominal pain, vomiting and diarrhea. Gastrointestinal effects are less severe but can occur even after cutaneous and respiratory exposure. Predominant immediate effects as seen in animal studies and in humans consist of increase in pulse rate and blood pressure. Nicotine also causes an increase in plasma free fatty acids, hyperglycemia, and an increase in the level of catecholamines in the blood. There is reduced coronary blood flow but an increased skeletal muscle blood flow. The increased rate of respiration causes hypothermia, a hypercoagulable state, decreases skin temperature, and increases the blood viscosity. (Jolma *et al*., 2002)

Nicotine is one of the most toxic of all poisons and has a rapid onset of action. Apart from local actions, the target organs are the peripheral and central nervous systems. In severe poisoning, there are tremors, prostration, cyanosis, dypnoea, convulsion, progression to collapse and coma. Even death may occur from paralysis of respiratory muscles and/or central respiratory failure. (Mishra *et al*., 2015).

1.6. Cigarette Filters

Cigarette filters were added to cigarettes in the 1950s, when it was becoming clear that smoking was seriously harmful to smokers' health, and nowadays most cigarettes possess filters. The idea that cigarette filters reduce the harm of smoking has now been refuted. Smokers compensate for the filtering action by increasing the number of cigarettes they smoke, inhaling more strongly, and covering ventilation holes in filters with their fingers to prevent loss of smoke. This is all subconscious behavior related to the need for more nicotine, to which smokers are addicted (Smith *et al*., 2011). The filter's cellulose acetate fibers can even enter the lungs of smokers (Novotny *et al*., 2009). In addition, the consumption of filter-tipped cigarettes has grown astonishingly to an annual amount of about 4.5 trillion in the world every year (Lee, 2012)**.**

Campaigners have sought to reduce the amount of nicotine in cigarettes as well, but the tobacco industry has tended to avoid this because it reduces addictiveness, and therefore profitability of cigarettes. (Keeler *et al*., 1993; Harris, 2011).

Over 95% of cigarette filters are made from cellulose acetate. Cellulose acetate is prepared by acetylating cellulose. Each glucose residue in cellulose can be acetylated at three possible hydroxyl residues (carbon atoms 2, 3, and 6), and commercial cellulose acetate varies in the degree of acetylation according to the properties required (Figure 4; Novotny*et al*., 2009; Xiao*et al*., 2011).

Cellulose is readily biodegraded by organisms that utilize cellulase enzymes, but due to the additional acetyl groups, cellulose acetate cannot degrade cellulose, and requires the prior action of deacetylases for biodegradation. Once partial deacetylation has been accomplished, the polymer's cellulose backbone is readily biodegraded by cellulases. Cellulose acetate is also photo-chemically degraded by UV wavelengths shorter than 280 nm, but has limited photo-degradability in sunlight due to the lack of chromophores for absorbing ultraviolet light. Photo-degradability is significantly enhanced by the addition of titanium dioxide, which is usedas a whitening agent in many consumer products. The combination of both photo-degradation and biodegradation allows a synergy that enhances the overall degradation rate of cellulose acetate filters.

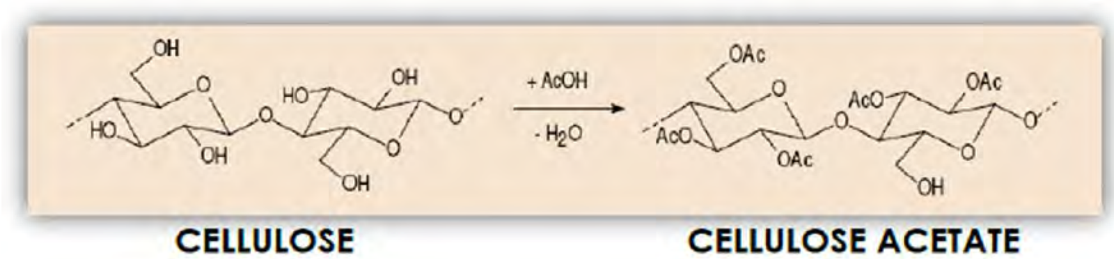


Figure 4 acetylation of cellulose to cellulose triple acetate

1.7. Environmental toxicity of cigarette butts

Cigarette butts are an environmental toxic waste due to the persistence of their chemicals and their cumulative effect when they are discarded into the environment. Most of these chemicals come from treatments used in growing tobacco. Heavy metals from soil, pesticides, insecticides,herbicides, and fungicides are present in tobacco products (Micevska*et al*., 2006).Processing ingredients such as brightening chemicals on cigarette paper also lends to the toxicity of cigarettes (Iskander*et al*., 1986). Smoked cigarettes butts contain numerous chemicals, such as ammonia, formaldehyde, butane, acrylonitrile, toluene, benzene, alkaloid nicotine (Schneider *et al*., 2011**)**. Moerman and Potts (2011) analysis on metals leached from smoked cigarette litter found a positive correlation between the concentration of several metal ions and the soaking time of cigarette butts. They concluded that cigarette litter is a point source of barium, iron, manganese, and strontium contamination for at least a month (Moerman and Potts, 2011). Cigarettes butts may seem harmless because of their size, but they are toxic and persistent in our environment. One cigarette butt may not do much damage, but trillions will. Cigarette butts are small in size but are packed with various chemicals that have consequences which are only recently being researched.

Small animals are often the receiving end of leached chemicals and toxins. Small animals may be especially harmed by nicotine, ethylphenol, and other organic compounds in cigarettes (Micevska*et.al..,* 2006).Ethylphenol, which is commonly used to flavor tobacco, can accumulate to such high levels in small animals that they exceed the concentration in the surrounding environment (Clark *et al.,*1996).

Cahn and colleagues examined the toxic effects of smoked cigarette butts (smoked filter and tobacco), smoked cigarette filters (no tobacco), and unburnt, unsmoked cigarette filters (no tobacco) on topsmelt and fathead minnows (Cahn *et al*., 2011; Slaughter*et al*., 2011).In this study, a single smoked cigarette butt leached into one litre of water was enough to kill a small fish. Unsmoked cigarette filters containing no tobacco were also toxic to fish, which indicates that chemicals used in manufacturing of filters are harmful even before the filters absorb chemicals when smoked.

Birds, especially in cities, are using cigarette butts to line their nests. A study of birds in Mexico suggests that there is a short-term benefit of using cigarette butts to line the nests, namely the reduction of ecto-parasites that affect birds, probably from the toxicity of nicotine to ecto-parasites. However, mutagenic chemicals in cigarette butts in the nest causes genotoxic damage and induced chromosomal breaks in the birds and their offspring. The greater the amount of cellulose acetate from cigarette butts in the nest, the more erythrocytes showed signs of such genotoxicity (Suárez-Rodríguez*et al*., 2014). Mutagens cause DNA damage and impairment of cytokinesis and is a frequent consequence of oxidative stress caused by other chemicals in cigarette butts (Fenech, 1993; Risom *et al*., 2005).

Smoked cigarette filters can cause harm in the marine environment in several ways. They present a vector for the transport and introduction of toxicants, including heavy metals, nicotine and known carcinogensto aquatic habitats (Moriwaki*et al*.,2009). Exposure to such toxicants in seawater could occur following the dissolution of compounds from the bioplastic filter to the surrounding seawater (leaching). Dietary exposure could occur through the ingestion of smoked cigarette filter microfibers due to filter degradation. If ingested, there is potential for the transfer of adhered toxicants to tissues. Thecellulose acetate microfibers of cigarette butts and their associated toxicants may persist in the marine environment and continue leaching chemicals for years, and are constantly being replenished as smokers discard their smoked cigarette butts (Novotnyand Slaughter 2014).

Exposure to cigarette butt and filter toxicants in seawater have a significant negative effect on the growth rateand weight of ragworms, and induce DNA damage in ragworms(Wright *et al.,* 2015).

1.8. Tobacco Regulation in Ethiopia

In Ethiopia there is a proclamation on Part three article 35 sub-article 1-3 and article 36 of Federal NegaritGazeta of The Federal Democratic Republic of Ethiopia describes the laws that should be implemented concerning 'Tobacco Packaging and Labelling of Tobacco Products and on the Places Prohibited for Smoking'(Federal NegaritGazeta, 2014). But regardless of the proclamation people smoke in public places such as hotels, cafeterias, taxis, schools and even in the hospital campuses outside of the designated smoking areas. It is not only the smoking which is bad, but also the trash which makes places look dirty and may cause environmental problems. It is illegal in some other parts of the world to throw cigarette butts / filters on the ground, and trash bins are widely available(Liu*et al*.,2015).In Ethiopia some rules relating to tobacco advertising, marketing and sales, smoking in public places, although they exist, there is a problem in the their enforcement and implementation.

1.9. Statement of the problem

Owing to the ubiquitous nature and magnitude of cigarette butts discharged into the environment, there are relatively few studies on environmental problems associated with cigarette butt waste, and no studies have been done in Ethiopia. Also, there are no published reports on the effect of environmental cigarette butts on mammals. So more studies are needed to determine whether cigarette butt waste can exert ecotoxic effects in the environment and whether they are toxic to small mammals. Therefore, this MSc project was done to initiate research into cigarette butts in Ethiopia specifically, and is also the first study done inside or outside of Ethiopia that is aimed at evaluating the toxicity of environmental cigarette butts to mammals

1.10. Objective of the study

General:

- To investigate the toxicity of leachates of cigarette butt on Swiss albino mice

Specific:

- To investigate the effect of cigarette butt Leachate on mice fluid and food intake
- To investigate the effect of cigarette butt Leachate on blood glucose and liver function tests
- To investigate the effect of cigarette butt Leachate on histology of mice
- To investigate the effect of cigarette butt Leachate on weight of the mice

Chapter 2

Materials and Methods

2.1. Study setting and time period

This study of the toxicity of cigarette butt leachates on laboratory Swiss albino mice, bred in the animal house of the Biochemistry Department, Addis Ababa University Medical School, was conducted from December 2014 to June 2015 G.C in the Biochemistry Department, Addis Ababa University Medical School. Serum tests were done in the Tikur Anbessa Hospital diagnostic laboratory. Cigarette butts were collected from the Tikur Anbessa hospital campus grounds and from the streets of Haya Hulett, Addis Ababa.

2.2. Laboratory mice

Laboratory Swiss albino mice were used for this study and were obtained from the Pasteur institute Addis Ababa. They were given two weeks of acclimatization period in the Biochemistry Department animal house, School of Medicine, Addis Ababa University. The animals were maintained under at ambient room temperature under natural day-night periods. The mice were randomly selected for study and housed in groups of five mice per cage with free access to water and standard mouse pellets. Standard mouse food was obtained from the Ethiopia Health Institution, Kality. Food and water were changed every two to three days. All ethical rules applying to animal safety and care were observed.

2.3. Ethical approval

Ethical approval was obtained from the Ethics Committee of the Department of Biochemistry, School of Medicine, Addis Ababa University (protocol number 04/2014).

2.4. Collection of cigarette butts and campus butt counts

Cigarette butts were collected randomly from two areas of Addis Ababa: the Tikur Anbessa Hospital campus designated smoking area and the streets of Haya Hulett in the vicinity of the Veronika Hotel. Butts at all levels of environmental decay were collected and stored for no longer than a week at room temperature before they were used to make leachates.

For determining Tikur Anbessa campus cigarette butt counts, the hospital campus was divided into five major areas (see Table 5, Results section) and each area was scanned by two individuals simultaneously, covering all ground possible, for visible cigarette butt counts. Counts from each area were added together to obtain a final campus butt count.

2.5. Preparation of cigarette butt leachates

Varying numbers of the cigarette butts, from 10 to 75, butts were used to make leachates. Debris such as small stones, grass and soil were removed manually from butts, then the appropriate number of butts was added to 250 mL of tap water in a glass beaker, shaken well and soaked for 24 hours at room temperature. After straining through a tea strainer to remove tobacco and butt debris, leachates were immediately given to the mice in place of their regular drinking water. Leachates in drinking bottles of the mice were freshly made and replaced every 3 days.

2.6. Measurement of fluid and food intake

For quantitative determination of fluid (water or leachate) uptake, fluid volumes in drinking bottles used by cages of five mice were accurately measured periodically and expressed as fluid drank per five mice (i.e. per cage) per day.

For quantitation of amount of food consumed, food pellets given to mice were weighed initially, then weights of food remaining were determined every two days, subtracted from the initial food weight, and expressed as grams of food consumed per five mice per two days.

2.7. Mouse necropsy

Mouse necropsy (autopsy) was performed at the end of each experiment, after mice were bled for serum tests. A horizontal incision in the lower abdomen area and a midline vertical incision were made to open up the peritoneal cavity and thoracic cavity and major organs (spleen, kidneys, liver, heart, lungs) as well as epididymal fat pads were removed, inspected for gross pathological abnormalities, and weighed. Tissue samples were also taken for basic histological processing and hematoxylin/ eosin staining.

2.8. Tissue histopathology

Mouse tissues (liver, kidney, lung and cardiac) were removed immediately from mice after terminal bleeding for blood tests. Tissues were sliced using a scalpel blade and fixed in 8 % buffered neutral formalin (formaldehyde solution). The specimens were treated with paraffin wax, then washed using standard histological procedures, then paraffin preparations were dehydrated sequentially in 70%, 80%, 95%, 100% ethanol for 1 hour at each step .

Tissues were then treated with xylene to remove ethanol from the tissue. The tissues were embedded in paraffin wax with the help of an Electro-thermal Wax Dispenser, to form tissue blocks in squared metallic plate block molds. A rotary microtome was used for sectioning tissue blocks manually at a thickness of 5μm and sections were transferred to microscope slides. Microtome sections of tissue were then stained using hematoxylin and eosin standard histological stains (Cardiff *et al*., 2014). A histopathologist, Dr Mulugeta Temesgen, from the Tikur Anbessa Hospital Department of Pathology helped with interpreting histopathology slides of tissues from mice in this study.

2.9. Blood collection and serum preparation

Mouse blood samples were collected directly from tail vein for blood glucose determination, by cutting, with a scalpel blade, a small amount (1 mm) of tissue from the end of the tail.

Larger amounts of blood were required for serum preparation and serum lipid liver enzyme tests. Blood was then collected from the facial/temporal vein, which allows 0.5 cc to 1.5 mL of blood to be obtained terminally (mouse will die usually from blood loss), but neck dislocation was performed after bleeding to reduce suffering. (Francisco *et al*, 2015).

The visible hairless freckle, corresponding to a sebaceous gland, was located on the side of the mouse’s jaw.A scalpel blade was used to make an incision about one third the way between the freckle and the inferior part of the ear, and drops of blood were collected by gravity into an Eppendorf tube. In this study, mice were terminally bled, allowing 0.5 to 1.5 mL of blood to be obtained. Mice were then humanely killed by neck dislocation.

Serum was prepared after coagulation of blood at room temperature for 45 to 60 min and centrifugation at 4000 rpm for 10 minutes. Serum was stored at -70 ^{o}C until blood tests were performed.

2.10. Blood glucose test

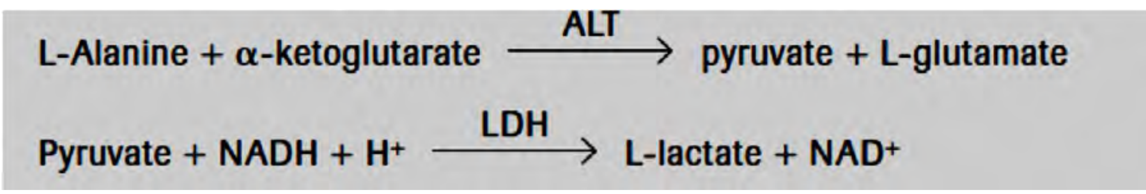
Fasting blood glucose was determined by tail vein venipuncture, using a commercial Sensocard glucometer.A drop of blood was obtained by tail vein bleed, after mice fasted for 4 hours, using a scalpel blade and cutting 1 mm off the end of the mouse tail, and was placed on a Sensocard test strip. Blood glucose concentrations were read from the glucometer.

The Sensocard glucometer is based on the principle that glucose oxidase, present in the test strip, converts blood glucose to gluconolactone and hydrogen peroxide.Electrons are generated by using an electron acceptor compound in the glucometer, generating an electric current that is converted to a digital signal that is proportional to the glucose concentration in the blood sample. The upper limit of detection of the Sensocard monitor is a blood glucose concentration of 600 mg/dL.

2.11. Serum alanine aminotransferase (ALT) test

Serum ALT level is probably the most specific test for liver damage; however, the severity of the liver damage is not necessarily shown by the ALT test, because with extensive liver fibrosis and loss of normal liver tissue, ALT levels can even be low. But ALT is widely used, and useful, because ALT levels are elevated in most patients with liver disease.

ALT catalyzes the transamination between L-alanine and α-ketoglutarate. The pyruvate formed is reduced by NADH in a reaction catalyzed by lactate dehydrogenase (LDH) to form L-lactate and NAD+. The rate of the NADH oxidation is directly proportional to the catalytic ALT activity. It is determined by measuring the decrease in absorbance at 340 nm (Ahn, J., 2011).



Procedure: Reagent and samples were pre-incubated at 37ºC. Serum samples (10 µL) and reagent containing necessary buffers and enzymes were mixed in an automatic chemistry analyser [Auto Lab 18 fully automated clinical chemistry analyzer (Italy)] that was standardized using known concentrations of ALT and absorbance decrease at 340 nm was measured automatically after 10 minutes. The analyser automatically calculates the ALT activity in the serum sample.

2.12. Serum aspartate aminotransferase (AST) test

Liver damage is also suggested by elevated AST even though it is less specific than ALT, because in may be raised with cardiac and skeletal muscle inflammation and with haemolysis.

Aspartate aminotransferase (AST) catalyzes the transamination of aspartate with α-oxoglutarate, forming L-glutamate and oxalacetate. The oxalacetate is then reduced to L-malate by malate dehydrogenase, while NADH is simultaneously converted to NAD^+.The decrease in absorbance due to the consumption of NADH is measured at 340 nm and is proportional to the AST activity in the serum sample (Ahn, 2011). The equation is illustrated as below.

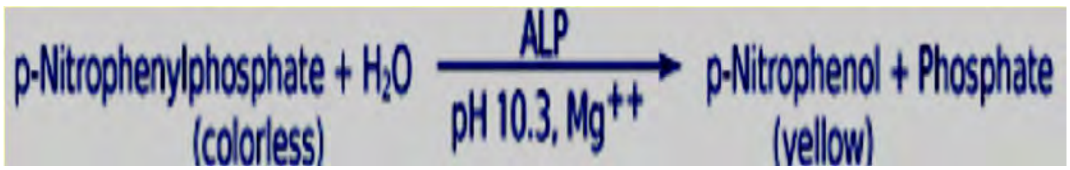
$$\text{L-Aspartate} + \alpha\text{-Oxoglutarate} \xrightarrow{\text{AST}} \text{L-Glutamate} + \text{Oxalacetate}$$

$$\text{Oxalacetate} + \text{NADH} + H^+ \xrightarrow{\text{MDH}} \text{L-Malate} + \text{NAD}^+$$

Procedure: Reagent and samples were pre-incubated at 37°C. Serum samples (10 µL) and reagent containing necessary buffers and enzymes were mixed in an automatic chemistry analyser [Auto Lab 18 fully automated clinical chemistry analyzer (Italy)] that was standardized using known concentrations of AST and absorbance decrease at 340 nm was measured automatically after 10 minutes. The analyser automatically calculates the AST activity in the serum sample.

2.13. Serum alkaline phosphatase (ALP) test

Alkaline phosphatase measurements are used in the diagnosis and treatment of liver, bone, parathyroid, and intestinal diseases. ALP is elevated significantly especially in hepatobiliary liver damage. ALP reagent is used to measure alkaline phosphatase activity by a kinetic rate method using a 2-amino-2-methyl-1-propanol buffer. In the reaction, alkaline phosphatase catalyzes the hydrolysis of the colorless organic phosphate ester substrate, p-nitrophenylphosphate, to the yellow colored product, p-nitrophenol, and phosphate. This reaction occurs at an alkaline pH of 10.3. The system monitors the change in absorbance at 410 nanometers (Ahn, 2011). This increase in absorbance is directly proportional to the activity of ALP in the serum sample and is used by the chemical analyser system to calculate and express ALP activity. The reaction is shown below:



Procedure: Reagent and samples were pre-incubated at 37ºC. Serum samples (10 µL) and reagent containing necessary buffers and enzyme were mixed in an automatic chemistry analyser [Auto Lab 18 fully automated clinical chemistry analyzer (Italy)] that was standardized using known concentrations of ALP and absorbance increase due to p-nitrophenol production at 410 nm was measured automatically after 10 minutes. The analyser automatically calculates the ALP activity in the serum sample.

2.14Statistical analysis

Values requiring statistical testing were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey's test. Graphs were constructed, andvalues of mouse body weights, fasting blood sugar and serum liver enzymes (ALT, AST, ALP) were expressed as mean ± Standard Deviation (SD), using SPSS statistical software Package Version 21.0. A p-value less than 0.05 was considered to be statistically significant.

Chapter 3

Results

3.1 Brands, types and abundance of cigarette butts collected

3.1.1. Brands and types of cigarette butt collected

Cigarette butts (a total of 342)were collected from the Tikur Anbessa hospital campus and from the streets of HayaHulett, and out of these, most (321/342, or 94%) of the cigarette butts with identifiable brand names (Figure 5 and Table 3) were those of the regular Nyala brand. Other brands found were Nyala Premium (8/342, 2%), Delight (7/342, 2%), Marlboro (3/342, 1%) and Rothman (3/342, 1%).No butts belonging to the other brand of cigarettes, Gisella, sold in Ethiopia were found: Gisella is not seen in the market in Addis Ababa. Somecigarette butts, chiefly ones where filters had decayed to their cellulose acetate remnants(about 17 %), were found without their brand labels being visible, so that their brand could not be identified. (See Figure 6 and Table 4).

Figure 5.Cigarette butts of different brands found on Tikur Anbessa campus and in HayaHulett area of Addis Ababa.

Table 3. Relative abundance of different cigarette brands among cigarette butts collected in Tikur Anbessa campus and HayaHulett.

Brand of cigarette	Count	Percentage (%)
Nyala	321	94 %
Nyala Premium	8	2 %
Delight	7	2 %
Marlboro	3	1 %
Rothman	3	1 %
Total	342	100 %

Different types of cigarette butts were found while collecting them, according to how much they were smoked before being discarded, whether tobacco had fallen out of them after being discarded, and to what extent they had decayed in the environment(Figure 5 and Table 4). Butts that had been smoked down to the filter without any substantial remnant tobacco left were the most abundant type of butt found (37% of total butts).Some butts (27.5% of total) had plenty of unburnt, unsmoked tobacco still associated with the butts.Some butts (18% of total) had white paper attached to the filter, but thetobacco had fallen out after the butt was discarded; indeed, just picking some butts up off the ground caused the dried tobacco to fall out easily. Others were smoked down to the filter but it appeared that the smoker had re-ignited the filter itself, presumably to get more smoking out of the cigarette, though these re-lit filters were rare (less than 1%

of the total butts collected). Some of the butts were well into the process of decaying (17% of total), and were at the stage of having lost their paper, orconsisted only of the cellulose acetate portion of the filter. At later stages of environmental decay, it was difficult to discern the cigarette brand.

Figure 6. Types of discarded cigarette butts, found in HayaHulett and TikurAnbessa Hospital Campus, Addis Ababa.

Cigarette butts were collected randomly. A, butts with unburnt, unsmoked tobacco present; B, decayed butt containing cellulose acetate component of filter only, with no remaining tobacco or paper; C, butt with attached paper, no tobacco present; D, butt smoked down to filter, then filter reignited; E, “end-on” view of butt with attached paper but with no tobacco; F, butts smoked down to filter. Note that brand name is missing from some butts (for example, B).

Table 4. Abundance of different types of cigarette butts, found in Tikur Anbessa campus and HayaHulett.

Type of cigarette butt	Count	Percentage of total butts
Butt smoked down to filter (no remaining tobacco or paper present)	127	37 %
Butt with unburnt, unsmoked tobacco still present	94	27.5 %
Butt with attached paper, no tobacco present*	61	18 %
Decayed butt with cellulose acetate only (no tobacco, no paper)	58	17 %
Cigarette butt smoked to filter, then reignited	2	0.5 %

*These were incompletely smoked cigarettes from which the tobacco had fallen out after they were discarded on the ground.

These butts were collected in February 2015 when the campus was not get officially smoke free. Results are for randomly picked butts.

Figure 7 shows the various stages of environmental decay of cigarette filters, as found in Tikur Anbessa campus's designated smoking area. Filters eventually lose their paper, leaving only the cellulose acetate component, and this gradually decays by photo-degradation and biodegradation. Cellulose acetate was previously thought not to be biodegradable, but recent evidence shows that it can be biodegraded by microorganismsin the environment that deacetylate the cellulose acetate using deacetylase enzymes, and then degrade the resulting cellulose using cellulases (Puls *et al*., 2011).

Figure 7. Various stages of cigarette butt decay in the environment.

From left to right, intact discarded cigarette butts showing decay to remnants of cellulose acetate component. These cigarette butts were collected from the designated smoking area of TikurAnbessa Hospital campus.

3.1.2. Survey of cigarette butt counts and distribution on Tikur Anbessa campus

A countof cigarette butts was done on Tikur Anbessa campus, Addis Ababa University. The campus was divided in to five main areas (Table 5), and cigarette butts were carefully counted in each area.

The total Tikur Anbessa campus count of cigarette butts was 3,747, as done in March 2015. Of these,2005 butts (53.5%) were counted in the grassy area opposite the

Emergency Room entrance, where many family members and friends of patients sit while patients arewaiting for evaluation and admission to hospital. A further 807butts (21.5 %) were inthe designatedsmoking area of the Tikur Anbessa campus, and 591 butts(15.8 %) were in the area around the soccer field and medical student dormitory area. The remaining butts were found on the pathway between upper and lower entrance gates (6.7%) and around the Orthopedic Clinic area (2.5%).

Table 5. Distribution and counts of cigarette buttson Tikur Anbessa Hospital campus.

Area of the campus	Cigarette butt count	Percentage of total (%)
Grassy area opposite to ER	2005	53.5
Designated smoking area	807	21.5
Student dorm and soccer field area	591	15.8
Main path between upper and lower gates	251	6.7
Orthopaedic Clinic area	93	2.5
Total	3,747	100

The hospital campus was divided into five main areas and cigarette butts were carefully counted in each of these areas in March 2015. At this time the campus was not officially smoke free. Areas were scanned visually for butts by two people and counts obtained. This butts are counted butts not collected.

3.2. Comparisons of effects of leachates of cigarette butts containing associated tobacco, with leachates of butts lacking tobacco, on fluid intake and growth of mice.

Cigarette butt leachates were made from soaking 10, 30, or 75 cigarette butts in 250 mL of water, then the debris was filtered out using a tea strainer (see Materials and Methods section). Leachates were given to mice in their drinking bottles in replacement of drinking water. Initiallya comparison was made between leachates prepared from cigarette butts with no attached tobacco and with butts containing associated tobacco.

3.2.1. Effect of leachatesof cigarette butts containing tobacco, and leachates of butts lacking tobacco, on oral fluid intake by mice.

In order to assess the effect of leachates on the volume of fluid drank by 12-week old mice, daily uptake of fluid from the drinking vessels was measured over time, to determine whether leachates might be aversive to mice because butts may make the water taste unpleasant.

The experiment shows that the mice preferred water over any of the tobacco butt leachates, whether there was tobacco associated with the leachates or not possibly because leachate tasted unpleasant. Mice also preferred to drink leachates made from butts that lacked associated tobacco over leachates made from butts with associated tobacco (Table 6 and Figure 8). Leachates made from lower concentrations of cigarette butts (10 butts/250 mL) were preferred by mice to leachates made with higher concentrations (30 butts/250 mL or 75 butts/250 mL), whether or not the butts contained associated tobacco.

Table 6. Volumes of water and leachates drank by mice (expressed as volume drank per 5 mice per day) after various time periods

Volume (mL/ 5 mice/ day) of fluid drank at different times			
Fluid in drinking bottle	6 days	14 days	28 days
Water only	35	50	35
10 butt leachate without tobacco	21.3	32	32
10 butt leachate with tobacco	10.3	25	18
30 butt leachate without tobacco	20	21.2	23
30 butt leachate with tobacco	9.5	18	12
75 butt leachate without tobacco	12.7	20	20
75 butt leachate with tobacco	9.3	12.5	10

Mice (starting at 12 weeks old) were given, in their drinking bottles, water alone or leachates made from 10, 30 or 75 cigarette butts/ 250 mL (with or without associated tobacco), and volumes of fluid drank at different times were measured over 24 hours.

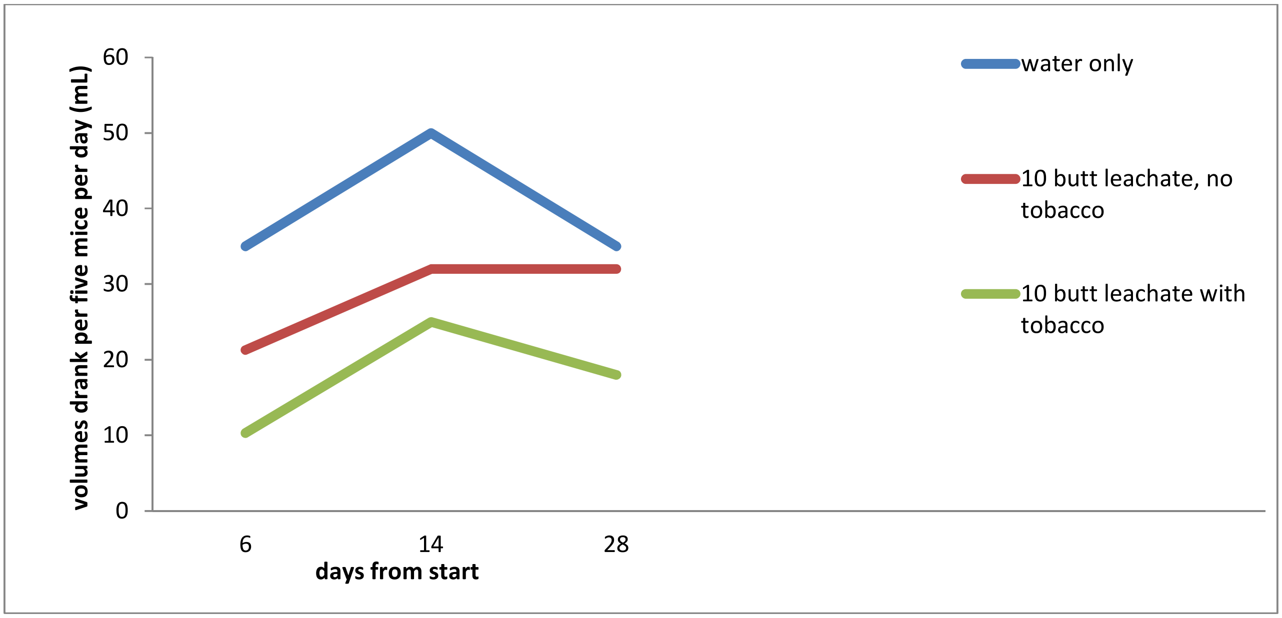


Figure 8a. Volume drank by mice drinking leachate made from 10 cigarette butts (with and without associated tobacco) per 250 mL water, compared with water alone.

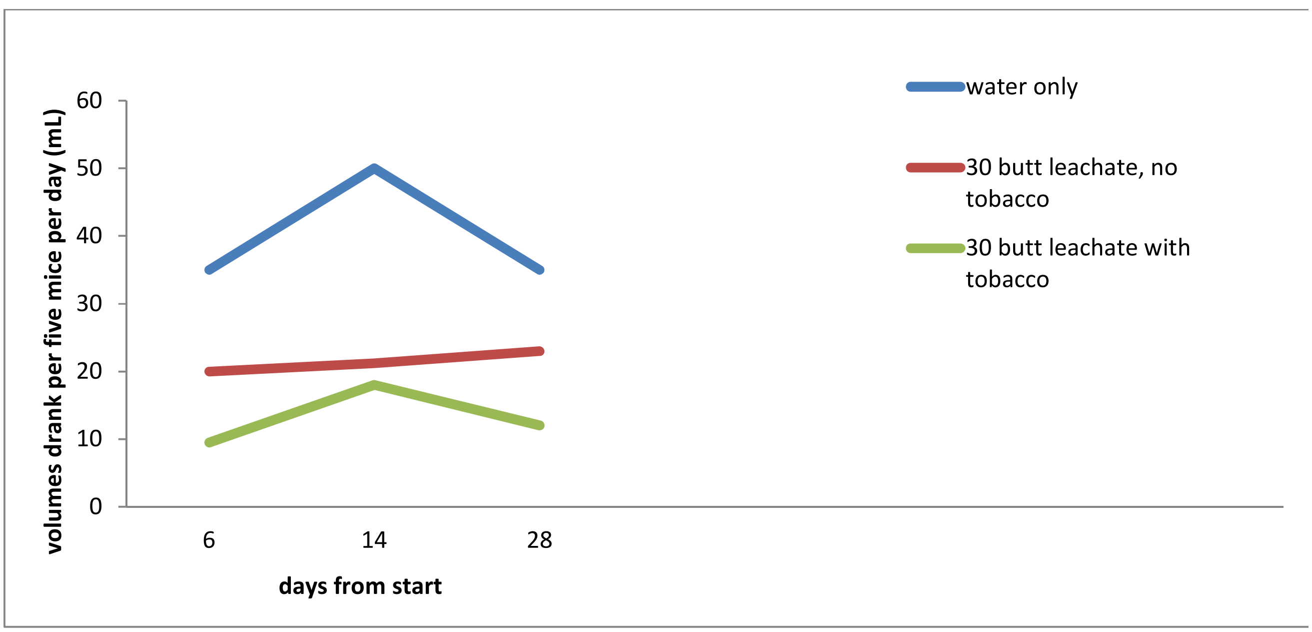


Figure 8b. Volume drank by the mice drinking leachate from 30 cigarette butts (with and without associated tobacco) per 250 mL water, compared with water alone.

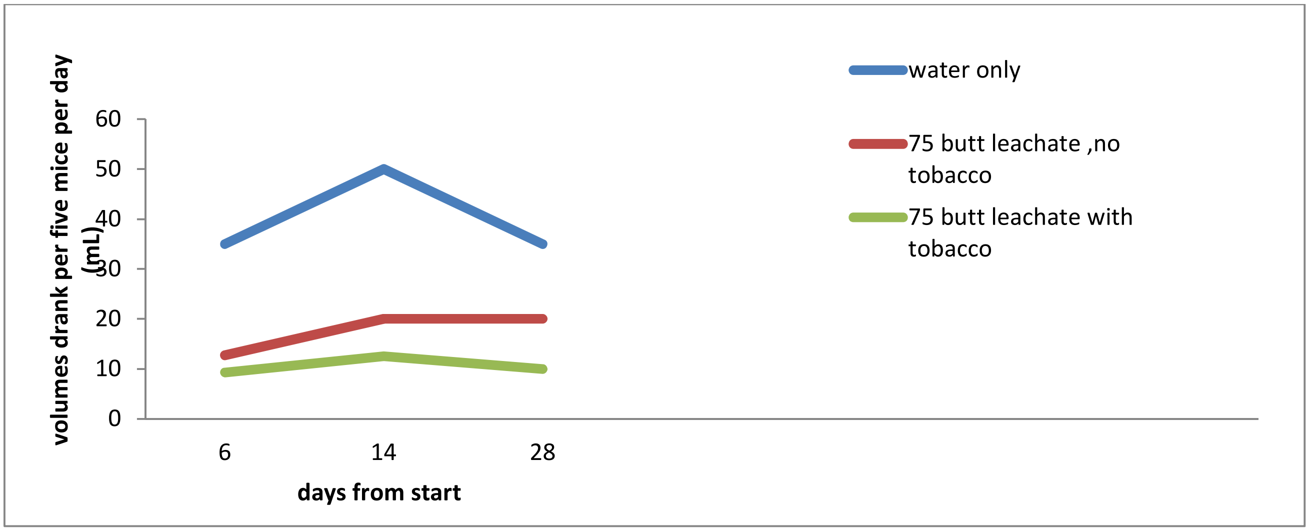


Figure 8c. Volume drank by mice drinking leachate from 75 cigarette butts (with and without associated tobacco) per 250 mL water, compared with water alone.

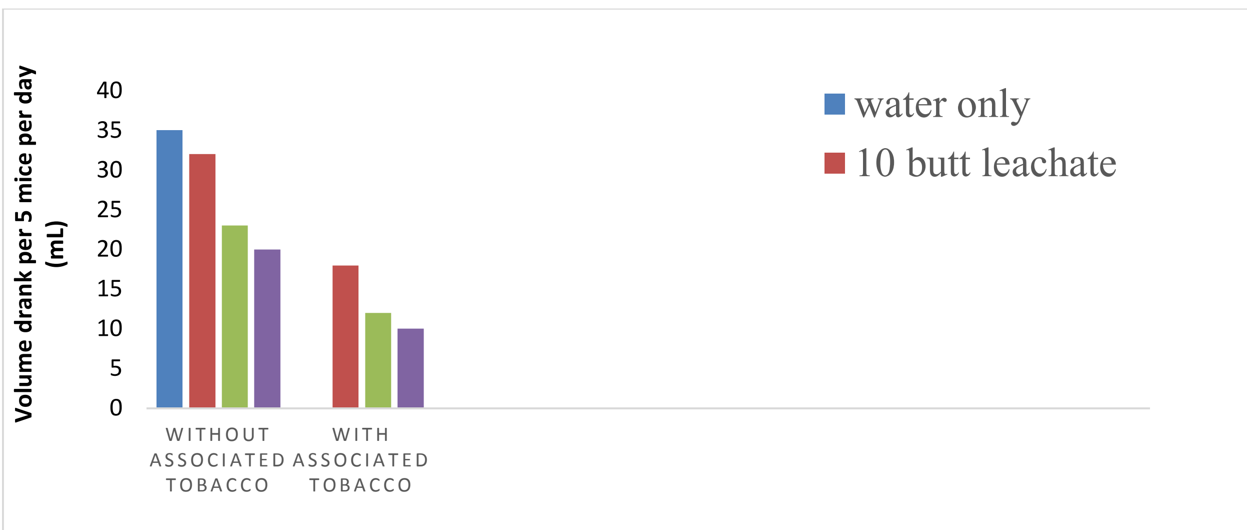
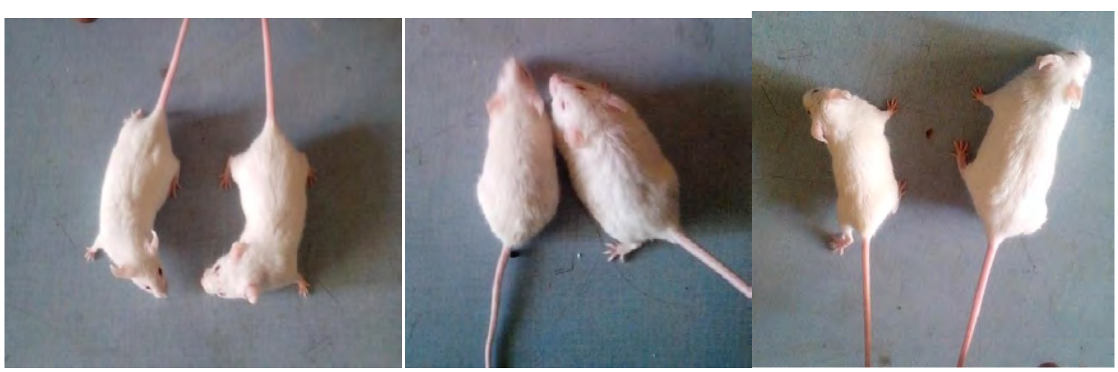


Figure 8d. Volume drank after 5 weeks by mice drinking leachate from 10, 30 or 75 cigarette butts (with and without associated tobacco) per 250 mL water, compared with water alone.

For experiments in Figures 8a, 8b and 8c and 8d, leachates were prepared from soaking 10, 30 or 75 cigarette butts, with or without tobacco in them, in 250 mL of water overnight, and then filtered to remove debris. The leachates were then placed in the mouse drinking vessels. Volumes of leachates were measured periodically to determine the amount of fluid that was drank. Results are expressed as volume of leachate drank per 5 miceper day.

3.2.2. Effect of leachates on weight of Swiss albino mice and on weights of various organs.

Mice drinking leachates made with the highest concentration of tobacco-associated butts (75 butts per 250 mL) for 5 weeks were visibly much smaller than the mice drinking water alone and also were visibly smaller than the ones drinking leachates made with cigarette butts only (without associated tobacco) (Figure 9).In agreement with their appearance, the weight of mice drinking leachates made from butts with tobacco were much lower, almost half, of the weight of mice drinking water alone (Table 7). The weight of the mice drinking the leachates made with butts containing no associated tobacco was about the same as the weight of mice drinking water alone.



A **B** **C**

Figure 9. Physical appearance of mice after drinking water alone (mice on right of each photo) or cigarette butt leachate containing 10 (photo A), 30 (photo B) or 75 (photo C) tobacco-associated cigarette butts (mice on left of each photo).

Mice were given either tap water or tap water that had been pre-soaked in cigarette butts with associated tobacco for 5 weeks. In all three photographs (A, B and C), the mouse on the right was given only tap water to drink. In A, mouse on left was given leachate made with 10 cigarette butts per 250 mL tap water; B, mouse on left was given leachate made from 30 cigarette butts per 250 mL tap water; C., mouse of left was given leachate made from 75 cigarette butts per 250 mL tap water.

Necropsy (animal autopsy) was performed on several mice and it was striking during necropsy that most organs and tissues, including skeletal muscle, heart, liver, lung, kidney, spleen and epididymal fat pads, were visibly grossly larger in mice that drank water alone than in mice that drank leachates made with tobacco-associated butts. This was confirmed by weighing various organs: heart, liver, kidneys, lungs, spleen and epididymal fat pads of mice that drank leachates made from tobacco-containing butts were lower in weight than the same tissues from mice that drank water alone (Table 7). This means that the mice drinking cigarette butt leachates were not simply less obese, despite their lower body weights, but showed visibly smaller organs, even when these organs had no obvious excess fat deposition in mice drinking water alone.

There was no difference in both the appearance and the weight between the mice drinking water alone and the mice drinking the leachates without associated tobacco. However, the mice drinking the leachates made with tobacco-associated butts were significantly smaller visibly and of lower weight than those drinking leachates made with butts containing no associated tobacco.

These results indicate that it is the tobacco component, rather than the filters, of tobacco-associated butts that is responsible for the lower weight of mice (and of the internal organs) drinking leachates made from tobacco-associated cigarette butts.

Table 7. Body weight, and weight of individual organs and tissues of Swiss albino mice, given leachates made from cigarette butts (with and without associated tobacco) to drink, compared with mice given water alone.

Organ/tissue mass (mg)			
Organ/ tissue	Drinking water only	Leachate made from 75 butts with no associated tobacco/ 250 mL water	Leachate made from 75 tobacco-associated butts/ 250 mL water
Heart	224mg	214mg	112mg
Liver	1,870 mg	2,300 mg	683mg
Lung	271mg	ND	217mg
Kidney	382mg	ND	224mg
Epididymal fat pad	182mg	242mg	36mg
Spleen	232mg	270mg	62mg
Mouse body weight	46 g	42.7 g	24 g

ND = not determined

Necropsies were performed on individual miceand the weights of the various dissected organs and tissues were determined.

3.3. Histopathological studies of the effects of cigarette butt leachates on various tissues of Swiss albino mice.

Standard histopathological staining with hematoxylin and eosin was done on several tissues of the mice drinking water alone and of the mice drinking the leachates made with tobacco-associated butts. The histological staining was done on the four organs; heart, liver, kidney and lung. The heart, liver and the kidney showed no histopathological difference between the two groups. Photograph of tissues other than the lung are not shown, because the photos taken were at different magnification and were not compatible. However, there were no histological effects of leachate on tissues other than the lung. The only tissue that showed a difference between mice drinking water compared with mice drinking cigarette butt leachates was that of the lung tissue. The primary finding was of increased alveolar wall thickness with mononuclear cell infiltrate, and increased irregular air space volumes, compatible with lung fibrosis and emphysema (Figure 10 and Figure 11).

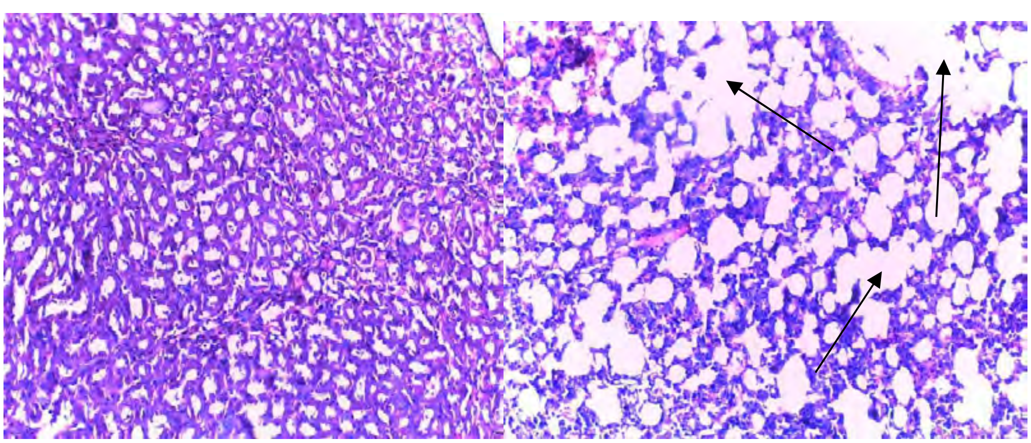


Figure 10 Histology (hematoxylin and eosin staining) of lung tissue from mice after drinking water alone (left) compared with mice after drinking leachate made from

tobacco-containing cigarette butts [75 butt/ 250 mL] (right) for 5 weeks. Arrows show irregular and enlarged air spaces. (Magnification 200X)

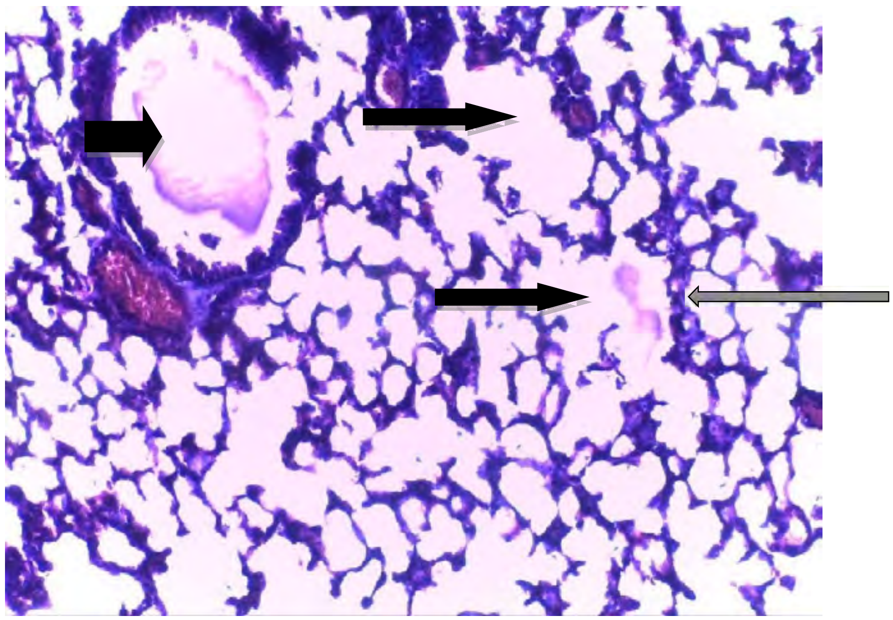


Figure 11. Histology (hematoxylin and eosin staining) of lung tissue from mouse given leachate made from 75 tobacco-associated ciagrette butts per 250 mL of water to drink for 5 weeks.Short black arrow shows a mucus plug inside a small airway; long black arrows show abnormal air spaces indicative of emphysema. Grey arrow points to thickened alveolar walls. (Magnification 200X)

3.4. Effect of leachates of cigarette butts on blood glucose levels of Swiss albino mice.

Fasting blood glucose levels from tail vein bleeds were measured using a glucometer after the mice had been drinking leachates of tobacco butts for 20 days. There were no differences between blood glucose levels of mice that drank water alone and mice that drank cigarette butt leachates (Table 8). All blood glucose levels were in the normal range for mice (less than 200 mg/dL).

Table 8. Blood glucose levels in mice after 20 days of drinking water alone or leachates made from tobacco butts (with or without associated tobacco).

Treatment (drinking fluid)	Mean fasting blood glucose (mg/dL)	
Water only		149 ± 40
Leachate made from 10 butts/ 250 mL water	Without tobacco	139 ± 30.3
	With tobacco	137 ± 32.4
Leachate made from 30 butts/ 250 mL water	Without tobacco	149 ± 31.5
	With tobacco	140 ± 30
Leachates made from 75 butts/ 250 mL water	Without tobacco	147 ± 32.6
	With tobacco	135 ± 29.8

Values are Mean ± SD of fasting blood glucose levels of five Swiss albino mice in each group.

3.3. Further studies of leachates of cigarette butts containing tobacco on fluid and food intake, growth and liver function tests of mice.

Further studies were done to investigate the effects of cigarette butt leachates on mice. In these experiments, mice were given leachates to drink that were made from different types and proportions of cigarette butts equivalent to the proportions found in the natural environment. Thus the mice received leachates prepared fromdifferent types of cigarette butts in the ratioof 8 butts without tobacco: 9 butts with tobacco: 3 butts decayed to cellulose acetate fibres, which is the ratio found in the butts collected from Tikur Anbessa campus.

In addition, mice were 6 weeks old at the start of these further studies, in order to follow their growth spurt more easily; also, these are equivalent to "adolescent" mice and would reflect more accurately the effect of leachates of butts on growing mice.

3.3.1. Effect of cigarette butt leachates on food intake of Swiss albino mice.

In order to determine if the reduced weight gain of mice drinking leachates made with cigarette butts containing associated tobacco was due to lower food intake (as a result of poor taste of the food or reduced appetite of the mice), a further experiment was done in which food intake was measured by weighing the mouse food periodically (Figure 12).

There was a similar increasedfood intake over time in mice of all three groups (those drinking water alone, those drinking leachatesmade with 20 butts per 250 mL, and those drinking leachates made from 40 butts per 250 mL) as they grew larger (Figure 12).

There was no difference in food intake between mice drinking cigarette butt leachates compared with mice fed water alone.

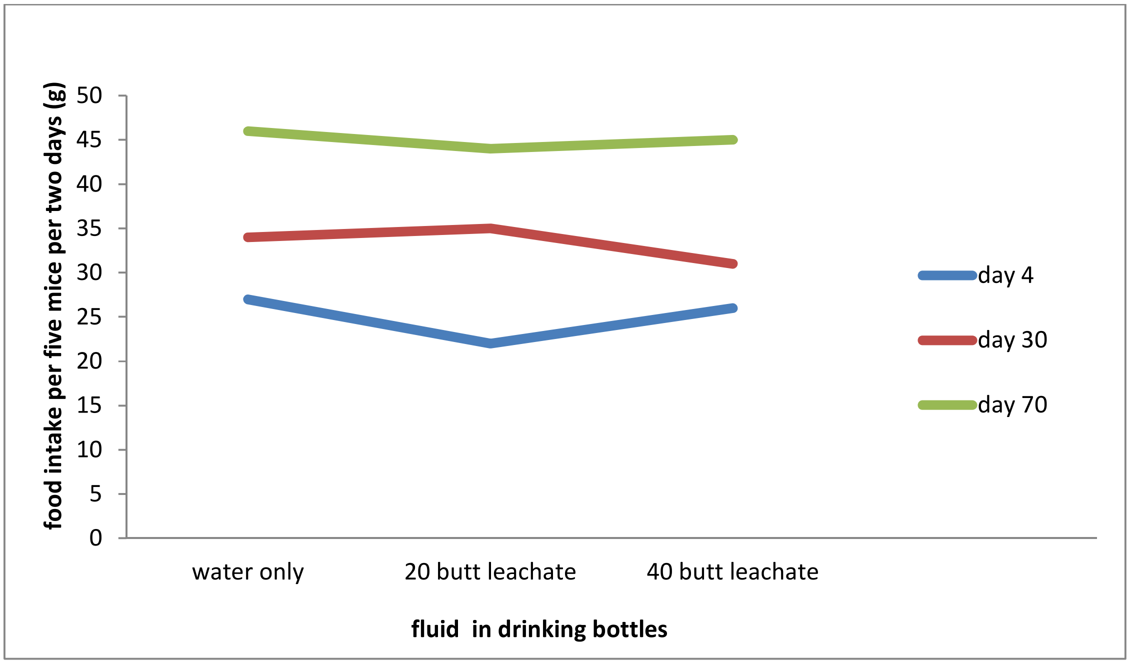


Figure 12. Food intake of mice given water alone or leachates made from cigarette butts over 40 days.

Mice given either water or cigarette butt leachates (made from 20 butts/ 250 mL or 40 butts/ 250 mL) to drink, and were fed with normal pellets and the food intake was measured per five mice per two days at various times.

In contrast with the experimental results shown in Table 6 and Figure 8, in which mice were 12 weeks old at the start of the experiment and a different sample (generally higher concentrations of tobacco-containing butts) of butt leachates were used, the results with 6-week old mice showed that there were no differences in volumes of leachate drank through the experiment (Figure 13), except for possibly reduced fluid intake early on (first 4 days) in the experiment in mice drinking butt leachates. The amount of daily fluid drank increased with time in mice drinking either water or butt leachates, as the mice grew, but there were no major differences between leachate or water intake throughout the 70 day experiment.

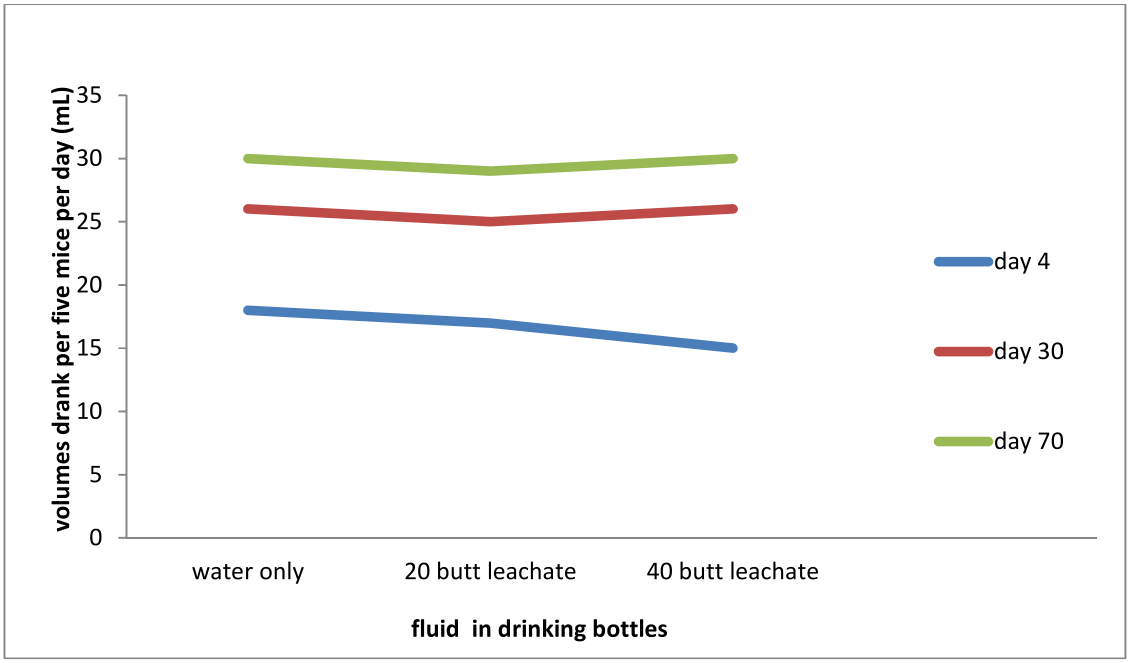


Figure 13. Volumes of fluid drank by mice given water alone or leachates made from tobacco butts at various times.

The mice were given different drinking fluids: water alone or leachate made from 20 cigarette butts/ 250 mL or leachate made from 40 cigarette butts/ 250 mL. Fluid intake was determined at different times by measuring the volumes of fluids in the drinking bottles.

3.3.2. Effect of cigarette butt leachates on weights of growing 6-week old Swiss albino mice.

Body weight increasedin 6-week old mice given water alone, 20 butt leachate, or 40 butt leachate to drink (leachates made from butts representative of their proportions in the environment) (Figure 14). In agreement with the experiment done on 12-week old mice (Figure 9, Table 7), mice drinking cigarette butt leachates weighed less throughout the experiment than those drinking water alone (Figure 14). After 70 days, mice drinking water weighed 41.7 g $\pm$ 1.03, mice drinking 20 butt leachates weighed significantly less (32.6 g$\pm$ 1.9; p-value < 0.001) and mice drinking 40 butt leachates weighed even less (29.3 g$\pm$ 1.6 ; p-value < 0.001). This suggests that there is a dose-response, with higher concentrations of leachates causinglower increments of weight gain.

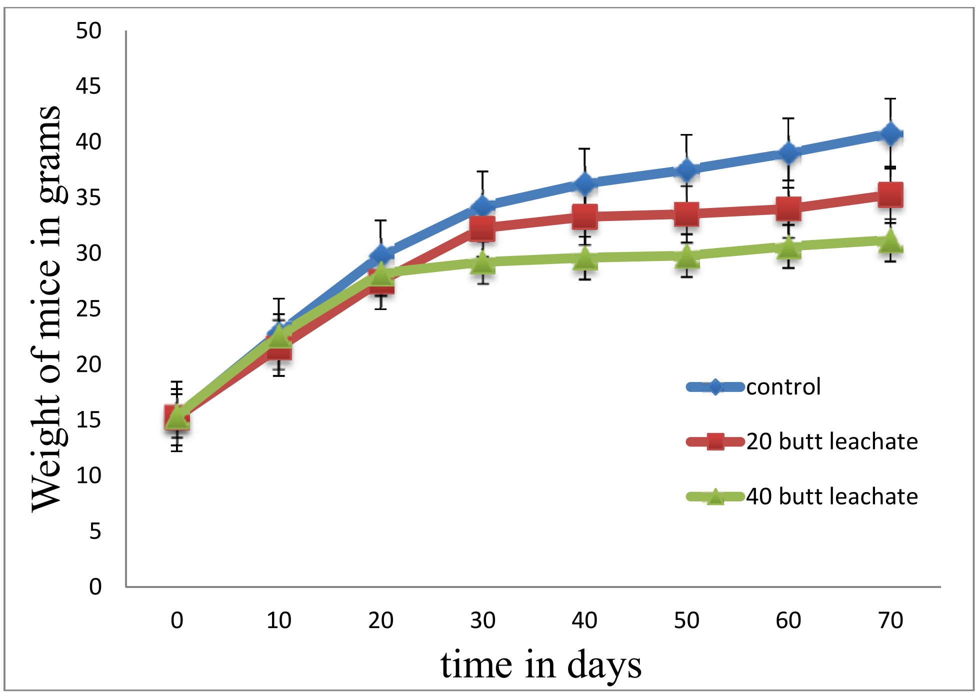


Figure 14. Growth of mice given drinking water alone or cigarette butt leachates, from 6-weeks old (time 0) to 16 weeks old (70 days).

Six-week old mice were given water alone, leachate made from 20 cigarette butts/ 250 mL, or leachate made from 40 cigarette butts/ 250 mL in their drinking bottles. (Butts were representative of their proportions found in the environment.) Weights of mice (5 mice per group) were determined after different times and are expressed as mean $\pm$ SD.

These data therefore show that the effect of cigarette leachates on lowering the weight gain of mice is not associated with decreases in fluid or food intake.

3.3.3. Effect of cigarette butt leachates on liver tests of Swiss albino mice.

In order to determine if there was any liver toxicity of cigarette butt leachates drinking on Swiss albino mice, serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) levels were measured after mice had drank water or butt leachates for 70 days. There were no significant increases (p-values are 0.09 for AST, 0.08 for ALT and 0.7 for ALP) in any of these serum markers of liver toxicity (Table 9), which, in conjunction with the normal liver histopathology seen, indicates that cigarette butt leachates caused no obvious hepatotoxicity in mice.

Table 9. Activities of serum AST (aspartate aminotransferase), ALT (alanine aminotransferase) and ALP (alkaline phosphatase) in mice given tobacco butt leachates or water to drink for 70 days

Serum enzyme	Water only	Leachate made from 20 cigarette butt/250 mL of water	Leachate made from 40 cigarette butt/250 mL of water
AST (U/L)	279 ± 70.2	209 ±17.5	201 ±31.3
ALT (U/L)	103 ±37.5	65 ±2.88	59 ±10.7
ALP (U/L)	196 ±66	219 ±14.5	184 ±3.5

.

Six-week old mice were given water alone, 20-butt leachate or 40 butt leachate to drink for 70 days, then serum levels of AST, ALT and ALP were determined. Values are Mean± SD of five Swiss albino mice in each group

Chapter 4

DISCUSSION

4.1. Distribution and types of cigarette butt found

The most frequently smoked cigarette, as found among collected cigarette butts in the Tikur Anbessa campus and HayaHulett area of Addis Ababa, was the Nyala brand. Nyala is produced in Ethiopia only for the local market and its frequent use might be due to the cheap cost of this brand since it is 20birr/pack of 20 cigarettes as compared to the 75 birr /packet of the Rothmans and 75 birr/packet of Marlboro. Delight cigarettes cost 20 birr/pack and Nyala Premium cost 25 birr/pack. Nyala is also by far produced in the highest quantity of all cigarettes sold in Ethiopia: about 5 billion Nyala cigarette sticks are produced yearly by National Tobacco Enterprise, the sole tobacco company in Ethiopia (Ashall, 2015).

When someone throws a smoked cigarette to the ground the cigarette starts to release its components to the environment. Tobacco falls out of some cigarette butts with unburnt, unsmoked tobacco in them, releasing the unburnt, unsmoked tobacco to the environment. However, this tobacco is not identical to the tobacco present in non-smoked cigarettes that have not been removed from a pack and ignited, because during smoking tobacco smoke toxins likely adhere to the tobacco of the smoked cigarette as the smoke passes through the unburnt tobacco. This means that the tobacco released into the environment releases many more toxins, including carcinogens, than are present in unburnt, unsmoked cigarettes, because tobacco smoke contains over 7000 chemicals whereas raw tobacco contains only about 800 chemicals.

From the cigarette butt analysis conducted in this MSc study, 45 % of the discarded cigarette butts had attached tobacco when thrown to the ground, and this tobacco will contain carcinogens and other toxins, from the cigarette smoke that had passed through it when it was smoked. It also contains substantial amounts of nicotine.

In order to estimate how much nicotine is discarded into the environment in cigarette butts, the average proportion of unburnt, unsmoked tobacco present in discarded butts with associated tobacco was determined by measuring the lengths of the unburnt, unsmoked parts of discarded cigarettes. On average, 21.6% of the length of these discarded cigarette butts (not including the length of the filter, which has no tobacco) was incompletely smoked tobacco when thrown to the ground. This means that, on average, 21.6 % of the tobacco portion is still present when one cigarette butt is thrown to the ground.

There are 2.5 mg of nicotine in 100 mg of tobacco in Nyala cigarettes **(**Kassa*et al.,* 2013), which means that in one Nyala cigarette, which contains 600mg of tobacco, there are 15 mg (2.5 X 6 mg) of nicotine. Since 21.6% of the cigarette tobacco is still present when one cigarette butt is thrown to the ground, then on average there are 3.2 mg(21.6 % of 15 mg) of nicotine in each tobacco-containing cigarette butt. And as noted previously, 45.5% of the total discarded cigarette butts contain tobacco, whereas the rest lack tobacco. This implies that an average cigarette butt contains about 1.5 mg of nicotine (45.5 % of 3.2 mg).

Therefore, for every 10 cigarette butts discarded, there are15mg(10 X 1.5 mg) of nicotine entering the environment. Since6 billion cigarettes are smoked in Ethiopia per year, this means that 6 billion cigarette butts are thrown away annually, which is equivalent to 6 $X10^9$ X 1.5 mg = 9 million grams, or 9,000 kg, of nicotine thrown in to the environment every year in Ethiopia.

A dose of nicotine of 3mg/kg is a lethal dose for a mouse. Therefore, for an average 30 g mouse, about 0.1 mg of nicotine is lethal. This means that one discarded cigarette butt containing an average of 1.5 mg of nicotine is enough to kill 15 mice, so ten cigarette butts could theoretically kill 150 mice. This illustrates how potentially toxic environmentally discarded cigarette butts could be tosmall animals.

In Ethiopia, particularly in urban areas, larger animals, including those used for human food sources, for example, chickens, sheep, goats and cows, rely on the streets for their food and water. The results of this study suggest the possibility that discarded tobacco butts may be toxic to these animals, and may reduce their weight and reduce consequently their yield of meat and other food products such as eggs and milk.

In humans it is generally stated that a toxic dose of nicotine is 60 mg for one individual, but Mayer evaluated studies done and concluded that the actual toxic dose of nicotine is 500 mg or more in humans (Mayer, 2014). This would mean that discarded cigarette butts are unlikely to be lethal to humans, if consumed accidentally (for example by children), but they nevertheless might be toxic, but not lethal, especially to children and pets.

The Tikur Anbessa campus tobacco butt count was conducted on 22ndMarch, 2015. The campus count shows that half the cigarette butts were present in an area close to the

Emergency Room and this is because many people sit in this area, for example people who come with patients.

In March 2015, the Ethiopian Food, Medicine and Healthcare Administration and Control Authority (EFMHACA) published their Ethiopian Tobacco Control Directive(Ethiopian Food, Medicine and Healthcare Administration and Control Authority, 2015). Part 4, article 14, sub-article 2, of this document states: “Smoking shall be prohibited in indoor work places of health and education institutions. But it allows smokers to smoke in designated smoking area” That was supposed to be enforced starting in April, 2015.

In fact, there are some changes around the Tikur Anbessa hospital compound, for example the area near to the Emergency Room can now no longer be accessed for smoking. In accordance with the EFMHACA Tobacco Directive (2015), smoking is now permitted only in the designated smoking area on the campus. However, in many countries where tobacco control is being well enforced and smoking rates are going down, hospitals have banned smoking anywhere on their campus, and have removed even designated smoking areas(Farrelly *et al*., 2012). This sends a positive message from hospitals to the public regarding their concerns about the health hazards of smoking.

4.2. Effect of cigarette butt leachates on growth of mice

Cigarette butt leachates with and without associated tobacco were, at high concentrations (75 butts per 250 mL) particularly aversive to 12-week old mice and reduced the volume of fluid that they drank, but did not reduce survival. However, when 6-week old mice were given leachates to drink that were made with a representative mixture of butts similar to that found in the environment, and a lower concentration of butts (20 or 40 butts/ 250 mL), mice drank the leachates over 70 days as well as they drank regular water (Figure 13). This indicates that changes in fluid intake due to leachates of cigarette buttswere not significant and did not cause the effects on weight gain and histopathological abnormalities seen in the lung tissue.

Mice gained less weight when they drank leachates made from cigarette butts than when they drank water. This lower weight gain was not a result solely of decreased fat deposition, and no gross pathology, other than small size, was seen on necropsy. The size of every organ including skeletal muscle, heart, spleen, lung, kidney, epididymal fat pads, and liver of the mice that drank leachates made from cigarette butts associated with tobaccowere much smaller than these organs taken from mice that drank water alone. Since this is not due to reduced food or fluid intake, it is unlikely to be due to a reduced appetite of the mice.

This result is partly consistent with the work on rats of Iranloye and Bolarinwa (2009), who reported that nicotine administration to female rats caused reduced weight of various tissues, including kidneys, uterus, ovaries, pituitary gland. However, contrary to this MSc study, they found weight gain of heart and liver tissue.

Reduced food intake, due for example to reduced appetite or aversion to food taste, was not an explanation for the lower weight of mice drinking cigarette butt leachates, because measurement of food weight showed no difference over time between mice drinking leachates and those drinking water alone.

One possibility is that it is the nicotine in leachates that is responsible for the lower weight of mice drinking leachates compared with those drinking water. Numerous published studies have shown that nicotine can induce weight loss or inhibit weight gain, despite there being no reduction in food or calorie intake. One study showed that nicotine reduced weight gain in mice, without reducing food intake, an effect that was much morepronounced in mice fed with the high fat diet compared with those on the normal chow diet (Mangubat*et al*., 2012). In another study, reduction in body weight after chronic nicotine administration also occurred in the absence of a significant decrease in food consumption (Schechter and Cook, 1976).

One mechanism why leachates reduced weight gain without reduced food intake is that a component, for example nicotine, might activate brown adipose tissue. Indeed nicotine is known to stimulate energy expenditure through involvement of corticotropin-releasing factor (CRF) in a mechanism by which nicotine stimulates thermogenesis in brown adipose tissue (BAT) (Mano-Otagiri*et al.*, 2009). Brown adipose tissue converts metabolic energy into heat through uncoupling protein 1 (UCP-1), which is located in the inner membrane of mitochondria, and makes up approximately5% of total mitochondrial protein in brown adipocytes of cold-acclimated rodents (Stuart *et al.*, 2001). This leads to the production of heat instead of production of ATP via ATP synthase, so that fewer calories are stored as fat.

Another possible mechanism by which butt leachates cause lower weight of mice is through interference of a cigarette butt component with basic hormonal growth mechanisms, in particular growth hormone and insulin-like growth factor-1 (IGF-1), which is produced by the liver and mediates the growth stimulating effects of growth hormone. In support of this idea, most tissues and organs were noted to be smaller in mice that drank cigarette butt leachates, as mice grew from 6 weeks to 16 weeks, a period during which growth hormone stimulation of growth is predominant. Again, nicotine may specifically be involved in this interference of growth.

One study showed that nicotine caused a marked hypersecretion of ACTH, vasopressin, beta-endorphin, prolactin and LH, showing that nicotine can acutely at least, affect pituitary function. Many of these acute stimulatory effects of nicotine rapidly disappeared, probably due to a desensitization of the central nicotinic cholinergic receptors involved (Fuxe*et al*., 1989).

GH levels are acutely increased by smoking (Markianos *et al*., 1993). Insulin-like growth factor-I (IGF-I) levels, however, show a downward trend with increasing smoking levels, especially as the secretion of IGF-I is largely dependent on GH, long-term smoking may lead to a down regulation of GH release(Landin-Wllhelmsen *et al*.,1994).

In another study (Coutant *et al*.,2001) IGF-1 was reduced in cord blood of neonates from smoking mothers compared to neonates from non-smoking mothers, and that this reduction was consistent with their lower birth weight percentile. Nicotine significantly reduced body weight gain in the fetus, but not food intake in the mother.

It is possible that some component or multiple components of cigarette butts other than nicotine are responsible for the effects on growth of mice and the reduced size of the tissues and organs examined.

The mean fasting blood sugar was not significantly different between mice drinking water and those drinking cigarette butt leachates. Also, liver function tests were not affected by leachates, the liver appeared grossly normal at necropsy, and liver histology was normal in mice drinking leachates, suggesting that there was no hepatotoxicity of the leachate components.

4.3 Effect of cigarette butt leachate consumption on lung histology

Notably, mice in this study were not exposed to cigarette smoke by inhalation, but were exposed to cigarette components by oral intake only, yet the lungs of mice drinking cigarette butt leachates showed significant fibrotic and emphysematous changes, involving alveolar wall thickening and expanded abnormal air spaces.

It has generally been accepted that emphysema is due to chemical and particulate components of cigarette smoke directly affecting lung tissue from inhalation, by increasing reactive oxygen species (ROS), enhancing inflammation, and reducing protease inhibitor activity and increasing elastases, metallo-proteases and other protease activities, leading to destruction of lung tissue and deposition of fibrotic tissue (Diamond and Lai, 1987; Diamond *et al.*, 1988; Piera-Velazquez *et al.*, 2011). Evidence is accumulating that nicotine itself, in addition to other tobacco smoke components, may cause fibrosis and emphysema of lungs as well as fibrosis of other tissues, even when administered orally or through other non-inhaled means, and in the absence of other tobacco components. (Chen *et al.*, 2007).

In agreement with this finding, intraperitoneal administration of nicotine caused thickening of alveolar septa in mice, with associated mononuclear cell infiltration, angiogenesis, and irregular areas of lung tissue collapse (Valença*et al.*, 2004)**.**This result is also similar to that obtained by (Braber*et al.*, 2010) who studiedhistological lung sections of nicotine-exposed mice and showed an increased air space enlargement and lung tissue destruction.

Also in agreement with the hypothesis that nicotine in leachates might cause at least partly, the lung fibrosis generally is that pregnant mice exposed to mothers who are administered nicotine show that total collagen was significantly higher in the lungs of the newborn mice, even though neither mother nor fetus was exposed to cigarette smoke (Huang *et al*., 2014)**.** Other studies confirm that nicotine exposure causes fibrosis in the lungs and other organs, including kidney, liver, oral mucosa, vascular endothelium and heart muscle, independently of being present in tobacco or of being inhaled into the lungs. For example, maternal nicotine exposure during gestation and lactation induces neonatal kidney fibrosis (Chen *et al*., 2007).

One study found a significant decrease in the alveolar count with an associated increase in the interstitial thickness in the lungs of adult offspring in nicotine-treated animals, and that fetal and neonatal nicotine exposure inhibits the alveolarization. These results suggest that fetal and neonatal nicotine exposure results in programming of lung fibrosis in adult offspring (Dasgupta*et al*., 2012).

4.3.1. Possible mechanisms by which nicotine may induce tissue fibrosis

Schwartz and Bond (1972) demonstrated *in vitro* that nicotine is chemotactic for human neutrophil leukocytes (a source of endogenous proteases) and that it enhances the responsiveness of neutrophilsto chemotactic peptides. Nicotine also has been found to promote the recruitment and release of elastase from pulmonary alveolar macrophages. In addition, nicotine has been shown to have detrimental effects on lung connective tissue repair processes. Low levels of nicotine are cytotoxic to fibroblasts (Chamsonand Auger, 1980), and high levels inhibit collagen synthesis (Hurst and Gilbert, 1979). A similar study also demonstrated that exposure of elastase-pretreated rats to cigarettes smoke containing a relatively high level of nicotine results in augmentation of emphysema, whereas similar exposure to cigarette smoke with a relatively low nicotine level does not. (Diamond *et al*., 1988).

Fibrosis is the pathophysiological response to chronic injury that results in excessive deposition of extracellular-matrix (ECM) proteins and scarring. In each organ system, the fibrogenic process has unique characteristics related to the function of the organ and the microenvironment created by the organ-specific epithelium. Nicotine induces fibrogenesis through different mechanisms. For instance, it may cause damage to epithelial and endothelial cells (Jain andVarga, 2006); release of transforming growth factor-β1 (TGF-β1),which stimulates the production of ECM proteins (including collagen in parenchymal cells, fibroblasts, and inflammatory cells) and plays a key role in the regulation of fibrosis (Jensen *et al*., 2012).

Recruitment of infiltrating inflammatory cells is also a key event leading to fibrosis. Changes in the activation status and the number of inflammatory cells play a vital role in a number of diseases associated with smoking.Inflammatory cytokines, including the prime neutrophil chemoattractant interleukin 8 (IL-8), leukotriene B_4 (LTB_4), monocyte chemotactic protein-1 (MCP-1), and a number of other factors attract inflammatory cells of the neutrophil and monocyte-macrophage lineage (Batallerand Brenner 2005). Production of reactive oxygen species (ROS) also has an important role in fibro genesis. Nicotine-stimulated ROS production has been linked to the damage of epithelial cells in an *in vivo* model of chronic kidney disease (Arany*et al*.,2011). In this model, nicotine exposure produced ROS through NADPH oxidase in the proximal tubules of the kidney; activation of collagen-producing cells; and ECM-dependent activation of myofibroblast cells (Yang *et al*., 2013).

Nicotine at levels in smokers' blood is pro-fibrogenic, through actions on nAChRs expressed on human hepatic stellate cells (hHSCs). Therefore, cigarette smoke, via its nicotine content, may worsen liver fibrosis. Moreover, nicotinic receptor antagonists may have utility as novel anti-fibrotic agents. Hepatic stellate cells express functional nAChRs and that nicotine directly stimulates these cells through their nAChR receptors. Additionally, we observed that nicotine induces hepatic stellate cell proliferationand up-regulation of the fibrogenic markers, TGF-b and collagen (Soeda*et al*., 2012)

4.4 Ecological significance of cigarette butts in Ethiopia

A cigarette butt contains the remnant tobacco portion of a cigarette, a cigarette filter usually made of cellulose acetate, a thermoplastic formed by the reaction of cellulose, acetic acid and acetic anhydride that takes months or years to degrade in the environment. It may take two months in favorable atmospheric conditions (it is both photo- and bio-degradable); and 3 years or more in dry conditions or seawater for a cigarette butt to degrade completely.

Cigarette butts contribute to water pollution, since cigarette butts are the most littered item accumulating in the environment waste stream. Worldwide, over 6 trillion are littered annually. Their chemicals contribute to non-point source pollution when carried through storm drains by rainfall and urban runoff to our lakes, rivers, wetlands, coastal waters, and even our underground sources of drinking water *(*Al-Khatib*et al*., 2009).

Cigarette butt litter also affects the food chain.Ingestion of cigarette filters is a serious threat to wildlife. A visible consequence is being witnessed higher up on the food chain by field biologists and wildlife rehabilitators who routinely find cigarette butts in the intestines, stomachs, and X-rays of dead or sick seaturtles, birds, fish, and dolphins (Litovitz*et al*., 1999).

When small animals are drinking this environmental cigarette butt waste, which is leached in to the ponds, lakes, rivers, they are exposed to the numerous hazardous chemicals in the leachate and the animals that are the prey of these small animals will be exposed too. Through this pattern the toxic chemicals from the leachate affects the food chain. In the case of Ethiopia, most of people live in the rural areas where the cattle are drinking from the lakes and ponds, which presumably contain numerous cigarette butts which may come from urban areas, where butts are washed away by rain. We humans also feed on thesecattle, which might expose us to the toxins, including nicotine and carcinogens and mutagens, from environmental cigarette butts.

Cigarette butts in different climates may also decay in a different way. For example, in Addis Ababa, there are eight dry months with little rain, so cigarette butts may accumulate in large numbers before they are washed into the water systems by the heavy rains of the rainy season, and this might cause seasonal variations in the occurrence of butt toxins in the waterways as well as on dry land. This might be an important area of further research.

A study done on snails showed that exposure to tobacco leaves for different periods of time caused significant DNA damage. Inhibition of cytochrome P450 enzymes occurred only in the tobacco exposed group. Chemical analysis indicated the presence of nicotine, coumarins, saponins, flavonoids and various heavy metals. These results show that tobacco leaves are genotoxic in the marine snail, *H. Aspersa,*and inhibit cytochrome P450 activity, probably through the action of the complex chemical mixture present in the plant. (Silva *et al*., 2013).

Lee and Lee (2015) recently reported that cigarette butt leachates prepared experimentally (as in this MSc study) suppressed development, lowered heart rate, and increased mortality of embryos of the fish species, *Oryziaslatipes*. In their study, effects were seen with leachates as made with as few as 0.2 butts per liter of water. This and other studies suggest that cigarette butt leachates are particularly toxic and mutagenic to small fish.

A study done on the house finch birds (*Carpodacusmexicanus*) revealed that birds use cellulose acetatefrom smoked cigarette butts as lining material for their nests and this reduce the number of ecto-parasites in their nests, probably because the nicotine repels arthropods. On the other hand, despite the benefits of having fewer ectoparasites, signs of genotoxicity in the blood cells also increasedwith the proportion of butt cellulose in the nests. (Suárez-Rodríguez *et al*., 2014).

Exposure to cigarette butt toxicants in seawater also affects ragworms, a common marine annelid. Cigarette filter chemical components caused weight loss, longer burrowing times, and significant DNA damage in these worms. Chemical toxins other than cellulose acetate fibers of cigarette butts were responsible for the damage to these worms (Wright *et al*, 2015). These studies also showed that nicotine concentrations were 500-fold more concentrated in seawater than sediments, showing that nicotine from cigarette butts can become concentrated in waterways as butts are washed away from their origins in the ground.

4.5. Regulations to reduce environmental cigarette butt contamination

The WHO Framework Convention on Tobacco Control (WHO FCTC) was adopted by the World Health Assembly in May 2003 and as of March, 2015 has been ratified (put into law) by 180 countries. Ethiopia is one of the countries that ratified (put into law) the WHO FCTC treaty, in June 2014 officially. Only 16 countries have not ratified the treaty, and interestingly one of these is the USA. The WHO FCTC document (World Health Organization, 2015) provides guidelines for governments to regulate such issues as tobacco advertising, promotion and sponsorship, sales of single cigarettes, sales to minors, protecting people from passive smoking, tobacco prices and taxes, health warnings on tobacco packs, reporting of tobacco constituents such as additives and nicotine levels, illicit trade of tobacco products and economically viable alternatives to tobacco.

However, little is covered in the WHO FCTC on regulations of environmental cigarette waste, including cigarette butts. Nevertheless, numerous proposals for reducing environmental cigarette wastehave been made. The most obvious way of reducing butt waste is to reduce cigarette smoking prevalence through other WHO FCTC regulations, such as putting warning signs on cigarette packs, increasing cigarette taxes and encouraging educational programs to the public and youth about the dangers of smoking. Below is a list of measures that might improve the problems associated with environmental cigarette butt waste (Novotny and Slaughter, 2014).

Feasible cigarette butt waste regulation measures

- Educating the public, especially smokers, about the hazards of littering cigarette butt on the ground.
- Holding tobacco companies responsible to pay for the cleanup of cigarette waste, including butts.
- Labeling the cigarette pack not just with health warnings, but also with environmental consequences.
- Creating a deposit /refund system by charging the consumers an extra cost, and this could be refunded when they return the smoked cigarette butts in their original pack.
- Charging an increased price, covered by smokers, on the cigarettes to cover costs of clean-up.
- Litigation against tobacco companies for selling products that cause environmental damage.
- Recycling of tobacco waste, including butts, for use in the production of other products, such as paper or furniture, but this requires removing toxins in the processing.

4.6. Conclusions

Leachates were made from cigarette butts collected on the ground in Addis Ababa city.Despite there being no change in food and fluid intake, mice that drank leachate prepared from the cigarette butts with associated tobacco showed significantly reduced body weight and also reduced weight of multiple organ and tissues. Gross examination of tissues showed no pathological abnormalities, except that all organs and tissues examined were smaller in size in mice that drank cigarette butt leachates containing associated tobacco. Examination of the histopathology of tissues from the mice that drank leachate prepared from cigarette buttscontaining associated tobacco indicated that drinking cigarette butt leachates caused emphysematous damage to lung tissue. Cigarette butt leachates showed no liver toxicity, according to histological examination of liver and measurement of serum liver enzymes.There was no effect of leachates on the blood glucose levels. In general these findings demonstrate that those mice that were drinking leachates prepared from cigarette butts that had associated tobacco, representative of tobacco butt distribution found in the environment, tended to gain less weight and also have emphysema in their lungs.

4.7. Limitations

This study has numerous limitations that should be considered, including

- The study may not reflect the real intake of cigarette butt leachate by mice or other animals in the real situation in the environment, though the exact situation in the real environment is unclear, and it is quite reasonable to consider that 20 or 40 cigarette butts in some areas may well form leachates with 250 mL of rain water. Also, as butts get washed into waterways from land, their concentrations increase.
- Mice were bred and studied in the laboratory and these might differ in susceptibility to cigarette butt leachate toxicity compared with wild animals.
- In this study the leachates were made with cigarette butts and tap water and this is different with the environmental leachates, which contain rain water and other environmental substances (such as soil), other than cigarette butts. Chemicals used to treat tap water may also interfere with the results.
- Inability to perform tests on the hormone level (GHRH, GH and IGF-1) levels of the mice and do other biochemical studies, due to limited costs and resources.
- Histology studies were done on limited tissues; for example, brain/ pituitary/ hypothalamus tissues were not examined.
- Limited numbers of blood tests (blood glucose, ALT, AST and ALP) were done.

4.8. Recommendations

- Further studies should be done on the GHRH, GH and IGF-1 levels, and their cellular receptors in mice after cigarette butt leachate exposure
- Further studies should be done to evaluatebrown adipose tissue activity in mice after cigarette butt leachate exposure, to determine if cigarette butt leachates reduce weight gain or cause weight loss through enhancement of brown adipose activity.
- Further study are recommended on the biochemical identification of cigarette butt leachate components, for examplebiochemical fractionation, chromatography, purification of components involved.
- Studies should be done on the toxicity of cigarette butt leachates to other mammals, including mammals larger than mice, and on possible toxicity to chickens, sheep and other animals that provide sources of food for humans.
- Studies could be performed on looking at nicotine levels and levels of other cigarette toxins such as carcinogens,in rainwater and waterways, especially because this MSc study showed that a substantial amount of tobacco (and therefore nicotine) as well as cellulose acetate filter, are released into the environment.
- Investigations are recommended of levels of cigarette butts in waterways (rivers, lakes) in Ethiopia, including in cities and rural areas and at different times of the year (rainy and dry seasons).

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