

Investigation on Toxicity of Leachate of Cigarette Butts Collected from Addis Ababa on Swiss Albino Mice

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Abstract

Background: Cigarette butts are the most common form of litter in the world. They are toxic to microorganisms, fish and other marine organisms and birds, but no studies have been done on their toxicity to mammals. The aim of the study was to test the toxicity of cigarette butt leachate on mice.

Methods: A total of 10,20,30,40 and 75 cigarette buttes with remaining tobacco at the end and/or without were used to prepare leachates. Two Swiss albino mice (adult and baby) groups were allowed to drink the prepared leachates by soaking in water with different number of buttes' according to study protocol used for adult and baby mice. The weights of the mice, as well as the amount of food and fluid consumed were followed and compared over time. Necropsies were performed and tissues were weighed and evaluated by histological staining with eosin and hematoxylin. Blood glucose and liver function tests (Alanine Aminotransferase, Aspartate Aminotransferase and Alkaline Phosphatase) were also measured. Data was collected from the respective experiments, and analyzed using SPSS version 21 software.

Results: The mean water only intake of the mice was 35; the mean fluid intake of 10 butt leachate with tobacco was 18; the mean fluid intake of 30 butt leachate with tobacco was 12 and the mean fluid intake of 75 butt leachate with tobacco was 10ml. All fluid intake values were reported in mL/5mice/day. The body weight of experimental mice has shown statistically significant difference in those arms, taking 75 cigarette butt leachate with associated tobacco (p-value, 0.00078). The lung tissue of the mice that drank leachates made with tobacco-associated butts showed increased air space volumes and alveolar fibrosis.

Conclusion: Cigarette butts resulted in reduced weight gain during growth, reduced mass and size of tissues and organs, and pulmonary emphysematous changes in mice. Since there are few studies on effect of cigarette butt on mice, more studies should be done in this area.

Keywords: *Cigarette Butt, Leachate, Nicotine, Lung Toxicity*

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Introduction

Cigarette butts are toxic environmental 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 leads to the toxicity of cigarettes (Iskander *et al.*, 1986). Smoked cigarette butts contain numerous chemicals, such as ammonia, formaldehyde, butane,

acrylonitrile, toluene, benzene, alkaloid nicotine (Schneider *et al.*, 2011). By the analysis, done on metals leached from smoking cigarette litter found a positive correlation between the concentration of several metal ions and the soaking time of cigarette butts (Moerman and Potts, 2011).

Approximately 5.6 trillion cigarettes are smoked yearly worldwide, which produced, ubiquitous cigarette butts discharged into the environment. This cigarette butts leachates are toxic to birds, fish, marine organisms and



microorganisms (Slaughter *et al.*, 2011). If ingested, there is potential for the transfer of adhered toxicants to tissues. These bioplastic microfibers and their associated toxicants may persist in the marine environment and continue leaching chemicals for up to 10 years (Novotny and Slaughter, 2014).

Exposure to cigarette butt and filter toxicants in seawater has a significant negative effect on the growth rate and weight of rag worms, and induces DNA damage in rag worms. However, there are relatively few studies on environmental problems associated with cigarette butt waste (Wright *et al.*, 2015). There are no published reports on the effect of cigarette butts on mice and environment in Ethiopia. Therefore, this research aimed at evaluating the toxicity of cigarette butts to mice.

Materials and methods

Preparation of cigarette butt leachates

Cigarette butts were collected from Addis Ababa, Bole Kefle Ketema around Haya Hullet area and from Black Lion Hospital designated smoking area. Debris on buttes such as small stones, grass and soil were removed manually. Ten, 20, 30, 40 and 75 cigarette buttes were used to make leachates. The leachate preparation and the measurement of the fluid drunk were done following standard methods (Moerman and Potts, 2011).

Leachates were prepared using cigarette butts, i.e. cigarette butt leachate with the associated tobacco and cigarette butt leachates without the associated tobacco. Leachate of 10 cigarette butts each with and without the associated tobacco, leachate of 30 cigarette butts each with and without the associated tobacco, leachate of 75 cigarette butts each with and without the associated tobacco were prepared for an experiment on adults 3 months mice. For an experiment on baby mice 20 and 40 cigarette butt leachate were prepared. 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. Straining through a tea strainer was used to remove tobacco and butt debris. The leachates were prepared every 2 days and were given to the mice every 3 days since the preparation needs to be leached out overnight.

Preparation of mice

Laboratory Swiss albino mice obtained from the Ethiopian Public Health Institute (EPHI) in Addis Ababa were used for this study. Two groups of mice were purchased on 2 different time frames. The first group of 175 mice were 3 months old and weighed 33-37mg.

Then in the second round 6 week aged 25 mice were purchased for the second experiment. They were given two weeks of acclimatization period and maintained at ambient room temperature under natural day and night periods. The mice used for both experiments were randomly selected for the study and housed in groups of five mice per cage with free access to water and standard mouse pellets.

Administration of leachate preparation

Two major experiments groups were conducted using a total of 200 mice (175 adult (3 months old) and 25 baby (6 week old) Swiss albino mice). The first experiment used 3 months old adult mice, consisting 7 experimental arms (each of the seven experimental arms had 25 mice, allocated in 5 cages) taking 6 different leachates prepared and one control arm (taking only water) conducted for 5 weeks to see the sub-acute toxic effect of cigarette butt leachate. The second experiment was conducted on baby mice to see if sub chronic cigarette butt leachate intake had effect on slowing growth, conducted for 10 weeks. It has 3 arms which consist of one control arm (taking only water), the second and third arm taking the two prepared leaches.

The leachates with and/or without tobacco attached butts were given immediately to the mice in place of their regular drinking water. The leachates in the drinking bottles were freshly made and replaced every 3 days. The mice were drinking the leachate were continuously monitored by measuring their fluid intake (Moerman & Potts, 2011).

Measurement of fluid and food intake

For quantitative determination of fluid (water or leachate) uptake, for both experiment I and II, fluid volumes in drinking bottles used by five mice in the cages were accurately measured every 3 days periodically and expressed as mean \pm SD fluid intake per five mice (i.e. per cage) per day. Fluid drunk per cage was measured in ml and then it was divided by five to get the amount of fluid taken up by individual mouse in that particular cage. Since there were five cages per experimental arm, the mean fluid intake from the five cages was calculated and expressed as the experimental group fluid intake. To quantify the amount of food consumed, food pellets given to mice were weighed initially, and 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. This means the food intake per cage was

measured and then it was divided by five to get the amount of food taken up by individual mouse in that cage within two days; the measurement per day was little, that is why expressed per two days. The weight gain was assessed differently for the two experiments. For the first experiment weight was measured at last before performing the necropsy. For the second experiment weight was conducted with 10 days interval.

Blood collection and serum preparation for Experiment I

Blood sample was taken directly from the end of the tail vein by cutting a small amount (1 mm) of tissue with a scalpel blade to determine the blood glucose level of each mouse. Blood glucose was determined by O-Toluidine method (Dubowski, 2008). The normal range for mice random blood glucose was 190-250 mg/dl (Itziar *et al.*, 2010). A larger amount of blood was required for serum liver enzyme tests. For this purpose blood was collected from the facial/temporal vein after neck dislocation of mice (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 way between the freckle and the inferior part of the ear, and 0.5 to 1.5mL of blood in which drops were collected by gravity into an Eppendorf tube.

Serum was prepared after coagulation of blood at room temperature for 45 to 60 minutes and centrifugation at 4000 rpm for 10 minutes (Baker, 2010). Serum was stored at -70°C until the blood tests were performed.

The laboratory tests were Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), Alkaline Phosphatase (AP) and blood glucose levels. The aforementioned tests were performed by laboratory technologist at Black Lion Hospital using BT-2000 chemistry analyzer which uses a spectrophotometric principle. The Biotechnica (Italy) Chemistry analyzer has a built in mechanism which enables it to detect the activities of ALT, AST and ALP enzymes in blood serum. The results were displayed on a computer output in IU/ml. (Ahn, 2011).

Mouse necropsy and tissue histopathology

Mouse necropsy (autopsy) was performed at the end of experiment I, after mice were bled for serum tests. Mouse tissues (liver, kidney, lung and cardiac) were removed immediately from the 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 (Cardiff *et al.*, 2014).

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 the tissue were then stained using hematoxylin and eosin standard histological stains (Cardiff *et al.*, 2014). The histology was performed by a pathologist from the Black Lion Hospital pathology department.

Operational definitions

Leachate: A liquid solution made by soaking cigarette butt overnight.

Toxicity: Altered lung histology

Data quality control

Internal quality controls were run for blood glucose and liver function tests. Weight and volume fluid intake was measured using calibrated weighing balance and flask respectively.

Data analysis

Data were analyzed using SPSS Version 21 statistical software package. Graphs were constructed, and the values of a mouse's body weight, random blood sugar, and serum liver enzymes (ALT, AST, and ALP) were expressed as mean ± Standard Deviation (SD), the difference between each mean was assessed using independent t-test (for comparing 2 means) and f-test (for comparing 3 means). A p-value less than 0.05 was considered to be statistically significant.

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).

Results

Effect of leachates on fluid and food intake for both experiment I and II

Cigarette butt leachates with and without associated tobacco prepared from 75 butts per 250 mL have lower average fluid volume intake (p-values of 0.008 and 0.0075 respectively) than similar comparison groups of lower concentration of cigarette leachates (10 butts/250 mL and 30 butts/250 mL) and water only at 28 days. This is also similar at 6 and 14 days. The results with 6 week old mice in the second experiment showed that there was no difference in the volumes of the leachate

drank throughout the experiment. In this experiment the amount of the daily fluid drunk increased with time in all experimental arms (**Table 1, Fig. 1, and Fig. 2**).

Measuring food intake was considered after seeing weight difference in the 2nd experiment among different experimental arms after taking different concentration of leachates. In this experiment, there was no difference in food intake between the mice drinking cigarette butt leachates compared with the mice fed water alone (**Fig. 2**).

Table 1. Volumes of water and leachates drank by adult mice (expressed as volume drank per 5 mice per day) after various time periods

Fluid in drinking bottle	Mean Volume (mL/ 5 mice/ day) of fluid drank at different times			P-values
	6 days	14 days	28 days	
Water only	35 ± 5	50 ± 5	35 ± 2.5	
10 butt leachate without tobacco	21.3 ± 4	32 ± 5	32 ± 3	0.012
10 butt leachate with tobacco	10.3 ± 3.5	25 ± 4	18 ± 3	0.02
30 butt leachate without tobacco	20 ± 4	21.2 ± 3	23 ± 3.5	0.001
30 butt leachate with tobacco	9.5 ± 4.5	18 ± 5	12 ± 4	0.001
75 butt leachate without tobacco	12.7 ± 3	20 ± 4.5	20 ± 4	0.008
75 butt leachate with tobacco	9.3 ± 3	12.5 ± 5	10 ± 4.5	0.0075

Values are Mean ± SD of volumes of drunk leachate by five Swiss albino mice in each group

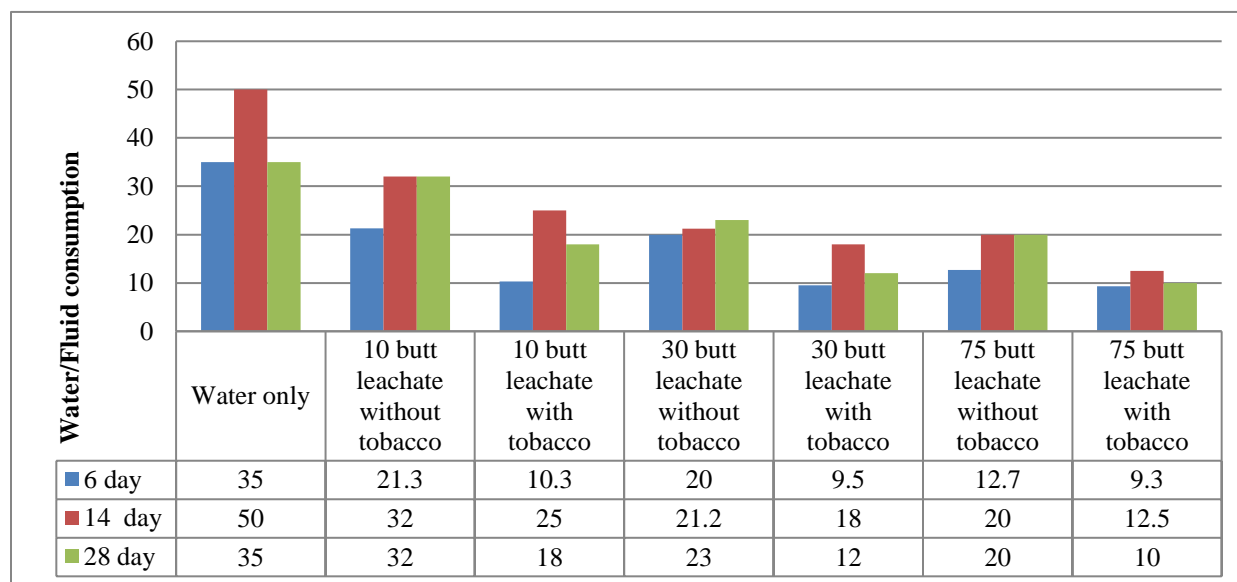


Figure 1: Summary of adult mice fluid intake of water (per day in mL), 10 butt, 30 butt and 75 butt leachate treatments with or without associated tobacco at 6th, 14th and 28th day (Experiment I).

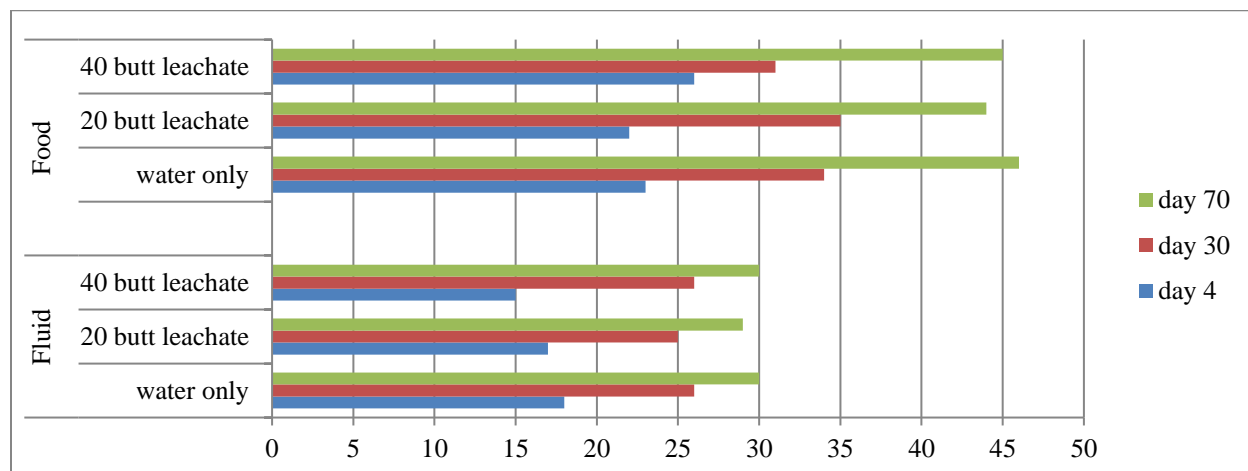


Figure 2: Summary of food and fluid intake of baby mice taking water, 20 butt and 40 butt leachate at 4th, 30th and 70 day (Experiment II).

Effect of cigarette butt leachates on liver tests and serum glucose level of Swiss albino mice for experiment I

There was no statistically significant difference in blood glucose level of the Swiss albino mice that drank only water and those drank cigarette butt leachates with and without tobacco (p-values > 0.05) (Table 2).

All the blood glucose levels were in the normal range for the mice (less than 200 mg/dL). Similarly, there was no significant increase (p-values for AST, ALT and ALP at 95% significance level was 0.09, 0.08 and 0.7 respectively) in any serum markers of liver toxicity (Table 2 and 3).

Table 2: Blood glucose levels in adult mice after 20 days of drinking water alone or leachates made from tobacco butts (with or without associated tobacco)

Treatment (drinking fluid)		Mean random blood glucose (mg/dL)	P value
Water only		149 ±40	0.645
Leachate made from 10 butts/ 250 mL water	Without tobacco	139 ±30.3	0.202
	With tobacco	137 ± 32.4	0.746
Leachate made from 30 butts/ 250 mL water	Without tobacco	149 ±31.5	0.563
	With tobacco	140 ± 30	0.5107
Leachates made from 75 butts/ 250 mL water	Without tobacco	147 ± 32.6	0.900
	With tobacco	135 ± 29.8	0.734

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

Table 3: Activities of serum AST (Aspartate aminotransferase), ALT (Alanine aminotransferase) and ALP (alkaline phosphatase) in mice given 75 tobacco butt leachates (with or without) or water to drink for 5 weeks.

Serum enzyme tests (U/L)	Water only	Leachate made from 75 cigarette butt without tobacco	Leachate made from 75 cigarette butt with tobacco	p-values
AST	279 ± 70.2	209 ±17.5	201 ±31.3	0.09
ALT	103 ±37.5	95 ±2.88	100±10.7	0.08
ALP	196 ±66	219 ±14.5	184 ±3.5	0.7

Effect of leachates on weight of Swiss albino mice for experiment I and experiment II

The mice that drinking leachates made with the highest concentration of tobacco associated butts (75 butts per 250 mL) for 5 weeks were visibly much smaller ($24 \text{ g} \pm 3$) than the mice drinking only water ($46 \text{ g} \pm 5$) and also were visibly smaller than the ones drinking leachates made with cigarette butts only (without associated tobacco, $42.7 \text{ g} \pm 3.5$) ($p < 0.05$) (Table 4).

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 75 cigarette butts associated with tobacco were much smaller than the organs taken from the mice that drank 75 cigarette butts associated with tobacco and only water. The differences was statically significant ($p < 0.05$) (Table 4).

The second experiment also showed that baby mice gained less weight when they drank leachates made from cigarette butts than when they drank water. The mice which drank 20 and 40 cigarette butt leachates weighed less throughout the experiment than those drinking only water. Those mice drank 40 cigarette

butt leachates weighed less than those drank 20 cigarette butt leachates after 20 days of 70 days of experiment. On the last date of experiment 2, the mice drinking water weighed 32.0 ± 8.264 , the mice drinking 20 butt leachates weighed significantly less (29.063 ± 6.7125 ; $p\text{-value} < 0.001$) and the mice drinking 40 butt leachates weighed even less (27.075 ± 5.07 ; $p\text{-value} < 0.001$) (Fig. 3 & Table 5).

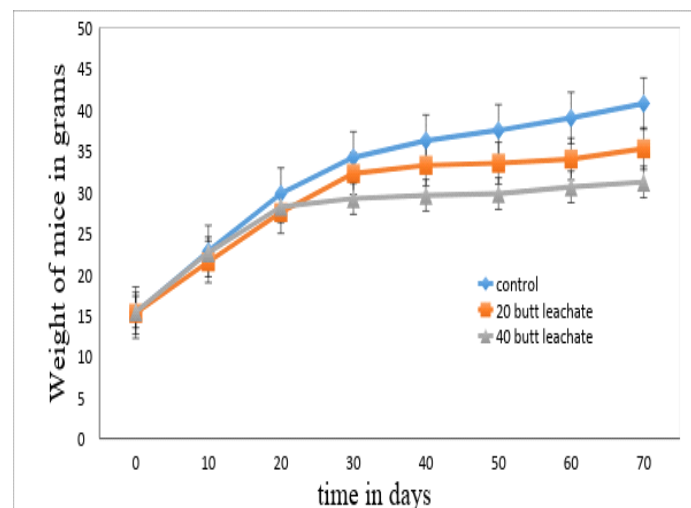


Figure 3: Weight gain of baby mice; given drinking water alone, 20 or 40 cigarette butt leachates, from 6 weeks old (time 0) to 16 weeks old (70 days).

Table 4: Weight of individual organs and tissues of Swiss albino mice drunk leachates made from 75 cigarette butts (with and without associated tobacco) compared with water alone at 35 days of treatment

Organ/ tissue	Mean Organ/tissue mass (mg) at different fluid intake			P-values
	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 \pm 18mg	214mg \pm 23	112mg \pm 18	0.0001
Liver	1,870 mg \pm 22	2,300 mg \pm 22	683mg \pm 15	0.0009
Lung	271mg \pm 22	ND	217mg \pm 15	0.00057
Kidney	382mg \pm 20	ND	224mg \pm 20	0.00012
Epididymal fat pad	182mg \pm 17	242mg \pm 20	36mg \pm 25	0.000145
Spleen	232mg \pm 18	270mg \pm 22	62mg \pm 22	0.00079
Mouse body weight	46 g \pm 5	42.7 g \pm 3.5	24 g \pm 3	0.00078

ND = not determined; Values are Mean \pm SD of tissue weight of five Swiss albino mice in each group

Table 5: Weight of mice in experiment II using baby mice conducted on the 70th day of experiment

Treatments	Weight (Mean \pm SD)	p-values
Water (control)	31.9525 \pm 8.264	
20 butt leachate	29.0625 \pm 6.7125	<0.001
40 butt leachate	27.075 \pm 5.06649	<0.001

Lung histological findings

The only tissue necropsy that showed a difference between the mice drinking water compared with the mice drinking cigarette butt leachates was that of the lung tissue. The primary finding was the increased alveolar wall thickness with mononuclear cell infiltrate, and increased irregular air space volumes, compatible with lung fibrosis and emphysema (**Fig. 4**).

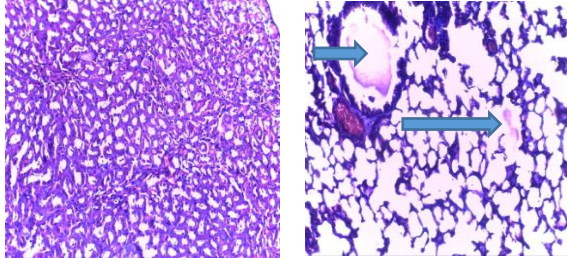


Figure 4: Histology (hematoxylin and eosin staining) of lung tissue from mice after drinking water alone (left) compared with mice after drinking leachate made from tobacco-containing cigarette butts [75 butt/250 mL] (right) for 5 weeks. Arrows show irregular and enlarged air spaces (Magnification 200X).

Discussion

One of the prominent finding was reduced weight gain of adult mice drank 75 cigarette butt leachate with associated tobacco receiving experimental arms, followed by 30 cigarette butt leachate and water in descending order. This lower weight was not a result solely of decreased fat deposition, and no gross pathology was seen on necropsy. It is not due to reduced fluid intake reported in the adult mice. It is unlikely to be due to a reduced appetite of the mice. This indicates that changes in fluid intake due to leachates of cigarette butts, although significant, did not cause the effects on weight loss and histological abnormalities of lung tissues. The weight of the mice at the end of the experiment II showed a significant difference between baby mice that drank leachate made from 4 cigarette (with associated tobacco) weighed much less than those drinking 20 cigarette butt leachate and water only. This suggests that there is a dose response relationship, with higher concentrations of leachates causing lower increment of weight gain.

One possible reason for the pronounced reduced body weight in the mice drinking leachate with associated tobacco without reducing food intake might be the nicotine in the leachates. The previous two studies has shown that nicotine can induce weight loss or inhibit weight gain, despite there being no reduction in food

or calorie intake (Mangubat *et al.*, 2012, Schechter and Cook, 1976).

Nicotine is known to stimulate energy expenditure through the 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 approximately 5% of the 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 Adenosine Triphosphate (ATP) via ATP synthase, so that fewer calories are stored as fat.

Another possible mechanism by which cigarette 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 (Fuxe *et al.*, 1989). In support of this idea, most tissues and organs were noted to be smaller in the mice that drank cigarette butt leachates.

The mice in this study were not exposed to cigarette smoke by inhalation, but were exposed to cigarette components by oral intake only. Lungs of the mice drinking cigarette butt leachates showed significant fibrotic and emphysematous changes, involving alveolar wall thickening and expanded abnormal air spaces. Evidence is accumulating that nicotine itself, in addition to other cigarette toxins, 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 was reported in other study (Chen *et al.*, 2007). There was report which indicates nicotine-stimulated Reactive Oxygen Species (ROS) production has been linked to the damage of epithelial cells in an *in vivo* model of chronic kidney disease (Arany *et al.*, 2011).

Limitation

The study doesn't reflect the real intake of cigarette butt located in real situations/environment. Inability to perform, fractionation tests on the chemical component of the leachate made from cigarette butts and tests for the hormone level of the mice. In addition, absence

of sophisticated laboratory set-up to perform genotoxicity were our limitations.

Conclusions

In both experiment I and experiment II, the mice that drank leachate prepared from the cigarette butts with associated tobacco showed significantly reduced body weight. Gross examination of tissues showed no pathological abnormalities except emphysematous damage to lung tissue. All the organs and tissues examined were smaller in size in the mice that drank cigarette butt leachates containing associated tobacco.

According to histological examination of liver and measurement of serum liver enzymes, cigarette butt leachates showed no liver toxicity. Similarly, there was no effect of leachates on the blood glucose levels. Further study should be done on the growth hormone releasing hormone, growth hormone, insulin-like growth factor levels with cellular receptors and activity of brown adipose tissue of the mice after cigarette butt leachate exposure. In addition, studies should also be done on evaluating brown adipose tissue activity in mice after cigarette butt leachate exposure. Studies on the biochemical identification of cigarette butt leachate components should be performed.

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Author contributions

TT designed the study, data collection, analysis, interpretation, and write-up, drafted the manuscript and critically revised the manuscript, read and approved the final manuscript. FA has been hugely participated in designing, data collection, analysis and write-up.

Competing interests

There is no conflict of interest related to the submitted research work.

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