

## A Simple, Inexpensive and Adaptable Smoke Extraction System

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### Abstract

Tobacco smoking and exposure to biomass smoke are the major sources of exposure to harmful chemicals and particulate matter which may lead to cardiovascular and lung diseases. Our understanding of the pathophysiology of these diseases and the role of smoke constituents in the development of these diseases has evolved over time, but is still limited due to the difficulty in mimicking human smoke exposure *in vitro*. In this study, we developed an adaptable smoke extraction system to generate smoke extracts from cigarettes, bidis (Indian cigarettes), and biomass (cow dung). These extracts were then standardized using UV spectroscopy. A strong positive correlation was observed between the number of cigarettes, bidis or amount of biomass burnt and the absorbance at 400nm. Reproducibility and internal consistency was confirmed between multiple preparations of the smoke extracts using Cronbach's Alpha ( $\alpha$ ) and intraclass correlation analysis (ICC) of the complete absorption spectra. Variation in the absorption spectra were mostly observed between 350 and 550 nm and thus a further analysis of the variation in the absorbance within this range was performed. Internal consistency was again found to be excellent as indicated by  $\alpha$  value of  $> 0.9$  for all the three extracts. ICC measurements also indicated high reproducibility for the absorbance of all three sources of extracts within this range. A dose-dependent decrease in cell viability was observed in RAW 264.7 and A549 cells after treatment with these extracts for 4h as measured by the MTT cell viability assay. Thus, we show the development of a simple smoke extraction system, method for extract standardization and its effect on cell viability with extracts from different sources with these data.

**Keywords:** Cigarette Smoke; Bidi; Biomass; Smoke Extract; Hookah

### Introduction

Inhalation of smoke generated from burning of tobacco (cigarettes, bidis, etc.) and biomass (cooking and heating) has been identified to be responsible for 6 million [1] and 4.3 million [2] deaths worldwide, respectively. Tobacco smoking with an estimated global prevalence of 18.7% in 2012 [3], is one of the leading causes of premature deaths worldwide and is an established risk factor for the development of several diseases including cardiovascular disorders, lung cancer and chronic obstructive pulmonary disease (COPD) [4]. The health consequences of smoking have been known for more than half a century [5], but the mechanisms for the pathogenesis of diseases caused due to tobacco smoking are still incompletely understood.

Cigarette smoke is composed of thousands of chemicals [6] and the biological pathways affected by the individual constituents of smoke add up to the complexity of understanding the development of disease. More than 75% of the global smokers come from the developing world [3] where apart from smoking they are also exposed to other toxic environmental pollutants. In countries like India and many others in South Asia, tobacco is smoked in the form of bidis which are similar to cigarettes and are indigenously made by rolling tobacco flakes in a tendu leaf [7]. Bidis are highly popular, inexpensive and differ from cigarettes in not having a filter which leads to higher exposure to tar and nicotine increasing their overall toxicity as compared to cigarettes. Bidi smoking also leads to higher deposition of the smoke constituents in the lungs as the tendu leaf constituting 60% of the weight of the bidi has poor combustibility, requiring higher frequency of puffs with deeper breaths to keep the bidi lit [8,9].

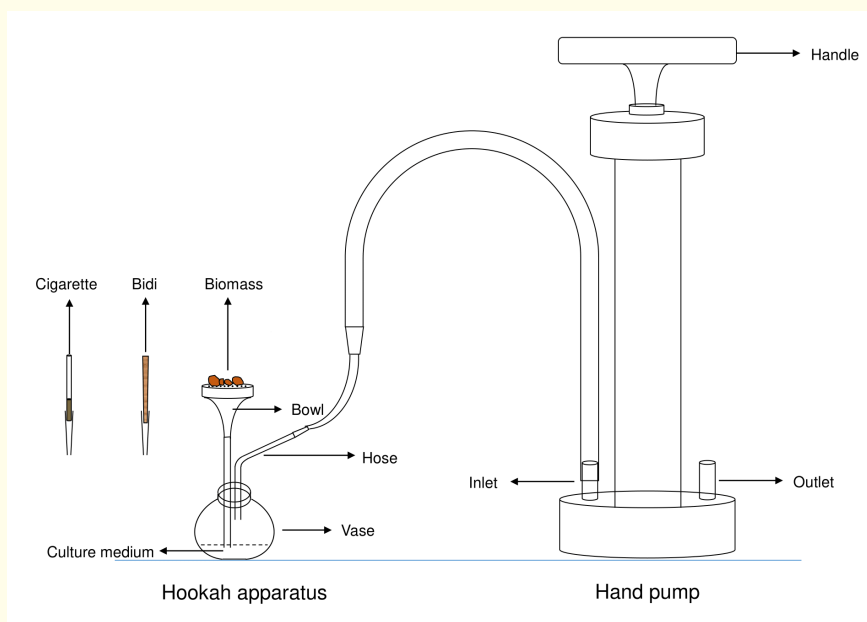
Half of the worlds' 7 billion people are dependent on biomass fuels for cooking [10,11], almost all of which reside in the developing countries. Biomass fuel usually comprise of animal dung cakes dried under the sun, crop residue, wood and kerosene or a combination of these based on availability [9]. The incidence of COPD in non-smokers has been historically, grossly underestimated [12] and burning of

biomass fuels may be the biggest contributing factor to the development of COPD in non-smokers [13]. Additionally, most of the published literature and the majority of clinical trials on pharmacotherapy for COPD has focused on the role of tobacco smoking in the development of the disease, the pathophysiology behind which too is still incompletely understood [14]. COPD in non-smokers has already been observed to clinically manifest differently [15-18] as compared to tobacco-smoking COPD [15]. The molecular basis for which is still incompletely understood.

Smoke generated from the combustion of cigarettes, bidis and biomass is composed of a particulate phase and a gaseous phase both of which contain chemical components that are either bound or free. The water soluble chemical components easily dissolve in the aqueous fluids covering the oral cavity, nasal cavity and the entire respiratory tract. The relatively insoluble chemical components are neither absorbed from the mouth nor retained by the lungs [19] as they are exhaled along with the substantial amount of smoke that is released into the air with expiration. It is important to understand the toxicological effects of the soluble components of the tobacco smoke as well as biomass smoke because they are the ones that are likely to cause greater tissue damage. Moreover, it is essential to understand the biological effects of the complete mixture of water soluble constituents of smoke rather than of individual chemicals as these chemicals may interact among themselves to form more potent toxicants.

The major impediment in studying the pathophysiological effects of cigarette smoke have been the lack of an animal model and the difficulty in mimicking human smoke exposure [20]. A number of *in-vitro* systems have been developed over the years to study the toxicity of cigarette smoke components [21]. The simplest system involves the bubbling of smoke through a liquid media and using the resultant extract to treat cells in an *in-vitro* environment. These systems require a pump to force the smoke through the liquid media and then blow it out. The pumps are mostly electronic which get clogged over time due to the moisture and the deposition of smoke components affects the pressure generated over time.

We have developed a smoke extraction system using a small hookah apparatus and a manual hand-pump (Figure 1) which could be opened and cleaned-up easily. These pumps are readily available and frequently used to inflate toys, air-beds, tyres etc. The hookah apparatus consists of a bowl to hold tobacco which is covered using aluminum foil and has perforations. Charcoal is usually kept above the foil and the bowl is connected to a vase holding water. An outlet from the vase is connected to a hose which is used to draw smoke through. The set-up can be easily adapted to generate smoke from cigarettes, bidis and biomass as we describe further.



**Figure 1:** Schematic of the smoke extraction system for preparing cigarette, bidi and biomass smoke extracts. A standard method for the preparation of smoke extracts involves combustion of 4 cigarettes or 4 bidis or 1g of biomass and pumping the smoke through the liquid medium held in the hookah base. The water-soluble components dissolve in the liquid medium and the smoke is then carried out of the hookah apparatus through the 2-way pump. The extract is then analyzed by absorbance spectroscopy before being filter sterilized and treated on cells.

## Experimental Procedures

**Preparation of cigarette smoke extract (CSE):** CSE was prepared by bubbling smoke from 4 commercially available cigarettes (Kings Gold Flake, ITC Ltd.) into 5 ml of serum-free cell culture medium (DMEM), using a hookah vase apparatus (Being Nawab, Moradabad, India) and a 2-way hand-pump (Intex Recreation Corp., Long Beach, CA) connected to the hose of hookah. Two rounds of pumping burnt one cigarette completely and the procedure was repeated with three more cigarettes. The resultant extract was then filtered using 0.2  $\mu\text{m}$  membrane filter (Pall Life Sciences, Port Washington, NY) for sterilization. The extract was analyzed spectrophotometrically (Multiskan spectrum, Thermo Scientific, Waltham, MA) by measuring the UV absorbance between 200 and 800 nm.

**Preparation of bidi smoke extract (BDSE):** BDSE was prepared by bubbling smoke from 4 commercially available bidis (Sambhaji Bidis, Sable-Waghire and Company Pvt Ltd) into 5 ml of cell culture medium held in the hookah vase as described above. Two rounds of pumping burnt one bidi completely and the procedure was repeated with three more bidis.

**Preparation of biomass smoke extract (BMSE):** BMSE was prepared by using the hookah bowl without the tobacco. 1 gm of cow dung cake (biomass) was placed over the foil where charcoal is kept in traditional hookah systems and lit by sprinkling 2 - 3 drops of kerosene as in traditional biomass cooking. The bowl was connected to the vase containing cell culture medium and the smoke pumped as described above. 15 rounds of pumping would burn the complete biomass and the extract was analyzed spectrophotometrically. The number of rounds required to pump biomass smoke is higher because of higher volume of room air being pulled along with the biomass smoke. In case of cigarette and bidi extract, there is no space of room air to be pulled in due to a better sealing which is not possible with biomass.

**Cell lines:** RAW 264.7 and A549 cell lines were obtained from National Centre for Cell Sciences, Pune. RAW 264.7 cell line, derived from murine monocytes, was cultured in DMEM media (Himedia Labs, Mumbai, IN) supplemented with 10% FBS. A549 cell line, derived from human lung carcinomatous tissue, was cultured in F-12K media supplemented with 10% FBS.

**Cell viability assay:** Cell viability assay was performed by measuring the ability of cells to reduce 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) after exposure to smoke extracts. RAW 264.7 cells were plated at a density of 60,000 cells per well in a 96-well plate and treated for 4h with extracts before washing with sterile PBS twice. MTT was dissolved in PBS (0.5 mg/ml) and then incubated with cells for 90mins. After incubation the cells were washed with PBS and the formazan crystals were dissolved in 100  $\mu\text{l}$  DMSO (Sigma Aldrich, St. Louis, MO). The plate was then read at 590 nm in a UV spectrophotometer (Multiskan spectrum, Thermo Scientific, Waltham, MA). A549 cells were plated at a density of 10,000 cells per well and the above procedure was repeated.

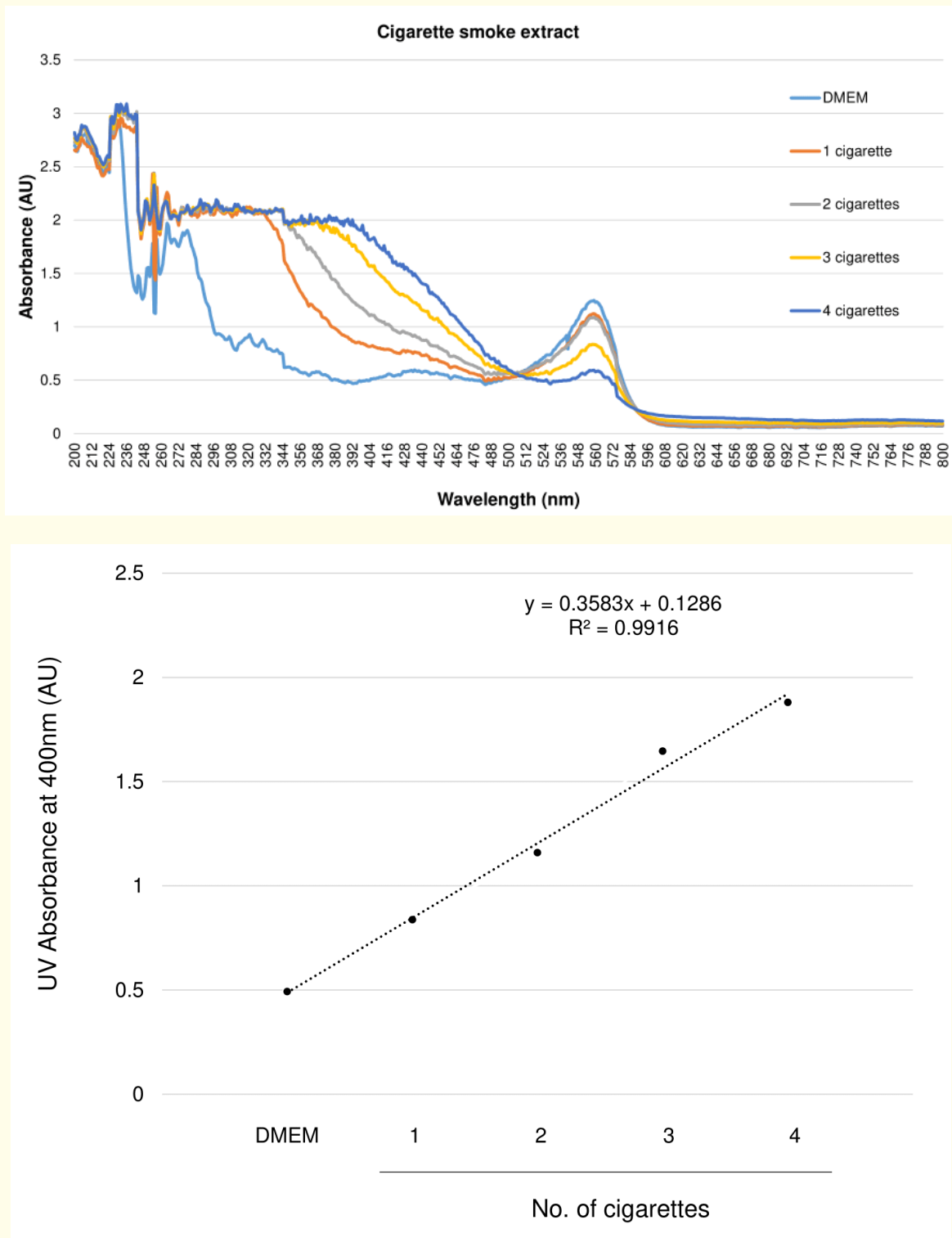
## Statistical analyses

Student t-test assuming unequal variances was performed as indicated in the respective figure legends. Results are expressed as mean  $\pm$  SD from a minimum of 3 experiments. Descriptive statistics was also used for a few experiments as indicated in the respective figure legends. The values were presented in the form of Mean  $\pm$  SD, (Min Max) and 95% CI. The repeatability of the experiment was analyzed using Reliability measure analysis and the results were presented in the form of Cronbach's Alpha and Intra class correlation coefficient i.e. (ICC). Intra class correlation coefficient (ICC) accounts for both consistency of performances from test to retest (within-subject change), as well as change in average performance of participants as a group over time (*i.e.* systematic change in mean). Two-way mixed effects model where people effects are random and measures effects are fixed was utilized. For single measures ICC calculation, the estimator is the same, whether the interaction effect is present or not. The average measures ICC calculation was computed assuming the interaction effect is absent, because it is not estimable otherwise. The analysis was performed using SPSS version 22.0. A p-value less than 0.05 was considered to indicate statistically significant differences.

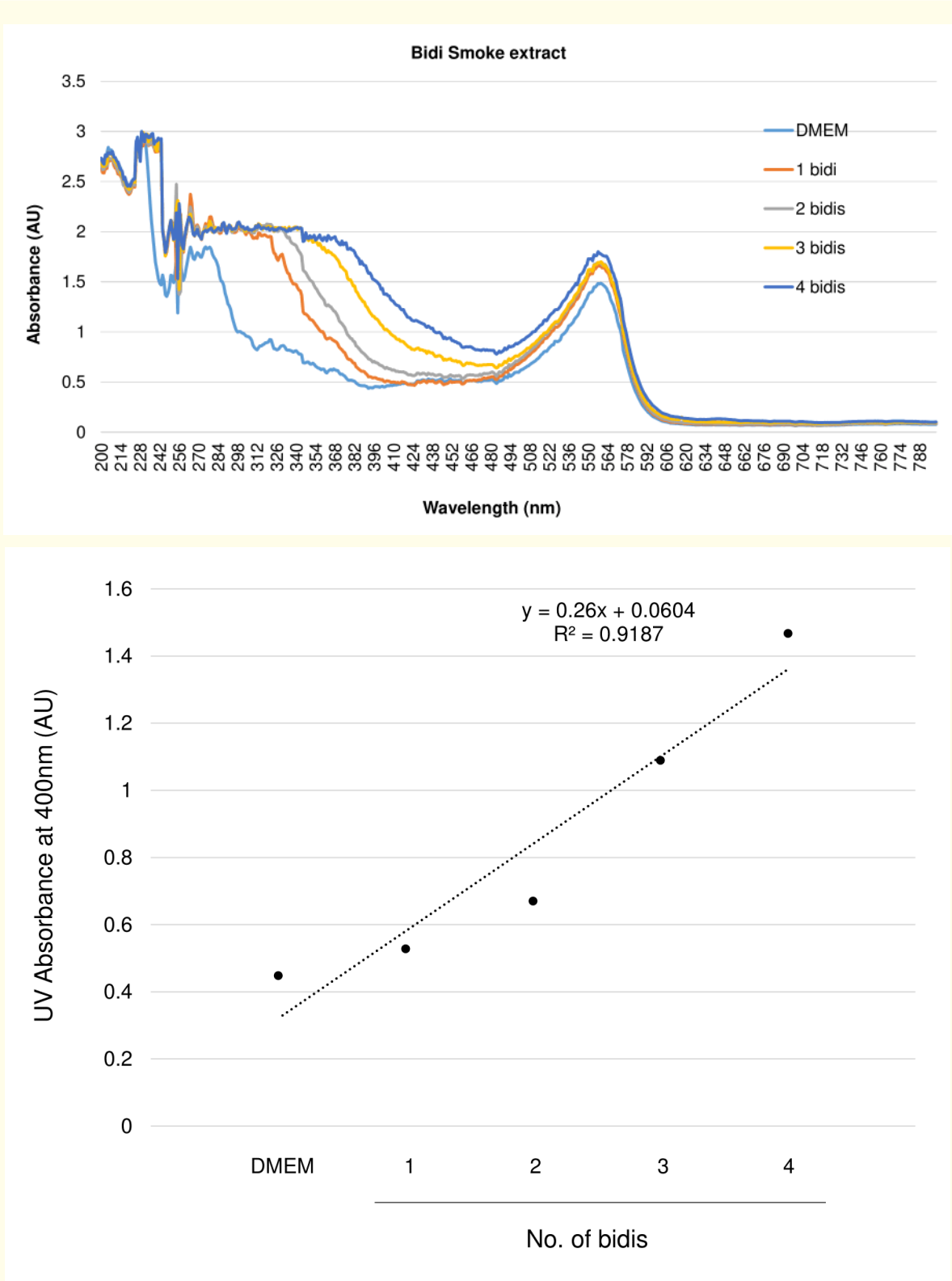
## Results

**Change in absorption spectra with increasing amounts of cigarettes, bidis and biomass:** Spectrophotometric analysis of smoke extracts revealed changes in the absorption spectra with increasing number of cigarettes (Figure 2A), bidis (Figure 3A) and amount of biomass (Figure 4A). The maximum changes in the absorption spectra were observed between the ranges of 350 to 500 nm. An almost dose-dependent increase in the absorbance within this range was observed with increasing number of cigarettes, bidis and amount of biomass. The correlation between the number of cigarettes smoked and the absorbance at 400 nm was also studied using the XY scatter plot and showed a strong positive correlation with an  $R^2$  value of 0.9916 (Figure 2B). Similarly, the number of bidis ( $R^2 = 0.9187$ , Figure

3B) and the amount of biomass ( $R^2 = 0.9717$ , Figure 4B) also showed a strong positive correlation with absorbance at 400nm. After bubbling smoke from 4 cigarettes, 4 bidis or 1 gm of biomass the absorbance at 400 nm was comparable from the three sources and thus, these number/amount of cigarettes, bidis and biomass were used for future experiments.

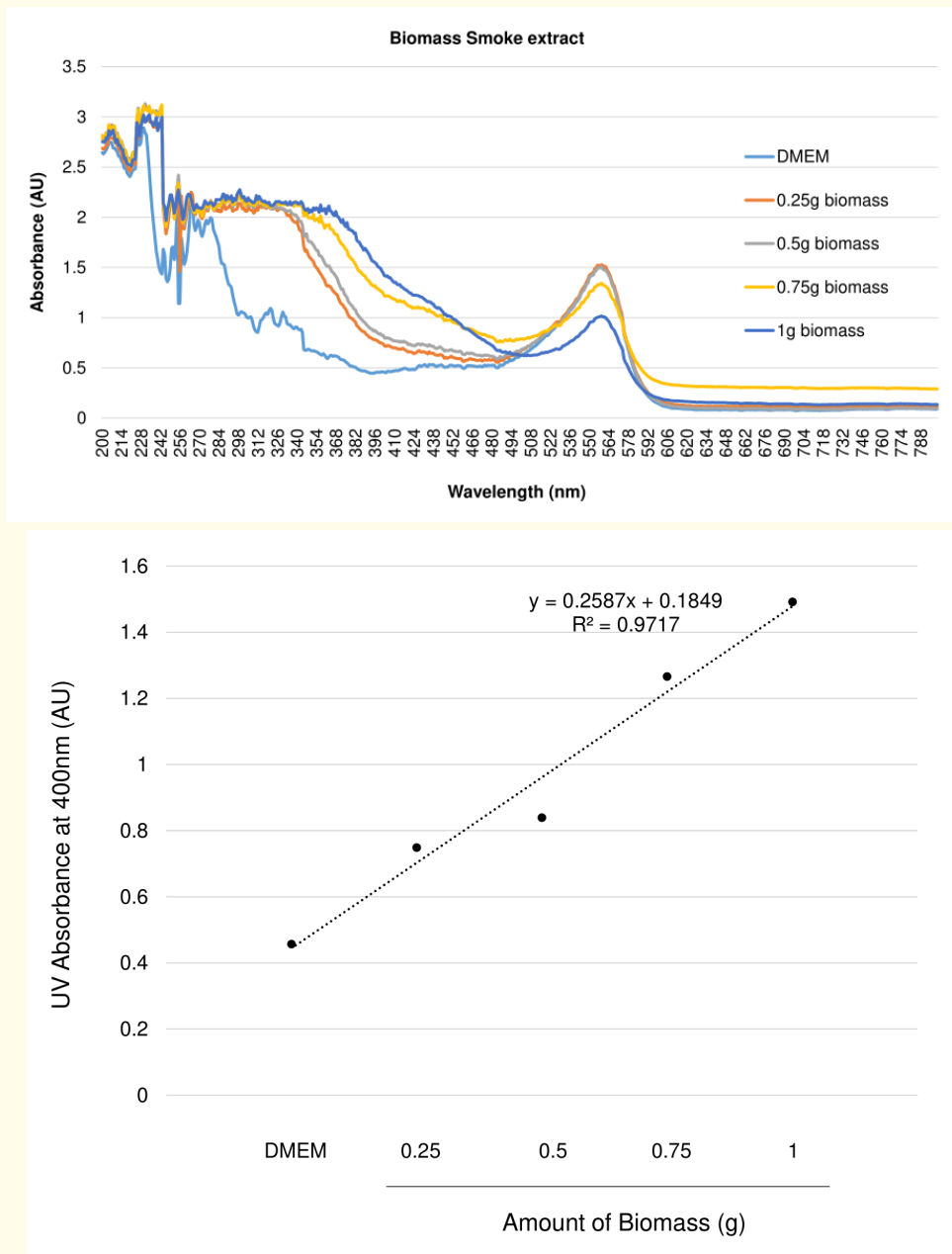


**Figure 2:** Effect of number of cigarettes combusted on the absorption spectra of the resultant smoke extract. (A) Complete absorption spectra of cigarette smoke extract with increasing number of cigarettes' smoke bubbled through the liquid medium. (B) Correlation analysis of absorbance of the smoke extract at 400 nm and the number of cigarettes burnt using an XY scatter plot.



**Figure 3:** Effect of number of bidis combusted on the absorption spectra of the resultant smoke extract.

(A) Complete absorption spectra of bidi smoke extract with increasing number of bidis' smoke bubbled through the liquid medium. (B) Correlation analysis of absorbance of the smoke extract at 400 nm and the number of bidis burnt using an XY scatter plot.

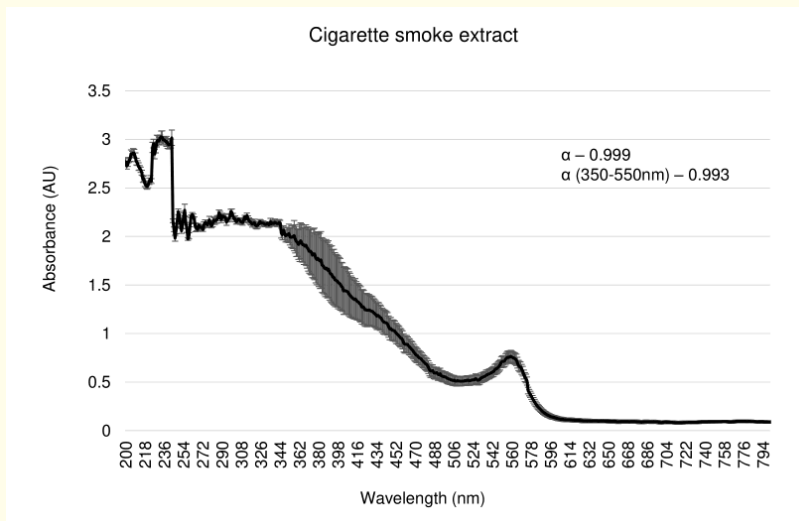


**Figure 4:** Effect of amount of biomass combusted on the absorption spectra of the resultant smoke extract. (A) Complete absorption spectra of biomass smoke extract with increasing amount of biomass' smoke bubbled through the liquid medium. (B) Correlation analysis of absorbance of the smoke extract at 400 nm and the amount of biomass burnt using an XY scatter plot.

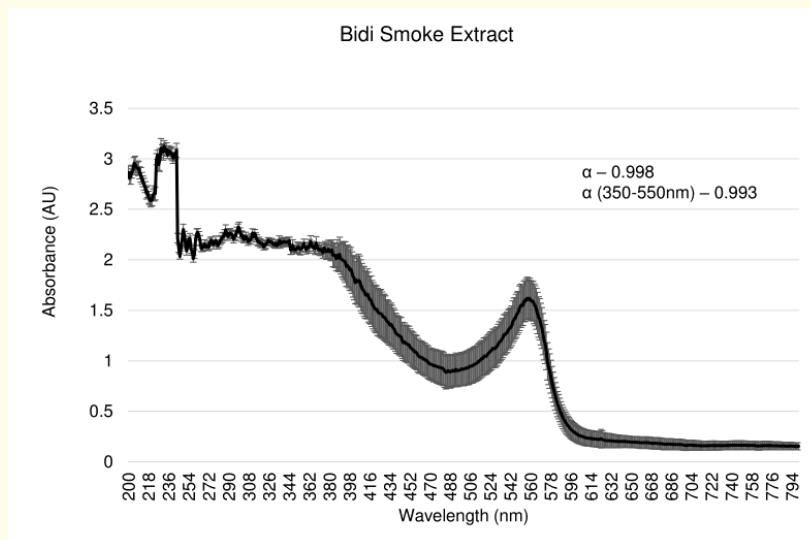
**Reliability of smoke extracts' preparation method:** The reliability of the extraction method to produce replicable extracts was measured by repeating the procedure multiple times and then analyzing the absorbance spectra for reproducibility and internal consistency. The internal consistency of the prepared extracts was excellent with Cronbach's alpha values of 0.999 (Figure 5), 0.998 (Figure 6) and 0.999 (Figure 7) for CSE, BDSE and BMSE respectively. As shown in figure 5-7 the variations were observed mostly between the wavelengths of 350 nm to 550 nm. Thus, a further analysis of the spectra from 350 to 550 nm was performed and the internal consistency confirmed with Cronbach's alpha values of 0.993 (Figure 5), 0.993 (Figure 6) and 0.994 (Figure 7) for CSE, BDE and BMSE, respectively. The reproducibility of the extracts was also measured using an intraclass correlation analysis. The correlation coefficient values of 0.990, 0.984 and 0.992 (Table 1) for CSE, BDSE, and BMSE, respectively indicates strong evidence for the reliability of the measurement at different time points. Further analysis of the variable region from 350 to 500 nm also indicated high reproducibility with ICC values of 0.915, 0.866 and 0.920 (Table 1) for CSE, BDSE, and BMSE, respectively.

	Intraclass Correlation	95% Confidence Interval		F Test with True Value 0			
		Lower Bound	Upper Bound	Value	df1	df2	Sig
CSE	.990	.986	.993	711.153	600	2400	0.000
CSE (350 - 550 nm)	.915	.897	.932	55.065	200	804	0.000
BDSE	.984	.981	.985	359.129	600	3005	0.000
BDSE (350 - 550nm)	.866	.839	.890	39.813	200	1005	0.000
BMSE	.992	.991	.993	732.538	600	3005	0.000
BMSE (350-550nm)	.920	.903	.935	70.193	200	1005	0.000

**Table 1:** Intraclass correlation coefficient analysis of the complete spectra and the variable region of the spectra (350-550nm) from a minimum of five preparations of each extract.

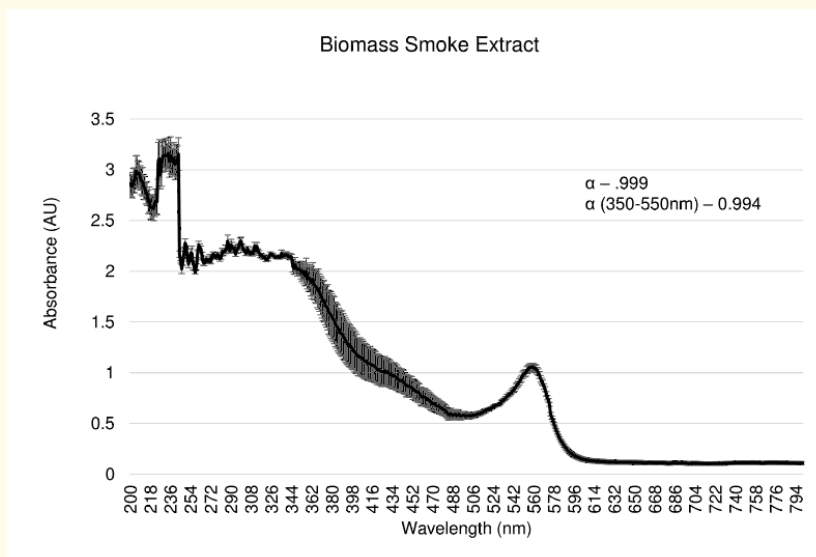


**Figure 5:** Variation in the absorption spectra of multiple preparations of cigarette smoke extract. The average absorption from five individual preparations of CSE was plotted across all wavelengths to measure the consistency of extract preparations. Reproducibility ( $\alpha$ ) measures are also indicated for the multiple preparations.



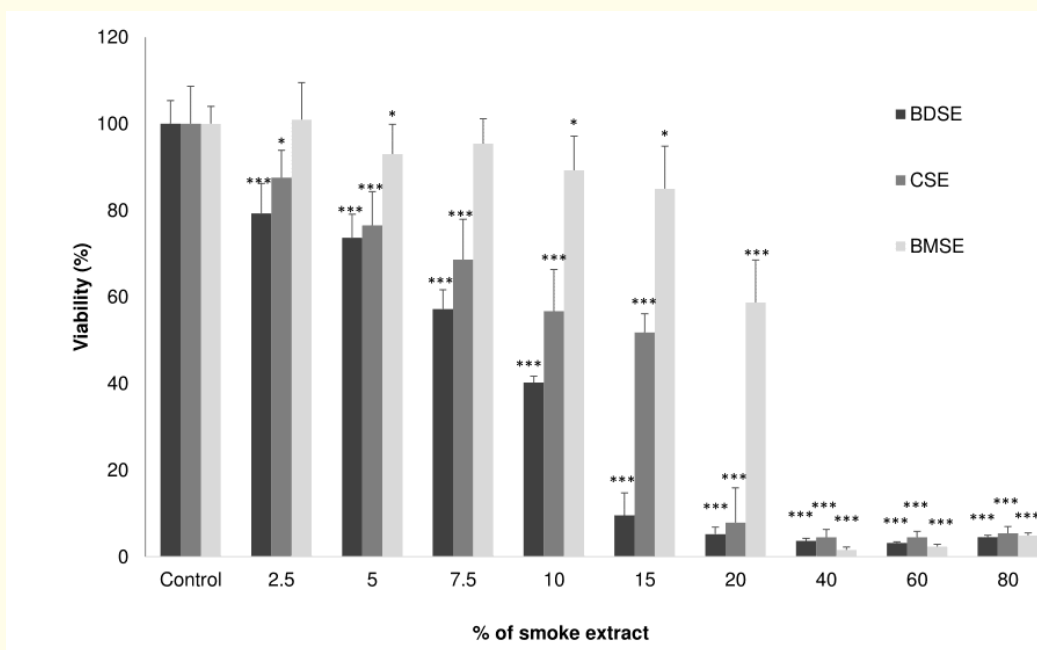
**Figure 6:** Variation in the absorption spectra of multiple preparations of bidi smoke extract. The average absorption from six individual preparations of BDSE was plotted across all wavelengths to measure the consistency of extract preparations. Reproducibility ( $\alpha$ ) measures are also indicated for the multiple preparations.





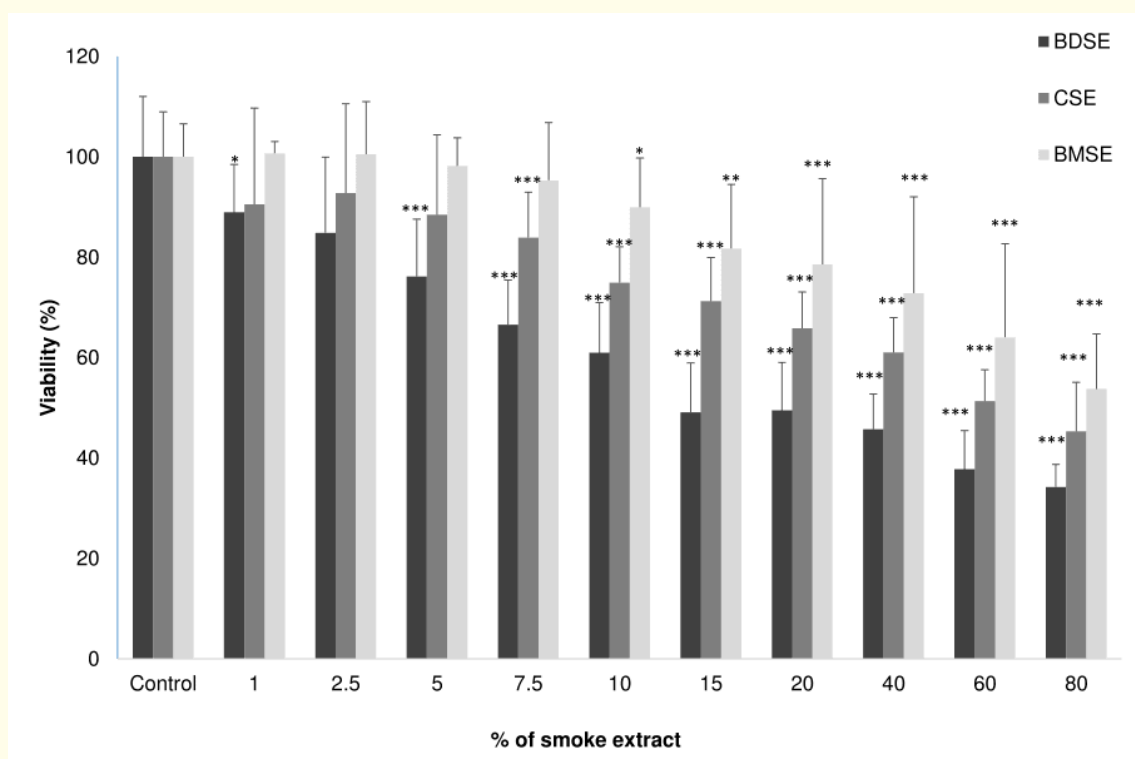
**Figure 7:** Variation in the absorption spectra of multiple preparations of biomass smoke extract. The average absorption from six individual preparations of BMSE was plotted across all wavelengths to measure the consistency of extract preparations. Reproducibility ( $\alpha$ ) measures are also indicated for the multiple preparations.

**Smoke extracts induced cytotoxicity in RAW 264.7 and A549 cells:** The effect of smoke extracts on cell viability was measured by studying the ability of cells to reduce MTT after 4h of exposure to smoke extract. A dose-dependent decrease in cell viability was observed in RAW 264.7 (Figure 8) and A549 (Figure 9) cells after exposure to increasing concentrations of smoke extracts. RAW 264.7 cells were more susceptible to smoke extract-induced cell death than A549 cells. The  $IC_{50}$  for RAW 264.7 cells treated with BDSE was between 7.5% and 10% and that for A549 cells was ~15%. The  $IC_{50}$  for RAW 264.7 and A549 cells treated with CSE was 15% and 60% respectively, whereas that for cells treated with BMSE were ~30% and 80%, respectively.



**Figure 8:** Smoke extracts induced cytotoxicity in mouse monocyte (RAW 264.7) cells. The cytotoxicity of BDSE, CSE and BMSE was studied by measuring the ability of the cells to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) as described in Experimental Procedures section. \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$  compared with control of the respective cells as evaluated using  $t$ -test.





**Figure 9:** Smoke extracts induced cytotoxicity in human epithelial (A549) cells.

The cytotoxicity of BDSE, CSE and BMSE was studied by measuring the ability of the cells to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) as described in Experimental Procedures section. \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$  compared with control of the respective cells as evaluated using *t*-test.

## Discussion and Conclusion

Historically, aqueous cigarette smoke extract has been prepared by pumping smoke from burning cigarettes into a liquid medium with different laboratories using syringes or electronic suction pumps [22-24]. This has led to enormous variability in the concentration and composition of aqueous CSE's used for research. This is indicated from the variation in the LD<sub>50</sub> values for cigarette smoke extract in different cell-lines from various studies. For example, the LD<sub>50</sub> for cigarette smoke extract in A549 cells ranges from 5% to 50% after 24 hr exposure [25-30]. The current study utilized a hookah assembly (Figure 1) in place of traditional laboratory conical flask with a rubber stopper for holding the liquid culture medium, as it allowed to adapt the same assembly for generating smoke extracts from other sources such as biomass. The other important component of a smoke extraction system is the pump used to force the smoke through the liquid medium and is also the most expensive one. Electronic pumps cost thousands of dollars and are difficult to repair and clean, whereas manual pumps usually cost ~20\$. Thus, using a two-way manual hand-pump provides an inexpensive alternative for the preparation of these extracts and the complete assembly cost us 25\$ as compared to at least 500\$ required for the electric pump assembly for smoke extraction. These pumps are easily available and can be readily opened and cleaned to avoid congestion. The pumps get clogged over time due to the deposition of smoke components and moisture which affects the flow rates over time and thus, manual pumps would be better suited for their ease in cleaning and to maintain consistency in the extract preparation. Thus, the method combines two readily available systems i.e. the hookah assembly and the hand-pump to generate smoke extracts from different sources of smoke.

The absence of any standardized method to prepare as well as compare the extracts from different laboratories [31] has further compounded the issue of variabilities in the prepared extracts. We propose using a UV spectrophotometry based method for standardizing the prepared extract. A number of groups have standardized the extract based on peaks obtained at a specific wavelength such as 320 nm [24,32], 302 nm [33] but do not show the complete spectra. As shown in figure 2A, 3A and 4A; a number of peaks could be obtained and a linear increase could be seen between the wavelengths of 350 to 550 nm. We selected 400nm as the reference wavelength to measure the correlation between absorbance and the number of cigarettes, bidis or amount of biomass as a dose-dependent increase could be seen in

the absorbance at this wavelength. A strong positive correlation was observed between the number of cigarettes (Figure 2B), bidis (Figure 3B) or amount of biomass smoked (Figure 4B) and the absorbance of the liquid medium. This indicated that the smoke extract system was reliably able to extract increasing amounts of smoke constituents in the liquid medium. The repeatability of smoke extract preparation was further confirmed using the test-retest reliability statistical measures. Multiple preparations were analyzed for the variations in the UV absorbance as an indicator for consistency in the preparations and excellent consistency was observed using reliability statistics (Tables 1). Further analysis of the variable region of the absorbance spectra from 350 to 550nm also showed excellent consistency (Tables 1).

The cytotoxicity of sterile filtered extracts was measured using the MTT assay. BDSE was found to be the most toxic among the three extracts in this set-up for both RAW 264.7 (Figure 8) and A549 (Figure 9) cells. We further probed if this toxicity was because of the difference in amounts of tobacco flakes in cigarettes and bidis. It was observed that the average amount of tobacco flakes and other constituents in a single cigarette was 2.6g whereas that for bidi was 0.8g. Thus, BDSE was found to be more toxic than CSE although having more tobacco product. This may also be because of the absence of a filter in bidis. Bidis differ from cigarettes in that they lack a filter which can reduce the exposure to tar, nicotine and other constituents of cigarette smoke. This may be one of the major reasons for the relatively lower toxicity of CSE. It could also be because of the difference in toxicity of the combustion product of dried tendu leaf compared with paper (cigarettes). BMSE which does not constitute any tobacco but mostly animal dung was found to be the least toxic among the three but the toxicity was fairly comparable with BDSE and CSE.

Toxicity differences were also observed between RAW 264.7 (Figure 8) and A549 (Figure 9) cells after treating with BDSE, CSE and BMSE. 10% of BDSE exposure for 4h reduced the RAW264.7 cells' viability to 40%, whereas the same concentration of BDSE exposure for the same duration reduced the cell viability of A549 cells to 60%. Similar differences were also observed with cigarettes indicating the sensitivity of smoke extracts to different cell lines. As A549 cells are structural (epithelial) cells and from human origin they could be expected to be stronger than RAW 264.7 cells which are circulatory and from mice origin.

The study, however; is not bereft of a few limitations which includes the inability to control for human error in the preparation of the extracts. As the pump is manually operated the differences in the effort exerted to pump the smoke may lead to errors in the preparation. If the pump is operated using a higher force the smoke will be exited faster providing it little time to dissolve the water-soluble components. On the other hand, if the pump is operated slowly, smoke may not enter completely into the liquid phase and have a very low surface area to dissolve the constituents before being sucked out. Another limitation is lack of extraction of the water-insoluble components of smoke using this method. The lipid-soluble components do not get extracted in the aqueous medium and are released with the smoke. Additionally, the sterile filtration step also removes the particulate matter > 0.2  $\mu\text{m}$ .

In summary, we have developed a standardized method for preparation of smoke extracts from different sources using the same extraction system. We also show that the extraction system makes possible the comparison of extracts from different sources. This should help laboratories to develop similar methods and streamline the investigation of toxicity from various sources of smoke. This is a simple and inexpensive method to generate smoke extracts for various *in-vitro* toxicological studies and could serve as an important tool to study the pathophysiological differences between smoking and non-smoking COPD which are yet unexplored.

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