



Original Article

Acute Toxicity of Cigarette from Vietnam on in Vivo Model Zebrafish (*Danio rerio*) Larvae

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Abstract: Smoking is widely known to be a major contributor to public health issues both worldwide and in Vietnam. At the same time, Vietnam has a large number of smokers and the market is filled with diverse brands of tobacco products. In this study, we sampled 6 types of cigarettes: ThangLong, Vina, Craven, Demi, Maxx and Bastos from local stores and conducted toxicological tests on zebrafish larvae. Our results show that there are variations in toxicological properties of total particle matter collected from different cigarette brands in lethal and morphological effects on zebrafish embryos. LC50 of 6 brands after 96 hours of exposure were: ThangLong = 48.7 mg/L, Vina = 45.96 mg/L, Craven = 80.52 mg/L, Demi = 30.91 mg/L, Maxx = 83.54 mg/L and Bastos = 74.92 mg/L. They are lower than 100 mg/L, which put them under Category 2 and 3 in the GHS classification criteria for acute toxicity.

Keywords: Zebrafish (*Danio rerio*), cigarette, toxicological test.

1. Introduction

The link between smoking and health concerns has been well established. Despite the scientific claim of an increased risk of pulmonary and respiratory diseases in smokers, over 1 billion people around the world are still using tobacco and 8.7 million people are killed each year, 7 million from direct uses and around 1.2 million from second-hand smoking [1]. WHO has launched many programs to educate the public and research about tobacco in multiple countries including Vietnam.

According to the latest Global Adult Tobacco Survey (GATS) in 2015, Vietnam is a country with high tobacco consumption, with more than 45.3% men and 22.5% overall (15.6 million adults) currently smoking tobacco. In addition, nearly 34.5 million non-smokers are at risk of exposure to secondhand smoking [2].

Throughout the world, tobacco products are consumed by various methods and in many forms, for example: snuff, cigars, cigarettes, bidis, kreteks, ... In Vietnam, cigarette is the most popular method of consuming tobacco in Vietnam with 18.2% overall (12.6 million adults) [2]. Currently, Vietnam has over 62 regulations (Vietnam standards) for tobacco and tobacco products, 9 of them are technical specifications for materials and production, 58 for testing

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methods and sample preparation. Notably, QCVN 16-1:2015/BYT regulates the maximum amount of Tar and Nicotine in each cigarette at 16 mg and 1.4 mg respectively. However, the cigarette market is a vivid place with many choices, various brands of cigarette with differences in flavor, packaging and prices competing in this highly profitable market. They introduce their products with claims on distinct tobacco origin, unique size, special designed filter,...

In the research community, zebrafish (*Danio rerio*) is a very popular model animal [3] and in recent years, it has been proven to be an excellent tool to study the effect of smoking on living organisms. In particular, zebrafish larvae have been used to compare the toxicity of different brands of cigarette [4], examine the toxicity of traditional tobacco (snuff) to modern one [5], investigate new types of nicotine products like e-cigarette [6] and elucidate the effects of tobacco products on the neuro system [7, 8], cardiovascular system [9, 10].

In this study, we investigated the effect of various brands of local cigarette in Vietnam on zebrafish by toxicological test on zebrafish larvae. By testing the total particle matter (TPM) from different cigarette brands and conducting standard toxicological test on the robust model of zebrafish larvae, we would provide insights on the diversity of cigarette products in Vietnam.

2. Methodology

2.1. Zebrafish Handling and Maintenance

Zebrafish (*Danio rerio*) were a kind gift from the laboratory of Life Science, GIGA, University of Liege, Belgium and were raised at our Zebrafish facility in Vietnam University of Science. The room temperature was maintained at 27 ± 1 °C, 14:10 light/dark cycle, fish water pH was between 7 - 7.5. Zebrafish were fed 2 times per day with a diverse menu consisted of: flake food (Tetramin), dried pellet (Hikari), boiled egg yolk or newly hatched brine shrimp.

Embryos were collected from multiple adult zebrafish couples to reduce genetic bias, they were sorted at 4 - 64 cell stage to remove unfertilized or defect embryos, cleaned and placed in petri dishes before further experiments.

2.2. Cigarette Selection

Six commercial brands of cigarettes were selected to represent popular cigarette brands in Vietnam. Our choices were based on the result of the Global Adult Tobacco Survey conducted in 2010 and 2015, in which WHO conducted interviews on a large scale to report about the trend in smoking across multiple countries including Vietnam. According to their results, the most popular brands of cigarette in Vietnam were: Thăng Long, Vinataba, Craven, Bastos and Jet. We have managed to purchase and tested 6 types of cigarettes under those brands: Thăng Long (ThangLong), Vinataba (Vina), Craven A (Craven), Craven A demi (Demi), Craven A maxx (Maxx) and Bastos blue (Bastos). Jet was excluded from the list because the brand has been forbidden in Vietnam and all Jet brand cigarettes in the market came from illicit routes. Most cigarettes in this selection have the typical size of 8mm width and 8 cm in length and equipped with filter. Two notable exceptions are Craven demi which has slim design (6mm in width and 7 cm in length) and Craven Maxx with advertised “flow filter” (advertised to increase the smoking sensation).

2.3. Cigarette Preparation

A simple cigarette smoking machine was built (Figure 1), consisted of a small mechanical vacuum connected to an air-tight chamber with a Cellulose Filter (Advantech 5C) installed inside to collect the total particle matter (TPM).

Cigarettes were smoked by machine with intact filter and without blocking the ventilation hole, in this study, instead of representing human smoking behaviors, we simply want to extract as much particle matter as possible from each cigarette. As a result, our smoking regime had only 1 puff at constant volume that last until the cigarette burnt to near the butt, which

was much more intense compared to ISO standard. At least 2 cigarettes were consumed for each filter. Subsequently, the filter would be transferred to a 11 cm autoclaved clean glass petri dish and dry in the incubator at 50 °C for at least 2 hours to evaporate any moisture left by the smoking process. We then measured the weight of filter (Weight 1), rinsed the filter with 10 mL DMSO 0.5% twice to get the TPM resided on the filter into the stock solution. The filter would be dried again at 50 °C again for at least 4 hours before being weighted again (Weight 2), the amount of TPM collected was calculated by subtraction of Weight 1 to Weight 2 and was used to calculate the concentration of the stock solution (Figure 1).

Stock solution would be aliquoted and diluted to suitable concentrations. The concentration range was discovered by another toxicological experiment to bracket the high concentration which caused lethal effects on all experimental subjects and the low concentration in which no effect would be found after 96 hours of exposure.

2.4. Zebrafish Toxicology Test (0h - 96h)

Our toxicology assay was based on the guideline of OECD to toxicology testing of fish (OECD 236 and OECD 203). In brief: Sorted embryos at 4 - 64 cell stage were put into 24 well plates at the density of 1 embryo per well. The A1 - D1 column is used as internal control and filled with 1 mL of DMSO 0.5% vehicle control, while the others contain concentration prepared from stock solution, experiment density was 20 larvae per concentration. In case fish couples did not produce enough embryos for this high density or we did not have enough supply of 24 well plates, the toxicological test would be conducted on 6 well plates, each well was filled with 4 ml of designated concentration and 10i - 20 embryos.

The lethal rate and malformation would be recorded every 24 hours to 96 hours after fertilization. Images are acquired by smartphone under stereo microscope (Leica MZ75). Experiments were replicated in triplication.

2.5. Statistical Analysis

Lethality was defined according to guidance in OECD 236, heartbeat was the main criteria to confirm the death of larvae at 48 - 96 hour after fertilization (hpf). LC50 - the concentration at which 50% of experiment subjects was dead by testing substance, was calculated by the dose response analysis in GraphPad 8 and fitting nonlinear dose response curve. The difference between brands of tobacco was examined by comparing the logLC50 value of fitted dose response curves by F test.

3. Results and Discussion

3.1. Acute Toxicology Effect on Zebrafish Larvae

In all cigarette brands, we could observe a dose dependent lethal effect on zebrafish larvae when exposed to total particulate matter (TPM) obtained from burning the cigarette. Most brands show no to little lethal effect in lower concentration range 10 - 50 mg/L and most embryos perished at concentration higher than 150 mg/L after only 24 hours of exposure (Figure 2 A-F). Major differences between toxicology of 6 cigarettes could be observed in the 50 mg/L - 100 mg/L and most relevant in 96hpf dose response curves (Figure 2 A - F, H). Notably, Demi had the highest toxicity at 50 mg/L at approximately 70% resulting in the lowest LC50 (Figure 2G) at 30.91 mg/L and 95% CI with undefined bottom and top at 67.86 mg/L. By the LC50 index (Figure 2G), we could divide 6 types of cigarettes into 2 groups with higher toxicity illustrated by lower LC50 ranging from 30.91 - 48.7 mg/L of Demi, Vina and ThangLong and the other safer group of Craven, Maxx and Bastos with LC50 between 74.92 - 80.52 mg/L. The logLC50 and Hillslope between 6 dose response curves are statistically different between groups (Extra sum of square F test, constrain bottom > 0, top < 1, p < 0.0001).

In summary, the super slim design Demi has the highest lethal effects on zebrafish larvae; the toxicology profile of the 2 most

popular brands ThangLong and Vina are comparable with LC50 at 48.7 mg/L and 45.96 mg/L respectively. Bastos, Maxx and Craven A are safer alternatives with LC50 > 70 mg/L. However, it is noted that even the safest cigarette brand Maxx has LC50 at 83.54 mg/L, which shows very potent toxic properties and should be put at the category 3 acute toxicity hazard by the GHS standard [11].

3.2. Morphological Effects on Zebrafish Larvae

During the experiments, various malformations in TPM-exposed zebrafish larvae have been observed. The most common phenotype is cardiac sac edema (Figure 3). Hemorrhage and tail defects could also be seen at a lower rate. Malformations could manifest simultaneously, but all three types rarely appear in one larva (Figure 3H). It is notable that at the same concentration of the one type of cigarette, different phenotypes could be observed simultaneously. For example, in the case of Vina,

at concentration of 50 mg/L, yolk sac edema and tail bend were observed in figure 3B, head hemorrhage and yolk sac edema in Figure 3C and hemostasis in the head and the extended yolk sac in Figure 3D, at concentration of 75 mg/L, more prominent edema and severe tail bend could be seen in figure 3E, while figure 3F shows an unhatched larva with hemorrhage near the yolk sac.

Generally, most abnormal morphologies are pictured at the middle range of the experimental concentration range: from 50 mg/L to 150 mg/L, especially 50 mg/L and 75 mg/L. The toxicity in this range was capable of altdevelopment of zebrafish larvae, but the disruptions were not enough to kill them. Larvae mortality could explain why less malformation could be captured at a higher concentration (> 150 mg/L) after 96 hours of exposure. In the case of a cigarette brand with lesser lethal effect like Bastos, malformations were recorded at higher concentration range (Figure 3H).

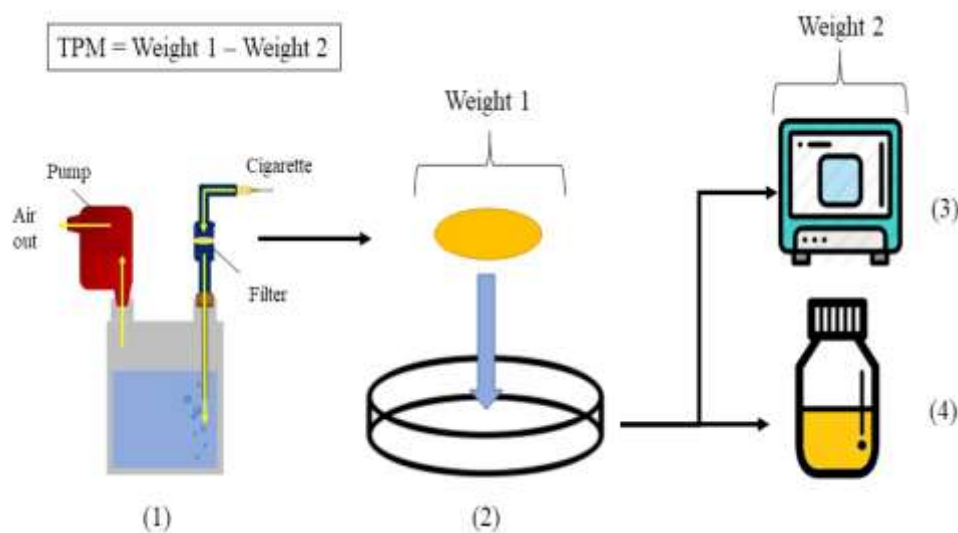


Figure 1. Stock preparation procedure.

- (1) TPM to a filter by cigarette smoking apparatus.
- (2) Dry the filter and weigh the 1st time to get Weight 1, then rinse the filter with E3 medium + 0.5% DMSO to make the stock solution.
- (3) Dry the filter and weigh the 2nd time to get Weight 2, mass of TPM = Weight 1 - Weight 2.
- (4) Calculate the concentration of stock solution and aspirate with E3 to desired concentration. Keep the stock in 4 °C and aliquot before use.

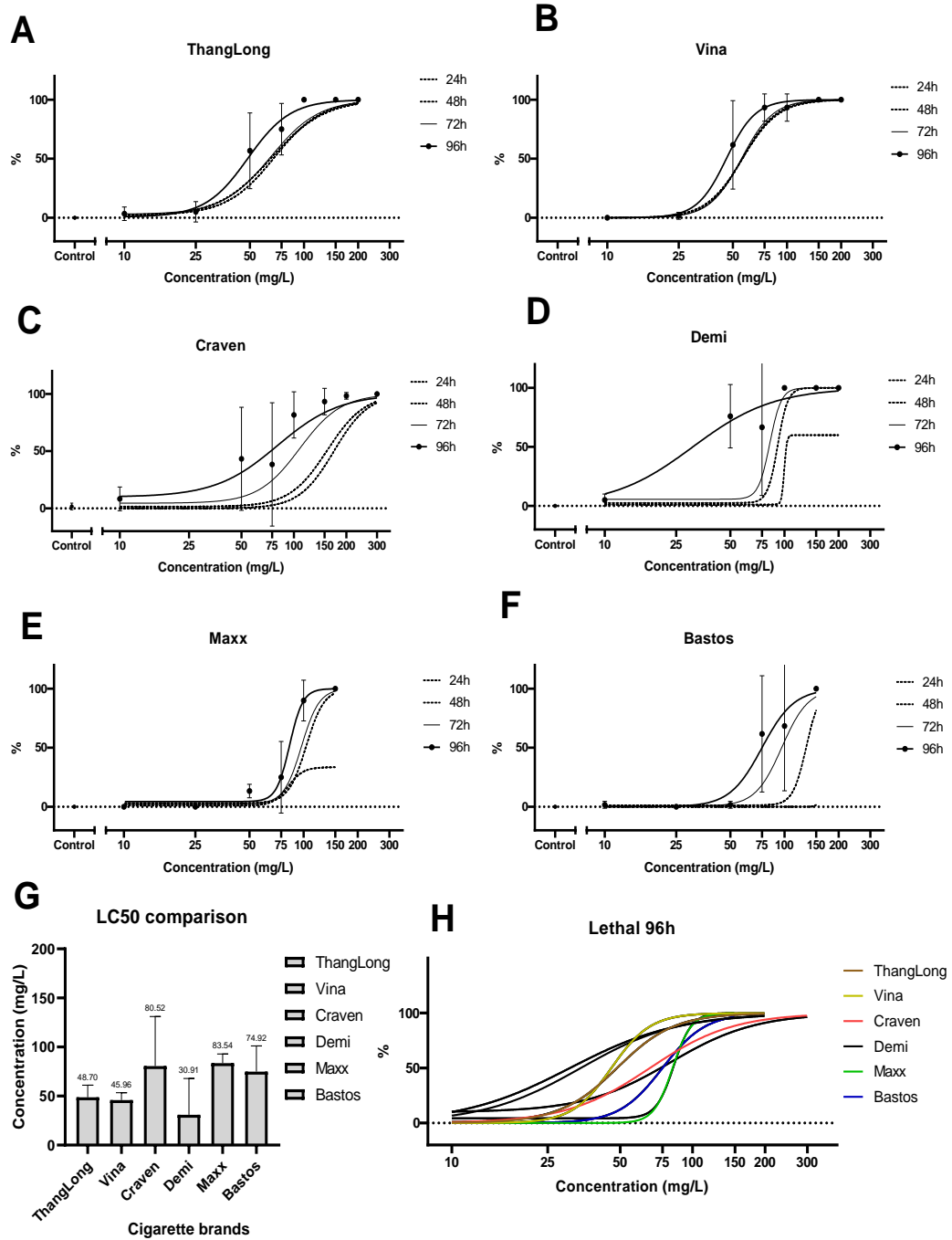


Figure 2. Dose response curves of 6 cigarettes, comparison of LC50 and Lethal curves at 96 hpf.

A-F: Dose response curves show dose and exposure length dependent lethality effect of TPM on zebrafish larvae. G. LC50 at 96 hpf of 6 cigarette brands, error bars show 95% CI. ThangLong, Vinataba and Demi have considerably lower LC50 compared to Craven Maxx and Bastos. H: Dose response curves of 6 cigarette brands, each color represented 1 type of cigarette. Most brands converge at 10 mg/L and concentration higher than 150 mg/L, Craven Demi (Black) shows higher toxicity at 25 - 50 mg/L concentration range while Craven Maxx had little lethal effect from 10 - 75 mg/L.

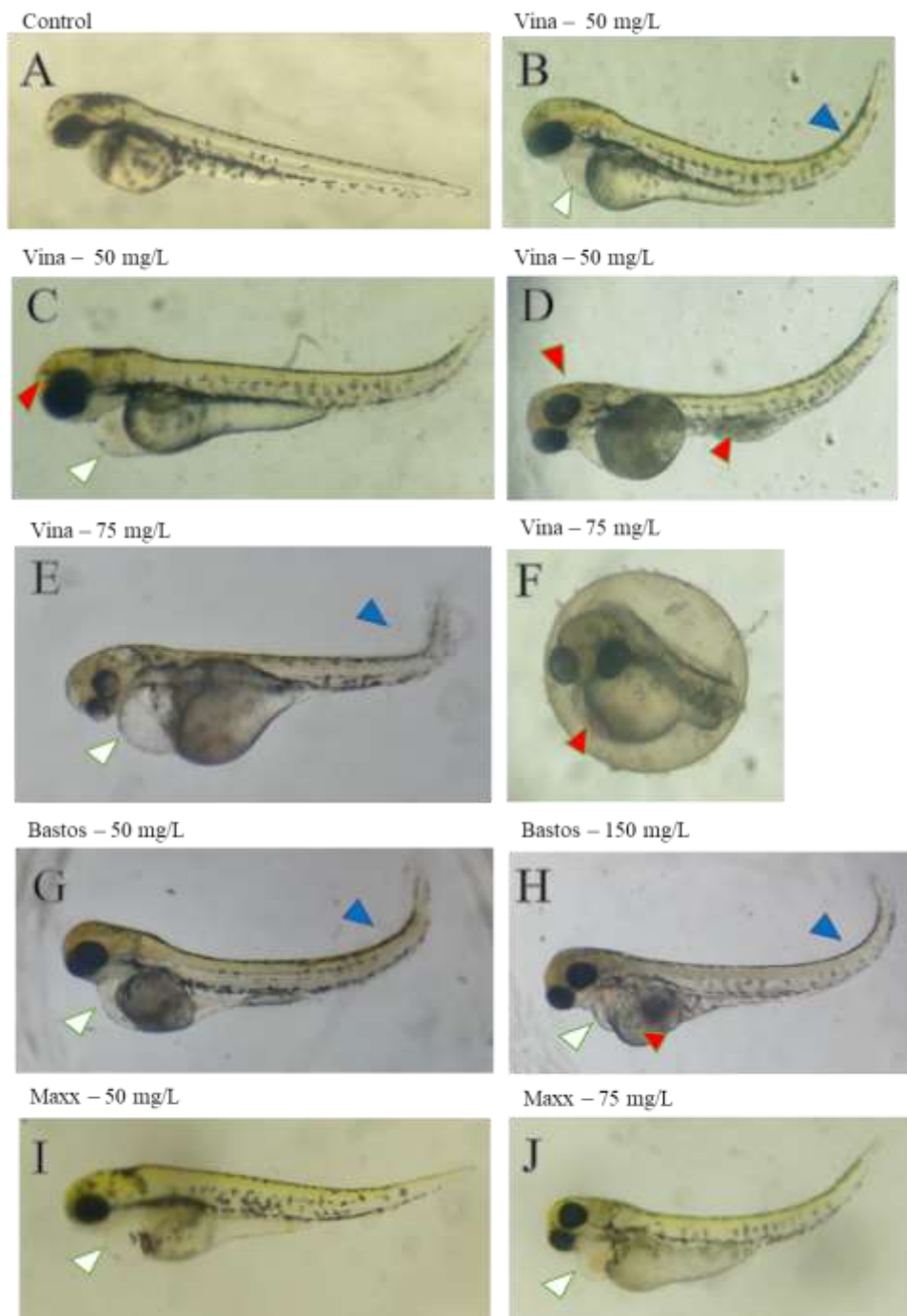


Figure 3. Effect of TPM on zebrafish larvae morphology. Common morphology defects are often detected at 50 mg/L and 75 mg/L and could also be observed in survived embryo of higher concentration. Malformations are mainly consisted of: Cardiac sac edema (White arrow), Hemorrhage (Red arrow) and Tail curve (Blue arrow). Despite the differences in brands, very similar malformations are observed across experiments.

On the other hand, in cigarettes with higher toxicity in the 50 - 75 mg/L range like Demi, embryos showed less malformation phenotypes for most of them had perished. The cardiac edema could be explained by the well proven effect of tobacco on zebrafish development and maintenance of the cardiovascular system [5, 9, 10, 12]. Hemorrhage occurred frequently in the head and yolk of larvae, identical observation could be drawn from other studies [4] and could also be the results of defects in the vascular system.

4. Conclusion

Our results have demonstrated the differences in toxicity of various cigarette brands in Vietnam market on *in vivo* model zebrafish larvae and confirmed the negative effects of tobacco smoke on living organisms. All cigarettes are considered highly toxic on zebrafish larvae and show that the differences in advertised flavors do not well correlate with toxicological effects.

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