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Cytotoxicity and Genotoxicity Assessments of Batik Industrial Wastewater on V79 Cells

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Abstract

Batik is well-known in Malaysian textile industry. It is one of the rapidly growing industries inherited from a generation to another. The manufacturer of batik industry usually discharges the wastewater containing hazardous pollutants (a mixture of chemicals especially reactive dyes, waxes, alum, resin, and sodium silicate) into the environment without any preliminary treatment. This study was conducted to assess the cytotoxicity and genotoxicity of batik industrial wastewater in three drums of wastewater on V79 cells as a preliminary study for a toxicity testing. The physicochemical properties of the wastewater were assessed. The cytotoxicity was assessed by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Meanwhile, the genotoxicity of the wastewater was evaluated via alkaline Comet assay. This study found that the wastewater from all the three drums demonstrated a cytotoxic effect toward the V79 cells at various concentrations, and drum A showed lower IC_{50} value compared to drum B and C. The IC_{50} in drum A are 8.8%, 8.0%, and 8.4% v/v for December 2014, January 2015, and February 2015, respectively. Meanwhile, for genotoxicity study of the wastewater samples on V79 cells, the study found that the value of tail moment for all samples were lower than 2 scores with the highest score is 1.842±0.150, while the results for the negative and positive controls were 6.5±1.079 and 0.436±0.012, respectively. In conclusion, the wastewater from all three the drums in this study had a cytotoxic effect but did not demonstrate a genotoxic effect on V79 cells, indicating no DNA damage inflicted.

Keywords: Batik Wastewater, Cytotoxicity, Genotoxicity, V79 Cells

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1. Introduction

Various industrial activities produce effluents often contaminated with hazardous or toxic materials. For example, the textile industry which uses enormous quantities of water and generates large amount of wastewater (Hai *et al.*, 2006). In this industry, the textile dyeing involves an intensive use of chemicals and a large quantity of water and produces large volumes of water from different processes (Babu *et al.*, 2007). According to Banat *et al.*, (1996) although colored organic compounds are just a minor fraction of organic waste in the wastewater, they could reduce the esthetic value of a water body.

Batik, which is the product of hand-painted and richly colored patterns, is a Malaysian-made textile inherited from generation to generation. Nowadays, this industry has become a commercialized industry and is a major contributor to the economy of several states especially in the east coast such as Kelantan and Terengganu (Noor and Rohasliney, 2011). This industry usually operates on a small scale, for examples at the backyard house, workshops, and small factories without proper waste management systems (Sridewi *et al.*, 2011). Due to that the operators of this industry usually release their industrial effluents into the environment without any proper treatment (Siti *et al.*, 2013), thus a causing a widespread pollution through the industrial wastewater containing dyes, waxes, and heavy metals with high Chemical Oxygen Demand (COD) and Total Suspended Solid (TSS) (Rashidi *et al.*, 2012). According to Rashidi *et al.*, (2012) initial treatment to the batik industrial wastewater should be carried out as the wax and resin released from the industry could harm the environment.

A very large quantity of dyes, wax, and chemicals

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including ludigol, sodium silicate (Na_2SiO_3), sodium carbonate (Na_2CO_3), sodium alginate, and calcium sulfate is used in batik industry (Wahid and Munaim, 2011; Ahmad *et al.,* 2012). The main problem of wastewater resulting from the textile industry is color and its toxic effects. According to Anjaneyulu *et al.*, (2005) although colored organic compounds are generally only a fraction of the organic load of the wastewater, but it can cause reduce the esthetic value of a water body. Besides being able to decrease the esthetic value, industrial wastewater also cause problems to aquatic organisms, any change in water quality caused disturbances in the physiology and biochemistry of aquatic organisms. Toxic compounds from industrial effluent enter the aquatic organisms through the food chain and eventually to humans by consumption of the seafood (Puvaneswari *et al.,* 2006).

Therefore, this toxicology study of batik industry wastewater is essential as a first step to identify potentially hazardous substances for better wastewater handling in the future and to ensure this wastewater does not degrade the quality of surface water, do not affect the environment, and do not directly affect humans. Furthermore, in the presence of the toxic component, this study served as a hazard identification for a better handling of the wastewater. The objective of this study was to determine the physicochemical properties, and cytotoxic and genotoxic effects of batik industrial wastewater on V79 cells.

2. Materials and Method

2.1 Sampling Location and Time

The sampling was done at one of the batik enterprises located at Hulu Langat, Selangor. Water samples were taken from each drum (A, B, and C) containing the wastewater from different batik making processes. Drum A was the first wastewater after the color turn off the process of the cloth by using Na_2SiO_3 to act as a fixer. Drum B was the wastewater point from the process of boiling the cloth with Na_2CO_3 (soda ash) to remove the waxes. Finally, drum C was the final wastewater point where the cloth was soaked and finally rinsed. Sampling was done thrice on December 2014, January 2015, and February 2015. All samples were collected, and direct reading was taken in triplicate.

2.2 Physicochemical Analysis

The physicochemical analysis was conducted based on the method by Hach (2002). The pH, dissolved oxygen, and temperature readings were taken using a multiparameter. Later, the wastewater samples from each drum were filled into a 1 L Schott bottle and Biochemical Oxygen Demand (BOD) bottle. Bottles containing water samples were placed in a cold box filled with ice all the way to the laboratory. The physicochemical analysis for the parameters namely TSS, BOD, and COD was made on the day of sampling. As for cytotoxicity and genotoxicity tests, the wastewater and genotoxicity, the wastewater samples were stored at a temperature of −20°C (Zegura *et al.,* 2009).

2.3 Cell Culture

This study used a V79 cells, that is, lung fibroblast cells derived from Chinese hamster male organism, *Cricetulus griseus* obtained from the American Type Culture Collection (CCL-93). Chaung *et al.*, (1997) stated that V79 cells are fibroblast cells that commonly used in studies on DNA damage and repair. These cells can be used to assess basal cytotoxicity (Ekwall and Ekwall, 1988) target organ toxicity (Balls and Fentem, 1992) and in some cases, it can provide information on the dosage of death in the *in-vivo* study (Shrivastava *et al.,* 1991). Furthermore, V79 cells were used because these cells are recognized and mostly used in studies on toxicity and mutagenicity (Cingi *et al.,* 1991).

These cells were cultured in a Dulbecco's Modified Eagle's Medium (DMEM) with L-glutamine supplemented with 10% foetal bovine serum and 1% penicillin/streptomycin. The cells were cultured in T75 or T25 culture flask with an ambient temperature 37°C, humidity 5% CO_2, and 95% air.

2.3.1 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide Assay

The cytotoxicity of the samples was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) according to Mosmann (Mosmann, 1983). This assay measures the conversion of MTT to insoluble formazan by dehydrogenase enzymes of the intact mitochondria of living cells. In this study, the V79 cells were seeded at a density of 1×10^4 cells/well into 96-well plates in three replicates. After 24 h of incubation at 37°C to allow attachment of the cells, the growth medium was replaced with a fresh medium containing 10%, 20%, 30%, 40%, and 50% v/v of water samples, and then the cells were incubated for 24 h. After treatment, 20 µL of MTT was added, and the cells were further incubated for

4 h at 37°C. The medium was removed, and the formazan crystals were dissolved in dimethyl sulfoxide. The amount of formazan crystals directly correlated to the number of viable cells. The Optical Density (OD) was measured at 570 nm using a microplate reader. Cell survival (viability) was determined by comparing the OD of the wells containing cells treated with wastewater samples to cells exposed to 50% v/v of distilled water in a growth medium. A 30% reduction of the viability by the sample was considered a cytotoxic response (Zegura *et al.,* 2009).

2.3.2 Alkaline Comet Assay

In this experiment, 5×10^4 cells/well was seeded in a 6-well plate, incubated for 24 h in DMEM medium, and then treated with each concentration of IC_{50} results from the MTT assay. The treatment time was 2 h. At the end of the treatment, the cells were washed with 2 mL of phosphate buffered saline at 37°C and trypsinized with 0.5 mL of trypsin. After 1 min, the cells were suspended in a complete medium, and the cells were centrifuged at 25 rpm in 5 min. These steps were repeated for 2 times before the cells were transferred into an Eppendorf tube. 100 µL of 0.6% NMA was embedded on the frosted slides while 80 µL 0.6% LMA was mixed with cells pallet and layered on the dry NMA on frosted slides. All the slides were placed inside a coplin jar filled with lysis buffer for 24 h. After 24 h of the lysis process, the slides were electrophoresed in an electrophoresis tank filled with electrophoresis buffer for 20 min. Then, the neutralized steps were taken where the slides were dropped with a neutralize buffer pH 7.5 in 5 min repeatedly. After the slides were dry, 45 µL of ethidium bromide was dropped on the slides, and the coverslip was placed. The slides were kept in an opaque box for 24 h at 4°C. The slides were analyzed via fluorescent microscope (Leitz Laborlux Epifluorescence Microscope, Germany) equipped with 515 barrier filter and 560 emission filter. Fifty cells per slide were scored, and the percentage Tail Moment (TM) of DNA was analyzed.

2.4 Statistical Analysis

The data were expressed as the mean±standard error of the mean. Statistical analysis was performed by Statistical Package for Social Sciences version 20.0 (SPSS Inc.), IBM Software by employing one-way ANOVA. From the analysis, the data were considered statistically significant when $P < 0.05$. One-way ANOVA was used to find the relationship between each drum.

3. Results and Discussion

3.1 Physicochemical Assessment

Table 1 shows the readings of the physicochemical parameter for the batik industrial wastewater during sampling in December 2014, January 2015, and February 2015. Each parameter in this study was compared with the parameters in the Malaysian Environmental Quality (Industrial Effluent) Regulation 2009 standard B, Environmental Quality Act 1974 to determine whether the effluent discharged into water bodies exceeded the prescribed limit (EQA, 1974). The pH readings were observed, and it can be concluded that this batik wastewater was alkaline as the average pH value ranged from 8 to 12.5. Five out of nine pH readings were observed to exceed the prescribed limit set by the regulation, namely 3.3-9.0.

Table 1. The readings of physicochemical parameter batik industrial wastewater during sampling in December 2014, January 2015, and February 2015

Drum	Month	Parameter					
		pH	Temperature (°C)	DO (mg/L)	COD (mg/L)*	BOD (mg/L)*	TSS (mg/L)**
A*	December 2014	11.2±0.0	27.9±0.0	0.5±0.1	4014.7±0.8	30.2±0.0	22.3±0.9
	January 2015	8.3±0.0	27.8±0.0	4.3±0.0	2179.0±8.9	30.3±0.1	20.7±3.2
	February 2015	8.9±0.00	28.7±0.0	3.3±0.0	2129.3±14.2	36.7±0.0	25.0±1.5
B**	December 2014	11.5±0.0	28.2±0.0	5.2±0.0	63.3±7.9	20.8±0.2	321.7±2.4
	January 2015	12.5±0.0	30.1±0.0	3.2±0.1	274.0±7.0	21.6±0.3	109.7±6.3
	February 2015	8.9±0.0	29.7±0.0	2.8±0.0	266.3±0.9	20.3±0.1	222.3±0.7
C	December 2014	9.9±0.0	28.0±0.0	4.5±0.0	37.7±3.7	21.0±0.4	5.3±0.3
	January 2015	10.4±0.1	27.5±0.0	4.7±0.0	244.0±3.0	20.2±0.1	8.0±0.6
	February 2015	8.8±0.0	28.2±0.0	3.4±0.0	206.7±3.7	26±0.0	7.3±0.9

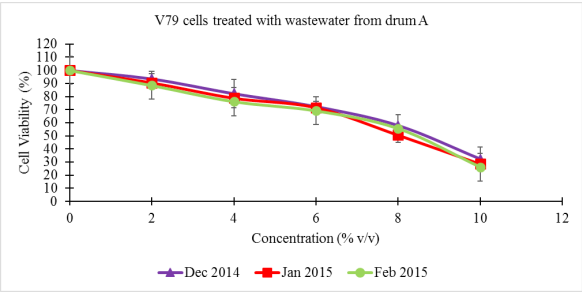
Each value is the average of triplicate expressed in average±SEM. *There is a significant difference between drum A with B and C with P<0.05, **There is a significant difference between drum B with A and C with P<0.05. SEM=Standard error of mean, DO=Dissolved oxygen, COD=Chemical oxygen demand, BOD=Biochemical oxygen demand, TSS=Total suspended solid

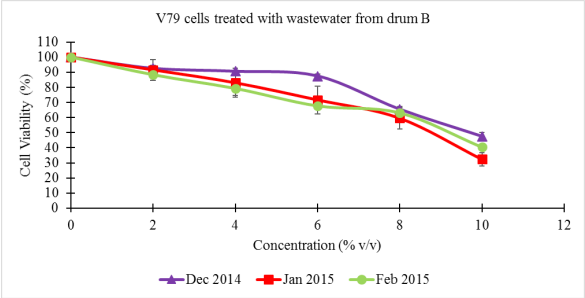
The prescribed limit for COD is 250 mg/L, while, for BOD, there is no prescribed limit suggested in the regulation. This study found that all COD readings from drum A exceeded the prescribed limit. In addition, statistical analysis showed there was a significant difference ($P < 0.05$) in BOD and COD parameters of the wastewater between drum A with B and C. BOD value is high due to the presence of organic materials that are not oxidized from sizing and desizing process which the textiles are treated with starch and enzymes (Hussain *et al.*, 2004).

For TSS, all the readings from drum B exceeded the prescribed limit, 100 mg/L. Statistical analysis showed a significant difference between drum B with A and C. The wastewater in drum B was a result from the process of heating of batik to remove the existing wax on the fabric. Therefore, the main source of suspended solids present in the wastewater was most likely from the wax in solid form at a room temperature.

3.2 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide Assay for Cytotoxicity

Figure 1 shows the percentage of cell viability when the V79 cells were treated with the wastewater samples from drum A, B, and C after 24 h of treatment. For drum A and B, the test concentrations were 2%, 4%, 6%, 8%, and 10% v/v, while for drum C, the test concentrations were 10%, 20%, 30%, 40%, and 50% v/v. Following 24 h treatment, the cytotoxic effects of the wastewaters against V79 cells were assessed using MTT assay, as shown in Figure 1. V79 cells showed a decrease in viability in a concentration-dependent manner following the 24 h treatment. IC_{50} value is defined as the concentration of inhibition when there is a 50% inhibition of cell viability at the concentration. For the sample from drum A, the IC_{50} values observed in December 2014, January 2015, and February 2015 were 8.8%, 8.0%, and 8.4% v/v, respectively, while for drum B were 9.8%, 9.2%, and 8.8% v/v, respectively.





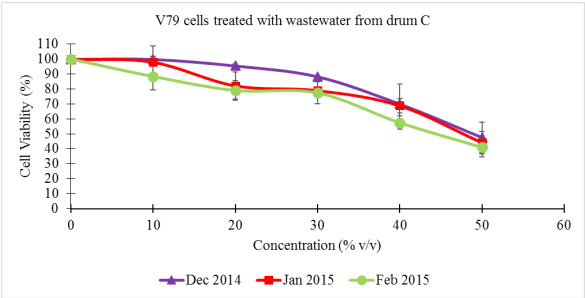


Figure 1. The percentage of V79 cells viability against the concentration of the wastewater in drum A, B and C after 24 h of treatment.

Based on the IC_{50} values observed, it can be summarized that the wastewater samples in drum A and B inhibited the V79 cells at low concentrations. This was probably due to a mixture of substances comprising vinyl sulfone (reactive dye), Na_2SiO_3, fixer substance, Na_2CO_3, resin, and other materials used throughout the batik making processes. The cytotoxic effects shown indicated that the concentration of the chemicals present in the wastewater was very high, strengthened by the high COD reading on drums A and B, which exceeded the guidelines set by the Environmental Quality (Industrial Effluent) Regulation 2009.

Next, the wastewater sample from drum C showed cytotoxic effects at high concentrations on V79 cells. The percentage of cell viability decreased slightly as the concentration increased from 10% v/v to the highest concentration 50% v/v. IC_{50} values were observed for wastewater sample from drum C at 49%, 48%, and 44% v/v for December 2014, January 2015, and February 2015, respectively. These results were most likely because all the chemicals used throughout the process of batik making were washed out in the wastewater from drum A and B, besides the chemical dilution that occurred. This is in line with Srebrenkoska *et al.*, (2014) who states that the nature of the wastewater from textile industry finishing process is less alkaline and has low BOD content.

Klemola *et al.* (2006) stated that dyes used in the textile dyeing process consist of a mixture of commercial dye and other chemicals including calcium stearate, therefore, most discussions and findings of the textile industry wastewater refer to the toxicity of the chemical mixtures rather than a pure chemical. The effects of pure dye are not easy to determine because originally it is in a powder form. However, since all these dyes are a part of the mixture in the wastewater, it is more effective to assess the toxicity of this mixture than the pure dye when assessing the safety of commercial dye formulation. *In vitro* toxicity studies on boar sperm cells against 3 monochlorotriazinyl reactive dyes namely yellow, red, and blue were conducted by Klemola *et al.* (2006). They found that all dyes showed toxic effects on the sperm cells in various concentrations.

In this present study, one-way ANOVA with the *post-hoc* Tukey test was used to compare the IC_{50} values of all the treatment groups. There was a significant difference ($P<0.05$) between wastewater samples from drum C with A and B. Based on the IC_{50} values of the wastewater samples, the sample from drum C was the least toxic. Although, there was no significant difference between drum A and B, the IC_{50} values for wastewater sample from drum A was clearly lower than the wastewater sample from drum B. Our results, therefore, demonstrated that the wastewater sample from drum A was the most cytotoxic among all the three wastewater samples studied.

3.3 Genotoxicity

Figure 2 shows the TM of the V79 cells for the negative control, positive control, and wastewater samples from drum A, B, and C treated with IC_{25} concentration. Alkaline comet assay was employed to detect the primary DNA damage induced by the wastewater in this study on the V79 cell line. IC_{25} concentration from MTT assay was chosen because it was a sensitive assay for the detection of genotoxic substance. If a higher concentration of inhibition was selected, the false positives results may exhibit because this assay would detect any materials that may cause damage to the DNA but could not differentiate it from other cytotoxic substances (Henderson *et al.*, 1998). Hartmann *et al.*, (2001) also suggest the use of 75% and above of cells viability in order to avoid false positive results. Table 2 tabulates the IC_{25} concentration used in this assay, while for menadione, the concentration used was 6 μM.

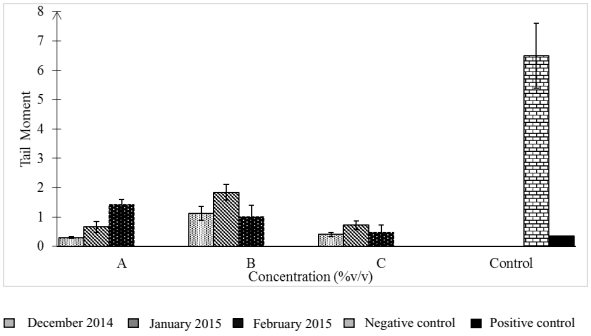


Figure 2. Tail moment of V79 cells for the negative and positive control and wastewater samples from drum A, B, and C treated with IC_{25} concentration.

TM is defined as the product of the distance between the head and the tail by the proportion of DNA in the tail. TM is used to evaluate the extent of DNA migration (Olive *et al.*, 1990). TM provides the most stable estimates for DNA damage because it has a large degree of uniformity in quartile dispersions. As shown in Figure 2, none of the wastewater samples showed any severe DNA damage compared to the negative control used, menadione. All of the TMs observed in this study were below 2, and the highest TM recorded was 1.842±0.150, while for positive and negative control, the readings were 6.5±1.079 and 0.436±0.012, respectively. These results clearly showed that there was no significant DNA damage. Based on one-way ANOVA statistical analysis, there was no significant difference between the TM of all wastewater samples from drum A, B, and C (P = 0.097). Cells treated with menadione demonstrated a significant DNA damage as compared to the treatment with the wastewater samples. Olive *et al.* (1990) suggest that a DNA damage is significant when the TM is higher than 5. Therefore, for this study, it can be concluded that there was no migration of DNA strands, and the wastewater samples from all drums of the batik industry did not cause a DNA damage. Table 3 shows all the TM results from the alkaline comet assay for genotoxicity assessment.

Table 2. IC_{25} concentration from MTT assay used in the alkaline comet assay

Month / Drum	December 2014 % (v/v)	January 2015 % (v/v)	February 2015 % (v/v)
A	5.6	5.4	4.2
B	7.2	5.4	4.6
C	38	35	32
Menadione		6 μM	

MTT=3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, IC25=Inhibition concentration

According to Ghaly *et al.*, (2014) during the dyeing processes, not all the dyes applied to the fabrics are fixed on them, and there is always a portion of these dyes that remains unfixed to the fabrics and is washed out. This is a contributing factor to the high concentration of dyes in wastewater that can cause toxic effects. In studying the harmful effects of textile wastewater, reactive dyes are among the key potential factors for the toxic effects to humans and the environment (Wang *et al.,* 2002). Moreover, the highly complex nature of textile wastewaters may prove to be a limitation factor for a complete assessment of toxicity with chemical analysis (Sharma *et al.,* 2007). Our study revealed that the batik industrial wastewater from drum A, B, and C resulted in a lower level of DNA damage in the V79 cells tested as compared to control.

Although, this batik industry wastewater is capable of causing cytotoxic effects on V79 cells and did not show any DNA damage, precautions must be taken to avoid this wastewater containing toxic chemicals harming the environment and health of local residents. Therefore, reducing the discharge of this textile industrial waste into variety pathway to the environment subsequently can reduce risks to the environment (Shaikh, 2009).

4. Conclusion

Our study found, all the wastewater samples from the batik industry in Hulu Langat, Selangor to be cytotoxic to V79 cells but were negative in the alkaline comet assay. In this study, physicochemical characterization of the wastewater samples served as an important aspect to understand the profile and quality of the wastewater. The study found that BOD, COD, and TSS parameters exceeded the Environmental Quality (Industrial Effluent) Regulation 2009. Early anticipation was made that the wastewaters were hazardous to human and the environment. The results of cytotoxicity assessment toward the V79 cells showed that all the three wastewater drums (A, B, and C) exhibited cytotoxic effects in various concentrations, which was in line with the anticipation made. However, genotoxicity assessment showed negative results toward the V79 cells, that is, there was no DNA damage shown, indicating the batik industrial wastewater did not cause genotoxic effects toward the V79 cells. This study is expected to be the pioneer in toxicity assessment studies on batik industrial wastewater.

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