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ENVIRONMENTAL HEALTH | RESEARCH ARTICLE

Downregulation of liver HSP70 in zebrafish exposed to *Jatropha curcas* seed cake extract

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Abstract: The use of *Jatropha curcas* in biodiesel production is one of the emerging alternative sources of renewable energy. However, the extraction process leaves a seed cake waste and the lack of disposal protocols poses serious threat to ecosystems. This study aims to determine the toxicity of the seed cake extract to *Danio rerio* (zebrafish) through the analysis of heat shock protein 70 expression. The median lethal concentration (LC50) was first determined through an acute toxicity test coupled with probit analysis. For the HSP70 induction test, fishes were subjected to a non-exposed setup and sublethal concentration (50% of LC50) of detoxified, washed, and untreated seed cake. Total RNA from liver samples was isolated and reverse transcribed followed by amplification through polymerase chain reaction (PCR) and gel profiling. Results indicate an LC50 of 0.172% (w/v) or 1.72 g/L. The heat shock induction test showed a significant downregulation of HSP70 expression in fishes exposed to washed and untreated setups. This underexpression may be attributed to phytochemicals such as lectins which have been widely reported to interact with HSP70. Furthermore, mRNA levels in the detoxified setup have no significant difference with the non-exposed setup, possibly indicating a successful inactivation of toxic phytochemicals in the seed cake. Moreover, the present study shows that downregulation of HSP70 is also an indicator of stress, and low HSP70 levels may lead to a decreased resistance of liver cells to stress-inducing agents.

ABOUT THE AUTHOR

Dr A.V. Hallare, the principal author, is an aquatic toxicologist and has been involved in a number of researches on the ecotoxicology of single chemicals or "cocktail" of contaminants present in environmental samples, such as water and soils/sediments. His expertise is on biomarkers of environmental pollution: fish embryotoxicity studies, histopathology, heat shock proteins (hsp 70/90), pollutant-induced DNA damage (comet assay, micronucleus, Ames test), dioxin-like activity (EROD Assay), endocrine disruption (YES Assay), and cytotoxicity assay; He is also involved on researches on bioassay-directed fractionation to characterize teratogens, mutagens, endocrine disruptors, and AhR-active substances in sediments. Results of his works have been presented in various international and national scientific conventions and have been published in internationally-refereed journals on environmental toxicology and aquatic ecosystem health.

PUBLIC INTEREST STATEMENT

Consequent to the growing demand for alternative sources of energy, *Jatropha curcas* seeds remain to be the favorite for biodiesel production. However, a significant volume of the residual organic mass (seed cake) is produced during the extraction process, which raises concerns on safe waste disposal. Thus, there is a call for international collaborative researches to address safety issues, such as verification of the environmental and health effects of alternative fuel sources, such as *J. curcas* seed cake for public welfare. The present study hopes to contribute by investigating on a mechanism of toxic response using a vertebrate model, the zebrafish. The obtained results recommend that proper handling, disposal methods, and detoxification of post-production seed cakes by energy firms should be mandatory in order to alleviate negative environmental impacts. Policies concerning the use of alternative fuels need to be developed before they are allowed to appear in the market.

1. Introduction

Due to the decreasing supply of petroleum and increasing oil prices, the search for renewable sources of fuel and energy is being profoundly conducted in recent years. One emerging source of renewable energy is the production of alternative fuel from the plant *Jatropha curcas*. A member of the family Euphorbiaceae and locally known as "tubang-bakod", *J. curcas* is being grown and its seeds are utilized to produce biodiesel through mechanical and solvent-aided oil extraction. The oil extraction process however leaves a pressed *Jatropha* seed cake as a waste material. *Jatropha* in general was reported to be toxic and poisonous and thus the seed cake is also effectively rendered toxic due to the presence of various secondary plant metabolites (Ahmed & Salimon, 2009; Makkar, Becker, Sporer, & Wink, 1997). Several studies have been conducted concerning the seed cake waste and some detoxification procedures have been proposed; however, there are still no clear protocols concerning its disposal. Furthermore, while detoxification studies have been able to determine and compare the levels of plant metabolites in both treated and untreated seed cakes, there is still limited number of studies with regard to actual testing of seed cake extracts on living organisms. In the Philippines, the use of *Jatropha* seeds as alternative source of biodiesel is just at its inception stage. The pilot testing is being carried out within the confines of the Department of Science and Technology-Biodiesel Pilot Production and Testing Plant. The voluminous seed cakes generated from this extraction are usually stored and used as soil additives in nearby agricultural lands. The operators have not looked into the potential environmental risks associated with this practice nor consider the potential toxicities of the seed cake extracts on living organisms. Since they are introduced in the soil matrices as fertilizers, natural degradation of the waste could happen. There is also a potential for leaching due to heavy rainfall events that is inherent in the Philippine climate system, especially during the wet season. Leaching to the lower soil levels may ultimately result in accumulation in nearby bodies of water, which may be harmful to the ecosystem. As aquatic ecosystems are the ultimate sink of contaminants due to direct discharges and the hydrological cycle (Stegeman & Hahn, 1994), assessing the risk of *J. curcas* seed cake through aquatic toxicology experiments would be beneficial in protecting the ecosystem and preserving biodiversity (Hallare, Carino, & Ruiz, 2014; Travis, Bishop, & Clarke, 2003).

In aquatic toxicology experiments, fishes have been traditionally and still utilized as test organisms due to a variety of reasons. These include their massive contributions in the food web and ecosystem conservation, extensive worldwide distribution, solid dependence on the environmental water quality, and inevitable response and sensitivity to water-borne toxicants in their own habitats (Van Der Oost, Beyer, & Vermeulen, 2003). Thus, in this study, we will make use of the zebrafish as the test organism since it is one of the most commonly used due to several advantages it gives to researchers (Mayden et al., 2007).

Studying gene expression in a test organism is regarded as an effective and accurate way of characterizing a chemical contaminant's toxicity in an environment since gene expression would signify changes in the organism such as cellular metabolism and protein expression (Lettiera, 2006). Analyzing fish gene expression of the test organism, zebrafish will accurately reflect the effect of the pressed cake toxicity in bodies of water. One type of protein frequently used in toxicology is the family of heat shock protein whose expressions are being used as biomarkers to evaluate environmental pollution levels (De Pomerai, 1996). The main objectives of the present study were (1) to evaluate the toxicity of the various treatments of *J. curcas* seed cake aqueous extracts through the determination of the LC50 and HSP70 induction in the zebrafish liver and (2) to determine the efficiency of the modified detoxification procedure we employed in the study.

2. Materials and methods

2.1. Procurement of seed cakes
The seed cake samples used in the present study were generously provided by the manager of the Biodiesel Pilot Production and Testing Plant of the Department of Science and Technology-Industrial Development Institute. They were stored at 4°C until used in the assay.

2.2. Procurement and maintenance of fish stock
Mature fishes were procured from Tropical Fish Farm (Purok V, Pansol, Pila, Laguna, Philippines). Three hundred (300) fishes were transported in high quality fish bags at a density of 5 fishes per liter of water with each bag about two-thirds full of oxygen. Upon the arrival of the zebrafish, they were carefully transferred and maintained at the Aquatic Toxicology Laboratory, Animal House, College of Arts and Sciences, University of the Philippines Manila and fishes were kept in 200-L glass tanks with the following controlled environmental settings utilized by Hallare, Nagel, Köhler, and Triebskorn (2006): temperature (24.0 ± 4.0°C), conductivity (745 ± 2 μS/cm), hardness (379 mg/L CaCO3 = 21.3°dH), pH (7.2 ± 0.4), dissolved oxygen (10.5 ± 2.0 mg/L O2; 95% saturation), and 12 h light/12 h dark photoperiod. 70 L of aquarium water was replaced weekly with the same volume of dechlorinated tap water. They were fed twice daily with commercially available diet (*TetraMin* Flakes). The fish were maintained for at least 2 weeks to give them time to acclimatize in the new environment.

Seed cakes were obtained from the Biodiesel Pilot Production and Testing Plant at the Department of Science and Technology–Industrial Technology Development Institute. Nine kg of samples were collected immediately after mechanical oil extraction and were transported to the laboratory. Seed cake aqueous extract was obtained by extracting 10 g of seed cake per liter of tap water through constant stirring. The mixture, which represents a concentration of 100% v/v or 1% w/v, was filtered (106 μm sieve) and the supernatant was decanted. The remaining liquid was collected and used immediately for the adult zebrafish bioassay. Leftover seed cakes and non-aqueous extract were properly bagged and stored in waste bins for disposal.

2.3. Preparation of seed cake treatments
The experiment was done in part to ascertain the effectiveness of the detoxification process which may render the seed cakes more useful for other purposes (e.g. as fertilizers, etc.). The detoxification procedure was a modified process derived from the technique currently available in the literature and invented by (Makkar & Becker, 2011). One hundred g of seed cake was placed in a round bottom flask. 500 ml of 70.0% ethanol containing NaOH at a concentration of 0.1 M (0.4% NaOH in 70.0% ethanol) was added to the flask to obtain a mixture in a ratio 1:5 (w/v). The flask was heated at 65–70°C for 30 min with a reflux apparatus above the flask. After heating, the mixture was filtered. The filtrate was discarded and the residue was washed with 70.0% ethanol. The washed residue was subjected to another round of the same procedure but this time using 70.0% ethanol without NaOH. The second residue was dried at room temperature by spreading on a tray. The moisture content of the dried residue was adjusted to 75% and the mixture was autoclaved at 121°C for 15 min. The mixture was dried to obtain the detoxified seed cake. For comparison, the same amount of seed cake was washed three times for 30 min each time with distilled water.

2.4. Acute toxicity test and HSP70 induction
Samples were prepared in logarithmic concentrations: 0, 3.125, 6.25, 12.5, 25, 50, and 100% (v/v) aqueous extracts. They were placed in 10-L glass aquariums under static, non-renewable systems to simulate micro-environments. They were maintained at a constant temperature (24.0 ± 4.0°C) and aerated regularly. Ten fishes per treatment were exposed to the seed cake extract microenvironment. Physicochemical factors were monitored throughout the duration of the study. Mortality of the fishes was monitored every 24 h post-exposure for 96 h. Dead fishes were removed immediately from the setups to prevent decomposition in the water. The mortality rate of the fishes and the medial lethal concentration (LC50) were determined. For the analysis of heat shock protein 70 induction or stress response, new sets of fishes were exposed for 7 days at a concentration of 50% of the LC50

of untreated pressed cake. Similar setups were done using the washed and detoxified seed cakes. A non-exposed control setup was also done. Surviving fishes were utilized for RNA isolation.

2.5. RNA isolation, reverse transcription, and PCR

The fishes were anesthetized in ice-cold water and sacrificed through decapitation. Liver tissues were excised and immediately immersed in an RNA stabilization solution, RNA later (*Qiagen*). RNA was isolated using TRIzol Reagent (*Ambion*) extraction protocol as provided by the manufacturer (*Invitrogen*).

Two μL resuspended total RNA, 4 μL 5x iScript reaction mix, 1 μL iScript reverse transcriptase, and 13 μL nuclease-free water were mixed in a polymerase chain reaction (PCR) tube and spun briefly in a vortex mixer. The mixture was incubated at 25°C for 5 min, 42°C for 30 min and 85°C for 5 min. Complementary DNA fragments of heat shock protein 70 (HSP70), and beta-actin housekeeping gene (ACTB) were amplified using DNA polymerase and HSP70 and ACTB specific primers. Polymerase chain reaction conditions were as follows: initial denaturation at 95°C for 1 min, followed by 35 cycles of amplification (95°C for 5 s, 60°C for 10 s).

Polymerase chain reaction products were electrophoresed in 1% agarose gel precast with ethidium bromide gel stain. The samples were electrophoresed at 100 V for 30 min. The gel was viewed using a standard transilluminator and the gel was imaged using an ethidium bromide filter. The electrophoresis bands were quantified using the *ImageJ* software. The change in expression levels of mRNA after exposure to the seed cake extract as represented by the electrophoretic bands were expressed as fold increase of the control values. Heat shock protein 70 (HSP70) electrophoretic bands were standardized using the ACBT electrophoretic bands. The fold increase values were obtained via normalization using the *ImageJ* software.

3. Results

Fresh *J. curcas* seed cakes collected right after mechanical press extraction are dark brown in color and slightly oily in texture. The seed cake is semi-compact and composed of hard, compressed, medium-sized slabs along with fragmented materials. The slabs, which are dry and brittle, can be crushed mechanically using the hand and further pulverized into a sand-like material using a mortar and pestle. The seed cake emits a strong odor similar to a decaying plant material, which is detectable when in close proximity to the sample.

3.1. Physicochemical characteristics of the extracts

After decantation and subsequent filtration of the seed cake & water mixture, the resulting aqueous extracts ranged in color from clear to brown. The color intensity of the extract generally increased as the concentration increases with a yellow color in the lower concentrations and a light brown color in the higher concentrations. The odor has similarities with that of the dry seed cake as it is also comparable to decaying plant material. The odor intensity also increased as the concentration increases. The conductivity, total dissolved solids (TDS) and NaCl levels (Figure 1) of the aqueous extracts increased as the concentration increases while the pH and resistivity decreased. The temperature levels of the extracts are consistent at all concentrations with readings nearly corresponding to that of the room temperature. Dissolved oxygen levels were not quantitatively measured; however, the aquariums were aerated regularly to ensure maximum oxygen dissolution through a 24-h continuous jet aeration by a submersible pump.

3.2. Acute toxicity test

The 96-h survival rates of the fishes exposed to 0.031, and 0.063 g/L aqueous extracts were very high (Figure 2). No mortality was observed at the two lowest concentrations while the 0.063 g/L aqueous extract setup exhibited a 97% survival rate. Significant reduction in survival rates were observed in the succeeding higher concentrations with the 0.125 and 0.25 g/L treatments posing 63 and 37% survival rates, respectively. A mortality rate of 100% was observed in fishes exposed to 0.50 g/L and 1.00 g/L aqueous extracts. Furthermore, 100% mortality was obtained as early as 24 h post exposure

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Figure 1. Physicochemical characteristics of the various concentrations of *J. curcas* seed cake aqueous extract.
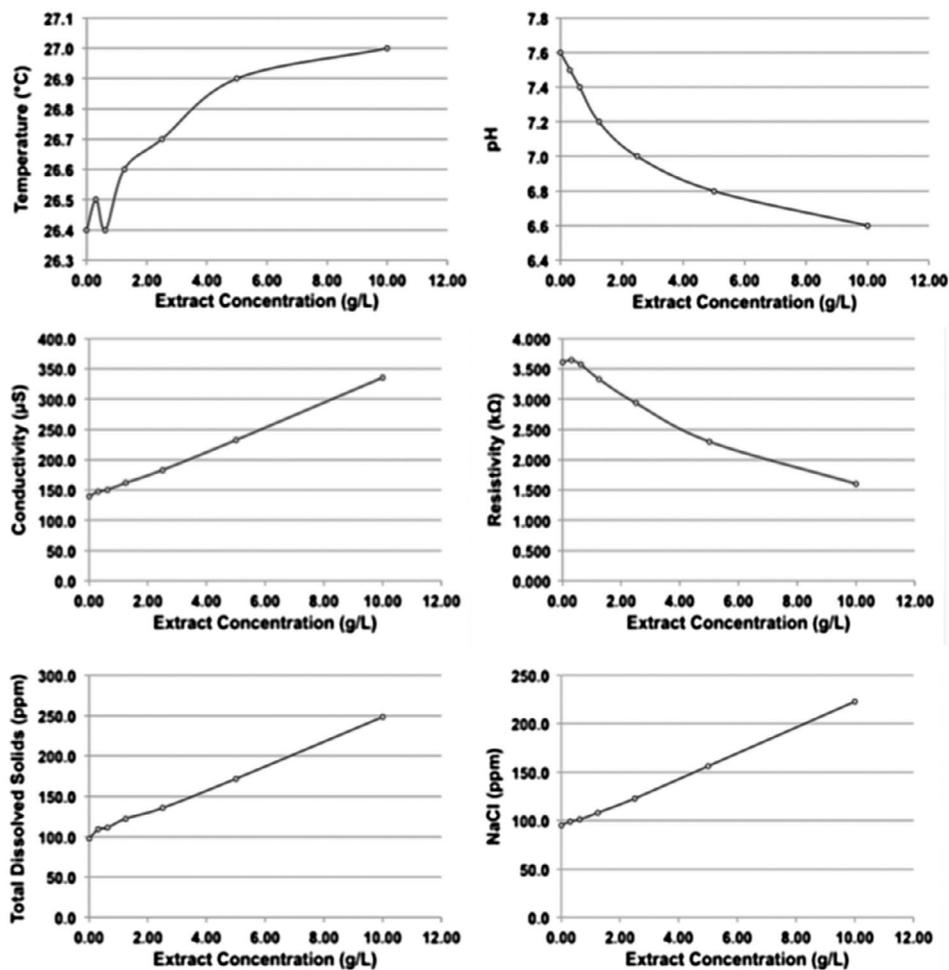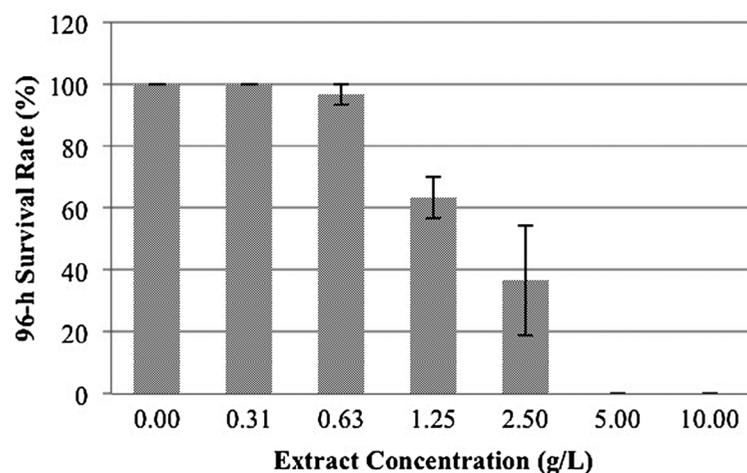

Figure 2. 96-h survival rate of zebrafish exposed to various concentrations of *J. curcas* seed cake extracts.

in the two setups with highest concentrations (Figure 3). Using probit analysis, the median lethal concentration of the *J. curcas* seed cake aqueous extract to adult zebrafish for 96 h was determined to be 0.172% (w/v) or 1.72 g/L (Figure 4).

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Figure 3. Time–response curve of zebrafish exposed to varying concentrations of *J. curcas* seed cake aqueous extracts.
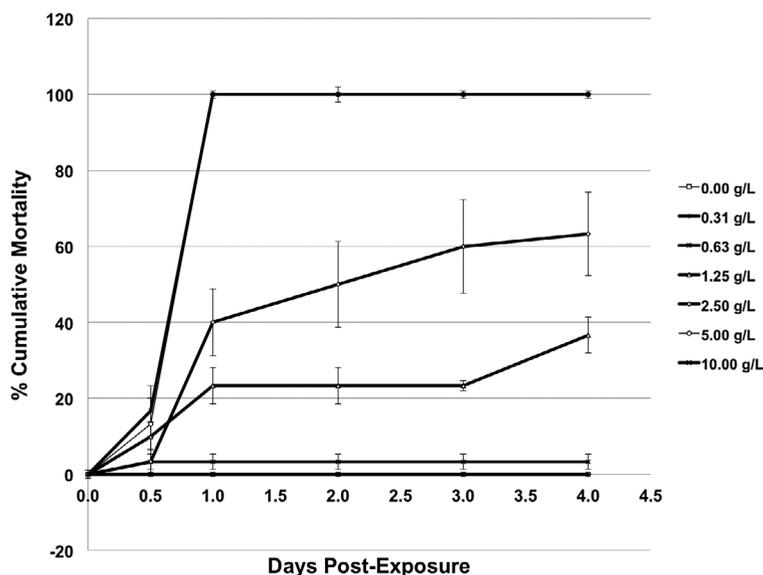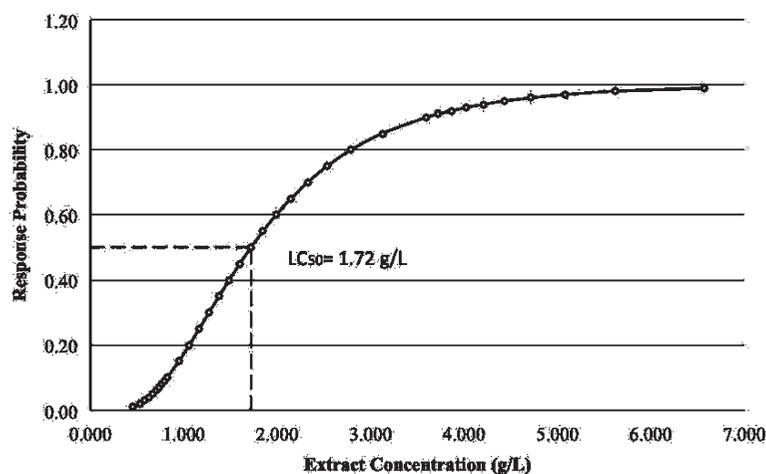

Figure 4. Response curve of zebrafish mortality upon exposure to various concentrations of *J. curcas* seed cake aqueous extracts for 96 hours.

Note: Obtained LC50 was 0.172% (w/v) or 1.72 g/L.

3.3. Induction of heat shock response

RNA was successfully extracted from the liver of the fishes exposed to all setups as shown by the gel profile (Figure 5). The two bands were expected to appear and were seen between the 650 and 1650 bp markers. These correspond to the total RNA with sizes that correspond to 28S (top band) and 18S (bottom band) rRNA.

After reverse transcription and PCR of the cDNA using HSP70 and ACTB primers, all of the samples exhibited clear banding in the ACTB profile and thus means presence of expression of the ACTB gene (Figure 6(a)) while in the HSP70 gel profile, only three clear bands were observed (Figure 6(b)). Clear bands were seen from the control and detoxified setups as well as the standard zebrafish cDNA. Light to no bands was observed in the washed and untreated setups indicating underexpression of HSP70 gene in those setups.

Subsequent standardization of the HSP70 bands against the ACTB bands using the *ImageJ software* indicated a decrease in HSP70 expression in fishes exposed to the washed and untreated setups as compared to the control, detoxified, and standard cDNA. The fishes posed to the washed and untreated samples respectively exhibited a fourfold and fivefold decrease in HSP70 expression as

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Figure 5. Gel electrophoresis profile of total RNA extracted from zebrafish liver.

Note: N-control, D-detoxified, W-washed, U-untreated (or undetoxified).
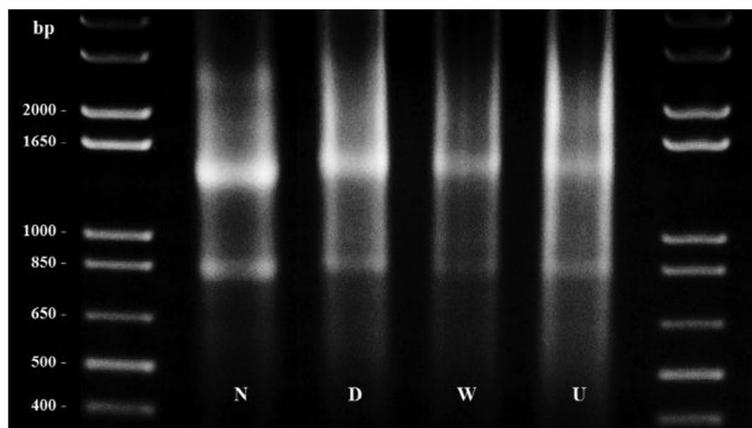

Figure 6. Gel electrophoresis profile of (A) beta actin gene (ACTB) and (B) HSP70 cDNA transcribed from extracted liver mRNA of adult zebrafish exposed to sublethal concentration (50% LC50) of untreated, washed, and detoxified seed cake aqueous extract. In (C), standardized liver HSP70 mRNA levels of adult zebrafish in non-exposed set up (0 g/L extract), and fishes exposed to sublethal concentration (50% LC50) of untreated (U), washed (W), and detoxified (D) seed cake extracts.

Note: Standard zebrafish cDNA sample loaded for positive internal control while no cDNA template was loaded for negative internal control.
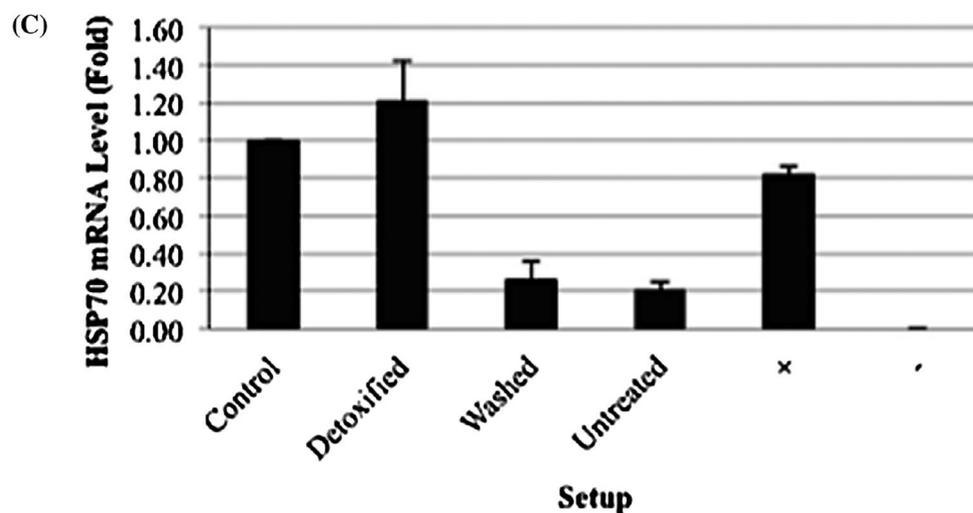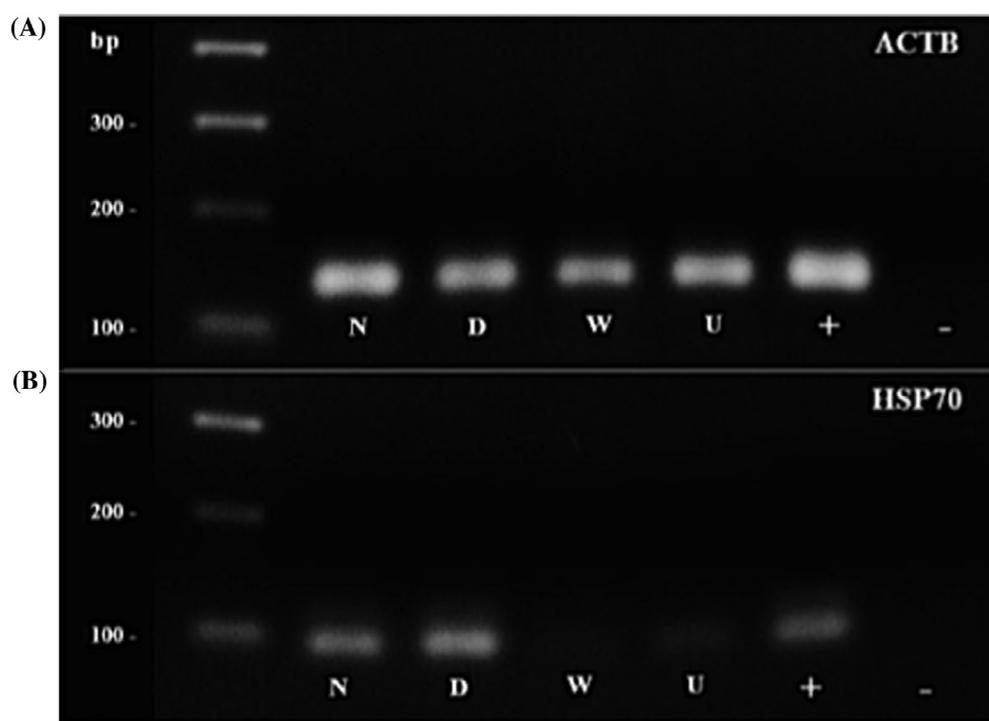
compared to the unexposed setup (Figure 6(c)). This indicates a general downregulation of HSP70 expression among the fishes exposed to those particular setups.

4. Discussion

Optimal water temperature range for zebrafish housing and breeding was generally known to be approximately at 26–28.5°C (Brand, Granato, & Nusslein-Volhard, 2002; Howells & Betts, 2009). In the experiment, the temperature of the setups ranges from 26.4–27.0°C which was inside the optimal range. Hence, temperature can be ruled out neither as a stress-causing factor triggering the induction of HSPs, nor a factor for the increase of fish mortality in increasing concentrations in the acute toxicity setup.

The pH tolerance range for zebrafish is 5.9–8.1 according to field observations; however optimal laboratory pH range is about 6.0–8.0 (Brand et al., 2002; Lawrence, 2007). It was observed that there was increasing acidity with increasing extract concentration; however in both the acute toxicity setup (pH range 6.6–7.6) and the HSP induction setup (pH range 7.4–7.7), the pH ranges fall within the optimal pH standards for the fish. Thus, the increase in zebrafish mortality may not be attributed to the pH. Further, it can also be assumed that pH may not be a factor in the induction of HSPs. Water conductivity, NaCl content, and TDS in the physicochemical analysis of water are altogether correlated with the increasing seed cake extract concentration. Upon obtaining the aqueous extracts, particles and solutes from the *Jatropha* seed cake were dissolved in the aqueous solution. Those molecular and micro-granular particles, organic and inorganic alike, which are able to pass through the sieve make up the TDS and accordingly the higher the concentration of *J. curcas*, the higher the TDS. Some of these particles may also dissolve in the water giving ions which cause the conductivity to increase, samples of which are Na+ and Cl-reflected by the increasing NaCl content.

The acute toxicity test showed a concentration-dependent toxicity and further reported a median lethal dose of 0.172% (w/v) or 1.72 g/L. This toxicity is attributed to the presence of phorbol esters and lectins in the seed cake. Several studies have already demonstrated the high levels of phorbol esters in the seed cakes of *J. curcas* (e.g. Ahmed & Salimon, 2009; Devappa, Rajesh, Kumar, Makkar, & Becker, 2012; Makkar et al., 1997; Makkar, Herrera, & Becker, 2008). Phorbol esters are amphiphilic diterpenoids that act as activator of protein kinase C, while lectins are sugar-binding proteins found in a variety of plants that are noted to be anti-nutritional factors and cause gastrointestinal damage (Miyake, Tanaka, & McNeil, 2007).

As stated previously, expressions of heat shock protein are being used as biomarkers to evaluate environmental pollution levels (De Pomerai, 1996; Rhee et al., 2009). To ensure that the heat shock responses in the fishes are due to the phytochemical constituents of the seed cake extract, several confounding factors were ruled out. The physicochemical factors are shown to be within normal limits. Tissues were also immediately immersed in an RNA stabilization solution to inactivate ribonucleases. The results of the heat shock stress response induction test showed a fourfold and fivefold decrease in liver HSP70 mRNA levels of fishes exposed to washed and untreated *J. curcas* aqueous extracts, respectively. The mRNA level of fishes exposed to the detoxified setup however is similar to the control and the standard zebrafish cDNA sample. These results indicate a general downregulation of HSP70 expression in the liver of fishes exposed to the washed and untreated setups. These were uncommon as heat shock proteins generally increase in response to stress (Cruz-Rodríguez & Chu, 2002; Hallare, Köhler, & Triebskorn, 2004; Hallare et al., 2006; Parsell & Lindquist, 1993). However, in certain studies, it has been shown that HSPs can be expressed differentially. For example endocrine disruptors such as 4-nonylphenol (NP) and 4-t-octylphenol (OP) caused HSP70 downregulation in the marine copepod *Tigriopus japonicus* (Rhee et al., 2009).

The results may be attributed to the fact that the main components and toxic constituents of the seed cakes are lectins and phorbol esters (Ahmed & Salimon, 2009; Devappa et al., 2012; Makkar et al., 1997). One major component of the *Jatropha* seed cake is curcin, a newly characterized toxic lectin (Lin, Zhou, Wang, Jiang, & Tang, 2010). Lectins are a family of sugar-binding proteins found in various

plants and which has several functions. Some lectins, such as phytohemagglutinin were noted to impair folding of newly synthesized protein (Ramadass, Dokladny, Lin, & Mosely, 2011). In a study by Ovelgonne et al. (2000), lectins have been shown to interfere with the levels of various HSPs in rat enterocytes. The HSP levels in the jejunum of lectin-fed rats were significantly lower than the control lactalbumin-fed rats. Meanwhile, legume lectins have been predicted *in silico* to interact with HSP70 since they are sugar-containing receptors. Some typical lectins can competitively bind with HSP70 and HSP90; and this direct binding could result to steric hindrance further disrupting the HSP70 interaction pathways (Shi et al., 2013).

Since quantification was done at the transcript or mRNA levels, several mechanisms for low HSP70 levels are plausible. This includes rapid translation and consumption without subsequent replenishment of HSP70 mRNA for repairing sublethal cell damage (Ovelgonne et al., 2000). Lectins have been noted to cause cytoskeletal instability and cytoskeletal lesions. A diversion of HSP70 function in stabilizing the cytoskeleton may have led to rapid translation of mRNA transcripts. This coupled with low transcription levels from the HSP70 gene (Ovelgonne et al., 2000) may have led to the low level of mRNA transcripts. Another mechanism may be a direct block at the transcriptional level. As the transcription of HSP genes are initiated by the activation and binding of heat shock factor 1 (HSF1) to heat shock elements (HSE) in the DNA, an interaction with HSF1 may lead to decreased transcription of HSP70 mRNA. For example, simple molecules such as deuterium oxide and glycerol can cause blocks directly at or before the transcriptional activation of HSP genes (Edington, Whelan, & Hightower, 1989). Since lectins are capable of interaction through its binding sites, it is possible that they have interacted with the HSP activation pathway. However, this can only be ascertained pending further experimentation.

The presence of phorbol esters may also have not affected the level of HSP70 expression. As noted by Holmberg, Leppa, Eriksson, and Sistonen (1997), phorbol esters, specifically 12-O-tetradecanoyl-phorbol-13-acetate (TPA), do not induce heat shock response but instead only enhances heat shock gene expression during heat stress. Since heat shock was not induced in the first place, the effect of phorbol esters on the heat shock response thus would not be apparent. As HSP70 expression is deemed to be a protective response to exposure to various chemicals (Rhee et al., 2009), an implication of HSP70 reduction by the washed and untreated seed cake aqueous extracts in zebrafish hepatocytes is that low HSP70 levels may lead to decreased resistance to stress. This leaves hepatocytes more vulnerable to stress induced by other plant metabolites that may be present in the *Jatropha* seed cakes. Furthermore, the present study also shows that downregulation of HSP70 may also be an indicator of stress. HSPs are generally present even in normal conditions as they assist in protein folding. Downregulation of HSPs signifies stress as the absence of molecular chaperones means impairment of the protein folding repair system and vulnerability of cells to stressors (Ovelgonne et al., 2000).

The results also showed that there was no significant difference among the liver mRNA levels of fishes in the control setup, the detoxified seed cake aqueous extract setup, and the standard zebrafish cDNA sample. This suggests that the detoxification procedure was successful in removing the toxic plant metabolites mainly phorbol esters and lectins, particularly curcin, which has been previously discussed as able to downregulate the expression of HSP genes. Studies have shown that heat treatment at 121°C effectively lowers the lectin content of the seed cake while treatment with NaOH and short-chain alcohol such as methanol removes phorbol esters (Aregheore, Becker, & Makkar, 2003). It is worth noting that the heat generated during the mechanical pressing was not high enough to inactivate lectins. A combination therefore of the heat and chemical treatment in the detoxification procedure for the *Jatropha* seed cake is essential since heat alone does not decrease the concentration of phorbol esters (Makkar et al., 1997). Futhermore, as shown by the close mRNA levels of fishes in the washed and untreated seed cake setups, water washing may not significantly reduce the toxic plant metabolites in the seed cake which stresses the need for a full detoxification procedure before the seed cakes are disposed or used for other applications.

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