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Evaluation of the hepatoprotective effect of methanolic extract of *Caulerpa lentillifera* against acetaminophen-induced liver toxicity in juvenile zebrafish (*Danio rerio*)

Kimberly D. Codorniz, Rose Emielle M. Marquina, Alexandra Dominique G. Nolasco, Paula Denise D. Palencia, Sigfredo B. Mata, RPh*

College of Pharmacy, De La Salle Medical and Health Sciences Institute, City of Dasmariñas, 4114 Cavite, Philippines
**Corresponding author:* sbmata@dlshsi.edu.ph

Abstract

Background: Liver injury is a common reason for drugs to be withdrawn from the market. Treatment options for common liver disease are limited, and therapy with modern medicines may lack effectiveness. *Caulerpa lentillifera* may have strong antioxidant systems that protect the plant from oxidative damage caused by the environment.
Objectives: The main objective of this study was to evaluate the hepatoprotective effect of the methanolic extract of *C. lentillifera* against acetaminophen-induced liver toxicity in juvenile zebrafish (*Danio rerio*).
Methods: Juvenile zebrafish (aged 1–3 months) were exposed to 10 μM and 25 μM acetaminophen (*N*-acetyl-p-aminophenol; APAP) to induce liver damage. *C. lentillifera* methanolic extracts (10 μg/L, 20 μg/L and 30 μg/L), were concomitantly added to individual tanks containing 10 μM or 25 μM APAP. The positive control group was treated with *N*-acetylcysteine/NAC (10 μM) and silymarin (10 μg/L, 20 μg/L and 30 μg/L). Hematoxylin and Eosin (H&E) staining revealed the extent of liver injury through the presence of hepatic necrosis, vacuolization, leukocyte infiltration, and ballooning. The antioxidant mechanism of hepatoprotective activity was assessed by a DPPH free radical scavenging assay.
Results: *C. lentillifera* extracts reduced the mortality of juvenile zebrafish when simultaneously exposed to 10 μM and 25 μM APAP. Upon histopathological examination of the liver tissue of juvenile zebrafish, the group treated with the 10 μM APAP together with the highest concentration (30 μg/L) of *C. lentillifera* extract showed minimal liver injury compared to the groups exposed to 25 μM APAP. However, the DPPH free radical scavenging assay performed using 24–36 mg/mL *C. lentillifera* extracts showed a minimal effect on the free radical scavenging activity.
Conclusion: The histopathological analysis of the liver showed that *C. lentillifera* extract prevented the progression of liver damage caused by APAP. The results of DPPH free radical scavenging assay indicated that the hepatoprotective activity of *C. lentillifera* extract might have other antioxidant mechanisms aside from free radical scavenging. In order to effectively assess the improvement in the survival rate of juvenile zebrafish, longer exposure in the treatments is recommended.
Keywords: *C. lentillifera*; juvenile zebrafish; hepatoprotective; drug-induced liver injury (DILI)

1. Introduction

Investigatory drugs are usually withdrawn in drug development and preclinical studies as well as after drug approval and marketing because of their ability to induce hepatotoxicity. Drug-induced liver injury results when the liver is unable to detoxify free radicals, such as reactive oxygen species (ROS), or other toxic metabolites from drug substances. This type of liver injury is a growing medical, scientific, and public health problem (Suk & Kim, 2012). Treatment choices for common liver injury are limited, and therapy with modern medicines may lack effectiveness. *N*-

acetylcysteine (NAC) is widely accepted in the prevention of hepatic injury due to acetaminophen overdose (Heard, 2008). A known hepatoprotective compound, silymarin from *Silybum marianum*, has an ability to inhibit the free radicals that are produced from the metabolism of toxic drug substances, including acetaminophen (Vargas-Mendoza et al., 2014).

Currently, there is a growing interest in the study of the antioxidant properties of marine species, such as algae, because of their inherent capability to withstand oxidative damage in the aquatic environment. *Caulerpa lentillifera*, known locally as *latô*, is commonly eaten as salad in the Philippines, and may have strong antioxidant systems that protect it from oxidative damage. Phenolic antioxidants found in *C. lentillifera* may become a possible agent used for the prevention of hepatotoxicity (Nguyen et al., 2011). Rodents are traditionally used in toxicological studies of the liver, but recently, small fish such as zebrafish (*Danio rerio*) have been used as an animal model as they present advantages, such as short generation time, high fertility, and low operational cost in terms of housing space and daily maintenance. In many liver toxicological studies, zebrafish larvae are utilized because they are optically clear and their internal organs can be directly observed without the need for dissection. Thus, real-time, simultaneous monitoring of livers in zebrafish larvae is easily achieved. Zebrafish therefore become an increasingly more valuable animal model than rodents in certain vertebrate toxicological studies (Asaoka et al., 2013). The main objective of this study was to determine the effect of *C. lentillifera* methanolic extract in reducing acetaminophen-induced liver toxicity in juvenile zebrafish (*Danio rerio*).

2. Methods

2.1. Collection and preparation of C. lentillifera extract

All seaweed specimens were collected from Barangay Talaba I in the City of Bacoor, Province of Cavite during the month of October 2018. They were immediately washed with tap water, dried, placed in wide-mouthed plastic containers covered with ice, and transported. A sample was authenticated at the Bureau of Fisheries and Aquatic Resources (BFAR) in Diliman, Quezon City. Each *C. lentillifera* specimen was washed *in situ* with distilled water, lyophilized at 70^{0}C for 7 days, and pulverized using a household blender. Methanolic extract was then prepared by maceration of the lyophilized and pulverized seaweed at 50^{0}C with sonication for 1 hour. This was then subjected to rotary evaporation to remove the solvent methanol at 40^{0}C and 70 rpm. The extract was dissolved in appropriate solvents for the bioassay and DPPH antioxidant assay.

2.2. Collection, acclimatization, and treatment of zebrafish

A total of 720 juvenile zebrafish (1 to 3 months old) was used in the study. Standard housing and treatment protocols were followed. The zebrafish were maintained in aerated water in the laboratory at 28 ± 2^{0}C in a 14 hr/10 hr light/dark cycle photoperiod and fed twice a day with fish food (sinking pellets) for 2 weeks. All zebrafish used in this study were healthy and free of any signs of disease.

After the acclimatization period, fish were randomly assigned into 24 experimental tanks, with a density of 10 zebrafish per 2 L. All treatments were done in triplicate and conducted for a total of 72 hours with twice daily feeding and regular fish tank maintenance. Stock solutions of treatments (APAP, NAC, silymarin, and *C. lentillifera*) were directly added into the fish tank water to make specified concentrations. In order to induce liver damage, the zebrafish were exposed to 10 µM and 25 µM APAP. *C. lentillifera* methanolic extracts (10 µg/L, 20 µg/L and 30 µg/L), were concomitantly added to individual tanks containing 10 µM or 25 µM APAP. Similar experiments were conducted for NAC (10 µM) and silymarin (10 µg/L, 20 µg/L and 30 µg/L) replacing *C. lentillifera* extracts. The groups were observed every 12 and 24 hrs for fish movement and mortality for 3 days. Live zebrafish were sacrificed through hypothermic shock in ice water for the histological examination.

2.3. Histological analysis

Whole body histological sections (7-µm sections) showing the liver were taken from the tail region behind the anus as prescribed by a histopathologist and using a standard protocol. Briefly, the fish was stored in Dietrich's fixative (28.5% ethanol, 1% formalin, 0.2% acetic acid) at a room temperature for several days and processed by a tissue processor (containing 70%, 80%, and 95% ethanol gradient) for dehydration. Ethanol was removed by immersing the cassettes in 100% xylene for 1 hr. The tissue was embedded in paraffin wax for 2 hrs at 56^{0}C and allowed to solidify. Sectioning was performed using Leica microtome. Tissue sections were stained using the hematoxylin and eosin (H & E) staining method by Ellis and Yin (2017).

2.4. DPPH free radical scavenging assay

The free radical scavenging activity of the *C. lentillifera* extracts were analyzed according to the method described by Müller et al. (2011) with the modifications from Osuna-Ruiz et al. (2016). DPPH free radical scavenging assay was also conducted to determine if the extract has the ability to scavenge free radicals as a hepatoprotective mechanism. *C. lentillifera* extracts (24 mg/mL, 27 mg/mL, 30 mg/mL, 33 mg/mL or 36 mg/mL) and L-ascorbic acid as the standard were separately incubated with 0.1 mM DPPH (2,2-diphenyl-1-picrylhydrazyl radical) in 1:1 hexane:methanol for 30

min in the dark and at a room temperature. The absorbance of the mixtures was determined at 540 nm using a UV/Vis spectrophotometer (Hitachi U-2910). When DPPH free radical scavengers reacted with the purple-colored DPPH, it was converted into its reduced form, which was yellow in color. This resulted in a decrease in absorbance at 540 nm. The percentage inhibition was determined using the formula:

$$\% \, Inhibition = \frac{A_{negative\ control} - \left(A_{sample} - A_{blank}\right)}{A_{negative\ control}} \times 100\% \qquad (1)$$

where:

$A_{negative\ control}$ = mean absorbance of DPPH solution in methanol

A_{sample} = mean absorbance of DPPH solution with *C. lentillifera* extract (or standard, L-ascorbic acid solution)

A_{blank} = mean absorbance of *C. lentillifera* extract (or L-ascorbic acid solution)

3. Results and discussion

The number of deaths in the APAP-treated control group doubled with the increase in the concentrations of APAP from 10 μM to 25 μM. When zebrafish were exposed to the negative control (the solvent used in preparing treatments), NAC, silymarin, or *C. lentillifera* extract, no zebrafish deaths were observed at the end of 72 hours. This means that exposure to the treatment groups alone and not in combination with APAP did not adversely affect the survival rate of the zebrafish. Similar to NAC and silymarin, which are known as hepatoprotective agents, *C. lentillifera* extracts reduced the mortality of juvenile zebrafish when simultaneously exposed to 10 μM and 25 μM APAP.

The histological characteristics of the zebrafish livers were assessed using H & E staining. Liver injury was indicated when hepatic necrosis, leukocyte infiltration and hepatocyte swelling were observed in the fish sections. The latter was seen as sinusoid compression due to swollen hepatocytes (Ellis & Yin, 2017). APAP treatment showed these signs of liver injury (Figure 1). All of the observed effects of acetaminophen in zebrafish hepatocytes were consistent with previously observed effects in human liver cells. APAP-induced liver injury is known to activate neutrophils, leading to neutrophil accumulation in the hepatic vasculature. Following APAP administration, a significant number of neutrophils are recruited into the liver resulting in subsequent development of hepatocellular injury between 4 and 24 hrs after drug treatment (Xu, et al., 2014).

To ascertain that NAC, silymarin, and *C. lentillifera* extract did not adversely affect the zebrafish liver when given in the absence of liver damaging APAP, controls were set up. Zebrafish groups treated with 10 μM NAC, 10 and 20 μg/L silymarin, and 10–30 μg/L *C. lentillifera* did not show any remarkable hepatocyte changes compared with the negative control containing only 0.1% by volume DMSO, the solvent used in preparing these solutions (Figure 2).

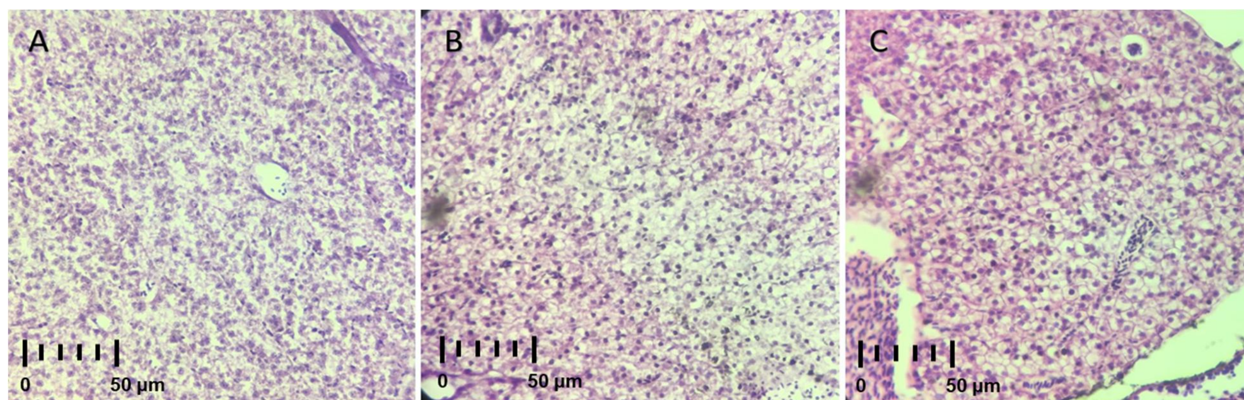

Figure 1. Exposure to APAP causing hepatic damage (400x magnification). Hepatic tissue of zebrafish from the negative control group (A), after exposure to 10 μM APAP (B), and after exposure to 25 μM APAP (C)

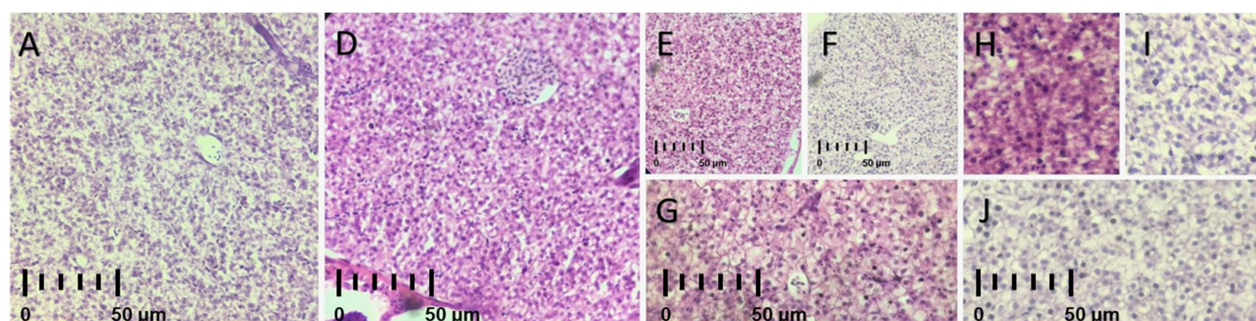

Figure 2. Treatment insignificantly affecting hepatic cells without exposure to APAP (400x magnification). Negative control (A), 10 μM NAC (D), 10 μg/L silymarin (E), 20 μg/L silymarin (F), 30 μg/L silymarin (G), 10 μg/L *C. lentillifera* extract (H), 20 μg/L *C. lentillifera* extract (I), and 30 μg/L *C. lentillifera* extract (J)

The liver histopathological features of juvenile zebrafish exposed to 10 μM APAP and concurrently treated with NAC (10 μM), silymarin (10–30 μg/L) or *C. lentillifera* extract (10–30 μg/L) showed a decrease in hepatic necrosis, leukocyte infiltration, hepatocyte vacuolization, and hepatocyte swelling in varying degrees consistent with their concentrations (Figure 3). However, hepatic tissues of zebrafish exposed to a higher concentration of APAP (25 μM) showed minimal changes on the hepatic cellular structures in the presence of the given treatments (NAC, silymarin, and *C. lentillifera* extract). These indicate that hepatic damage from exposure of zebrafish to 25 μM of APAP is irreversible with any of the known hepatoprotective agents (NAC and silymarin) and the investigational extract, *C. lentillifera*.

Treatment of zebrafish exposed to 10 μM APAP with 30 μg/L *C. lentillifera* extract showed no hepatic necrosis but minimal leukocyte infiltration and vacuolization. These were similar to those observed in 30 μg/L silymarin, possibly indicating that these plant extracts might share a similar hepatoprotective mechanism. The hepatoprotective properties of NAC and silymarin are well

established and appear to be partly related to their antioxidant activities. NAC is thought to reverse APAP-induced hepatotoxicity by replenishing glutathione, reducing the hepatotoxic metabolite of APAP, *N*-acetyl-*p*-benzoquinone imine (NAPQI), and effecting nonspecific hepatoprotective actions related to its antioxidant properties (Tardiolo, 2018). Silymarin extract contains a mixture of isomeric flavonolignans. Due to their phenolic structures, silymarin flavonoids have been reported to have antioxidant properties, which can control or inhibit free radicals produced by the hepatic metabolism of toxic substances such as APAP. In addition, the hepatoprotective activity of silymarin is shown to be caused by the maintenance of hepatocyte membrane integrity, affecting intracellular glutathione inhibition of leukotrienes and cyclooxygenase (Vargas-Mendoza, 2014). Flavonoids, which are previously reported to be present in *C. lentillifera*, may also be responsible for the observed hepatoprotective property of *C. lentillifera* (Nguyen, et al., 2011).

To determine if the hepatoprotective property of *C. lentillifera* was mediated by a free radical scavenging mechanism, the DPPH assay was performed. However, the DPPH free radical scavenging assay performed using 24–36 mg/mL *C. lentillifera* extracts showed a minimal effect on the free radical scavenging activity (Table 1). This was found to be consistent with a previous study by Nguyen, et al. (2011), indicating that the hepatoprotective activity of *C. lentillifera* extract might have other antioxidant mechanisms aside from free radical scavenging.

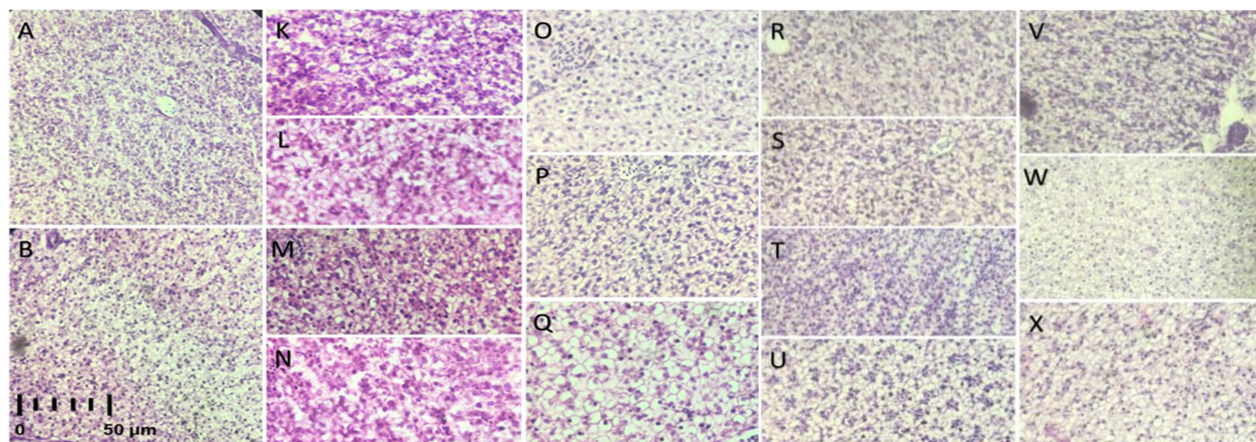

Figure 3. *C. lentillifera* reducing hepatic tissue injury after exposure of zebrafish to 10 μM APAP (K–Q) and 25 μM APAP (R–X) (400x magnification). Negative control (A), 10 μM APAP (B), with 10 μM *N*-acetylcysteine (K), with 10 μg/L silymarin (L), with 20 μg/L silymarin (M), with 30 μg/L silymarin (N), with 10 μg/L *C. lentillifera* extract (O), with 20 μg/L *C. lentillifera* extract (P), with 30 μg/L *C. lentillifera* extract (Q), with 10 μM *N*-acetylcysteine (R), with 10 μg/L silymarin (S), with 20 μg/L silymarin (T), with 30 μg/L silymarin (U), with 10 μg/L *C. lentillifera* extract (V), with 20 μg/L *C. lentillifera* extract (W), with 30 μg/L *C. lentillifera* extract (X)

Table 1. Percentage Inhibition of Free Radical Activity Using DPPH Assay

Concentration (mg/mL)	Percentage Inhibition	
	L-Ascorbic Acid Standard	*C. lentillifera* Extract
24.0	96.1	29.7
27.0	95.9	17.1
30.0	97.5	20.4
33.0	97.5	5.6
36.0	97.3	43.2

4. Conclusion

After 72 hours of exposure to 10 μM and 25 μM APAP, zebrafish showed an increased mortality rate with increasing APAP concentrations. Concurrent treatment with NAC, silymarin, and *C. lentillifera* extract for 72 hours resulted in zero deaths. *C. lentillifera* might have a potent hepatoprotective property similar to known hepatoprotective agents, NAC and silymarin. The histopathological analysis of the hepatic tissues showed that *C. lentillifera extracts* (at 10–30 μg/L) prevented the progression of hepatic damage caused by 10 μM APAP. The results of DPPH free radical scavenging assay indicated that the hepatoprotective activity of *C. lentillifera* extract might have other antioxidant mechanisms aside from free radical scavenging. In addition, the concentration of the extract might be insufficient to show its antioxidant activity.

In order to effectively assess the improvement in the survival rate of juvenile zebrafish, longer exposure in the treatments is recommended. Additional antioxidant assays may be performed on the methanolic extract of *C. lentillifera* to determine its mechanism of hepatoprotective activity.

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