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Hepatoprotective Effect of Aqueous Extract of *Gyrocarpus asiaticus* on Paracetamol Induced Hepatotoxicity in Zebra Fish

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A B S T R A C T

Paracetamol induced hepatotoxicity is a common model for accessing the hepatoprotective nature of new molecules. This study was conducted to evaluate the potential of aqueous extract of *Gyrocarpus asiaticus* on paracetamol induced hepatotoxicity in zebra fish. Fish were challenged with 20mM concentration of paracetamol followed by treatment with aqueous extract of *Gyrocarpus asiaticus.* Serum was evaluated for alanine transferase (ALT) concentration and liver samples were collected for histopathological analysis. Result of this study showed that, there was marked elevation of ALT in induced group and compare to paracetamol induced toxicity group, *Gyrocarpus asiaticus* treated group has got marked reduction in ALT which also confirmed through histopathological analysis, where the treatment group showed normal morphological features. Hence further study can be done to elucidate the mechanism of action of *Gyrocarpus asiaticus* through molecular level to have a confirmation of hepatoprotective nature of this plant.

Introduction

Drug induced liver injury (DILI) is a major challenge in pharmaceutical industry and drug development (Heidari *et al.,* 2015). DILI due to paracetamol overdose is dose dependant and to an extent, predictable from the dose ingested (Vliegenthart *et al.,* 2014). Marzilawati*et al.,* (2012) documented the significant association of 3.8% of ethnicity with hepatotoxicity in Indians. Therefore, improvised models are necessary for predicting DILI and to evaluate alternate hepato-protective agents. The zebrafish is a promising animal for assessing drug induced toxicity in variety of organ systems including liver as zebrafish metabolize drugs using similar pathways as human (Vliegenthart *et al.,* 2014). N-acetylcysteine (NAC) is used as specific antidote for paracetamol induced poisoning but is having some adverse effects like life threatening anaphylactic reactions (Algren, 2008). So as an alternative, natural product can be experimented to sort out the probable hepato-protective effect in a dose dependant manner. Among natural

compounds, silymarin is a well-known hepatoprotective drug used for the treatment of many liver diseases (Pradhan and Girish, 2006).

Gyrocarpus asiaticus belongs to family Hernandiaceae and found in various parts of India including Eastern Ghats of Tamilnadu (Jayakumar *et al.,* 2008). This plant is reported to have antidiabetic (Yelchuri and Yajaman, 2014), antibacterial (Kanthal *et al.,* 2014), ant helminthic (Kanthal *et al.,* 2013) and anticancer activities (Vithya *et al.,* 2013). According to the perusal of literature there are very scanty reports on hepatoprotective effects of this plant. Vithya *et al.,* (2012) reported on the free radical scavenging activity of this plant which signifies its antioxidant effect.

Hence keeping these criteria in mind this research is undertaken to explore the effect of Gyrocarpus *asiaticus* on paracetamol induced hepatotoxicity.

Materials and Methods

Animals

Adult zebrafish of both sexes (Danio rerio) were purchased from a local pet shop and acclimated in aerated tanks containing distilled water. Zebrafish were fed with commercial fish food twice a day and kept at approximately 28ºC with a 14hr: 10 hr light dark cycle. Each zebrafish weighed 0.2-0.3g and each ten zebrafish were treated in static tanks filled with 2.0 litres of water.

Preparation of aqueous extract of *Gyrocarpus asiaticus*

Stem bark portion of *Gyrocarpus asiaticus*was collected from the Azhagar Kovil hills, Madurai District, Tamilnadu, India. Twenty gram of powdered plant material was taken in 200 ml conical flask and 100 ml of water was added to it. The mouth of the conical flask was covered with aluminium foil and kept in a orbitals shaker for 24 hours for continuous agitation at 150 rev/min for through mixing. Then the extract was filtered by muslin cloth followed by Whatman no. 1 filter paper and finally filtered by using vacuum evaporator with the water bath temperature of 50ºC. Finally, the residues were collected and used for the experiment.

Acute toxicity study

Acute toxicity test was performed following OECD guideline no. 203. Aqueous extract of *Gyrocarpus asiaticus* was dissolved in water. Five ascending concentrations of *Gyrocarpus asiaticus* aqueous extract (10, 50, 100, 500, 1000 mg/l) were used for the study. After 7 days of acclimatization, seven fish were kept in each 4 litre glass aquarium. A total of 42 zebrafish (including control) were used in this test. During the test fish were not fed. The test was performed using a semistatic method with the solutions renewed every 24 hours. Fish condition and mortality were checked every 24 hours. Experiment was conducted for 96 hours. Water temperature (23 ± 0.5ºC), pH (8.3 to 8.61) and oxygen saturation (above 60%) were monitored every 24 hours.

Experimental design

Zebrafish were exposed to paracetamol (20-40mM), dissolved in system water for 3 hours (or system water alone for negative controls) (Fig. 1). At 40 mM concentration 80% mortality was seen within 2 hours of exposure and 60% of mortality at 30 Mm exposure. So the hepatotoxicity was induced by 20Mm concentration of paracetamol. This was followed by a change of system water for 2-21 hours with or without treatment (Table 1). This short exposure to high concentration of

paracetamol followed by delayed treatment was used to replicate a human single, acute overdose. Experiments were terminated 5-24 hours after the start of paracetamol exposure (Vliegenthart *et al.*, 2014). After treatment for 24 hours, zebrafish were anaesthetized using melting ice. Blood was collected using established lateral incision (LI) technique. Then fish were euthanized in melting mice. The liver tissues were separated and used immediately for biochemical analysis. Serum was separated by centrifuging the blood at 2500 rpm for 15 minutes and analysed for acute marker for cellular integrity i.e., alanine aminotransferase (ALT) was estimated in the serum by kinetic method using stanadard kits (Agappe Diagostics).

Histopathology

After sacrifice, portions of the liver were collected and fixed in neutral buffer formalin (NBF) for histopathological examination. Sections were cut at 5 µm thickness, stained with haematoxylin and eosin (H & E). The sections were then viewed under light microscope for histopathoogical analysis.

Statistical analysis

The results were expressed as mean± standard error (SE). The differences between groups were determined by using the statistical package for social sciences (SPSS) software package for windows. The effects of treatments were determined by analyzing the data using one-way ANVA followed by Duncan's multiple comparison tests. P values < 0.05 or < 0.01 were considered as statistically significant.

Results and Discussion

Treatment with 1000mg/ml resulted in death of 15% fishes in that group in 96 hr toxicity study whereas in other groups death was invariably low. So it was suggestive of LD_{50} value is higher than 1000mg/ml. As per the OECD guideline, at a limit dose 100mg/l fish showed no mortality indicating that at 99% confidence level, can also be concluded that LD_{50} value is higher than 100mg/ml. As the dosing is concern, 1/10th of the highest dose tested was used as the therapeutic dose in the study. In the present study, paracetamol treated group showed an increase in serum ALT activity (Fig. 2). Administration of *Gyrocarpus asiaticus* revealed a decrease in serum ALT activity up to normal level, which is similar to that of the standard drug silymarin at the given dose. As per histopathology reports, untreated zebrafish liver showed normal tissue and cell structure, tight cell contact and liver was filled with polygonal cells with well-preserved cytoplasm and prominent nucleus (Fig. 3A.). Paracetamol treatment increased vacuolar degeneration, hepatocyte necrosis and area of focal congestion and haemorrhage (Fig. 3B). As demonstrated in the Figure 3B1, paracetamol treated liver shows loose cell-to-cell contact with dissociated and irregular cells with various grades of small and large vacuoles and amount of hepatic parenchyma decreased.

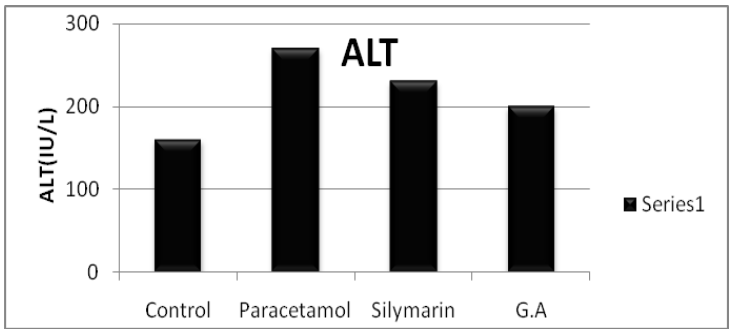
Table.1 Experimental design

GROUPS	TREATMENTS	No. of fish
I	Untreated control	24
II	Paracetamol Control	24
III	Paracetamol + Standard drug (Silymarin)	24
IV	Paracetamol + *Gyrocarpus asiaticus*	24

Fig.1 Zebrafish and its liver after induction with hepatotoxicity

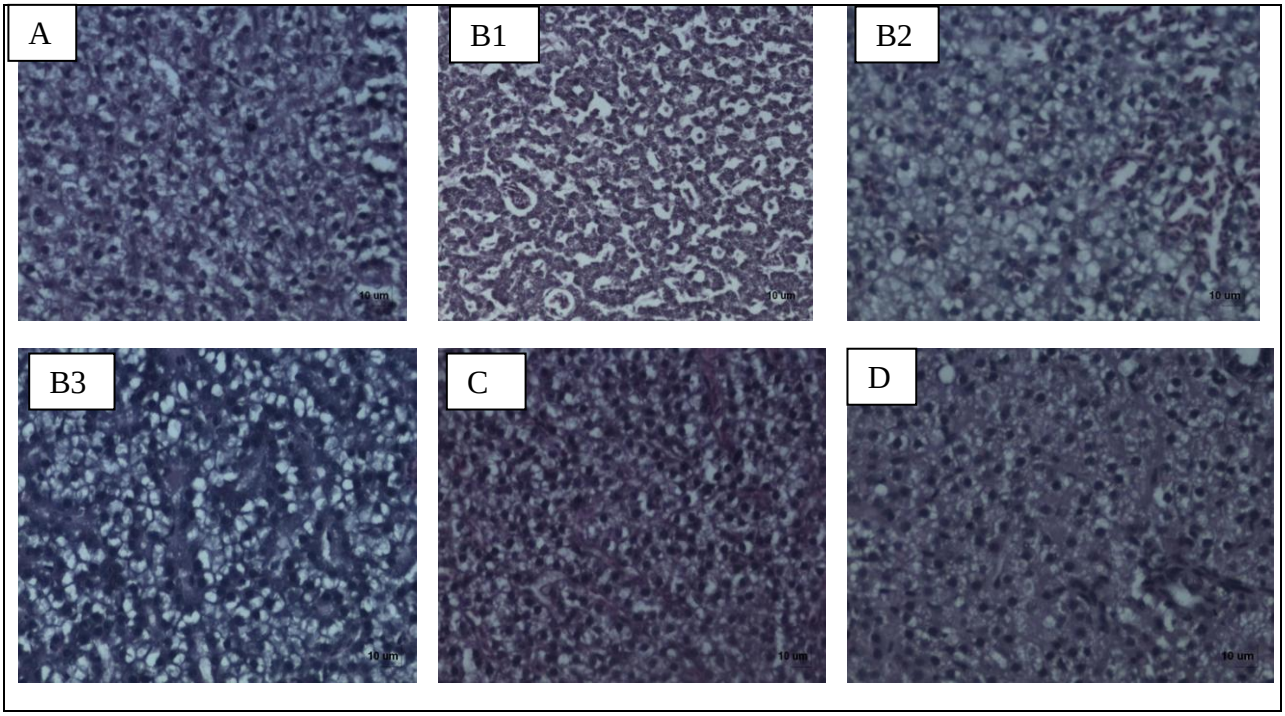
Liver- Paracetamol induced hepatotoxicity-Haemorrhage

Fig.2 Serum ALT



Histogram shows effects of paracetamol, silymarin, *Gyrocarpus asiaticus* (G.A) on ALT level pooled sample of serum

Fig.3 Histopathological study of liver



Histological images of adult zebrafish liver, A- control-Liver- normal, B- 1:Paracetamol-Liver loose cell-to-cell contact-diffuse,

2: Vacuolar degeneration- congestion-haemorrhage, 3: Vacuolar degeneration, C- Silymarin-Liver- Mild change, D- G.A -Liver- Mild vacuolar degeneration

Treatment with silymarin causes mild changes in hepatocytes (Fig. 3C) whereas treatment with *Gyrocarpus asiaticus* caused vacuolar degeneration with mild congestion (Fig. 3D).

For patients presenting with paracetamol-induced liver failure, the only reliable parameters to provide therapeutic guidance is ALT serum level. North *et al.,* (2010) and Shivashri *et al.,* (2013) also found an increase in ALT value in paracetamol induced hepatotoxicity in zebra fish.

Increase in ALT activity in blood of paracetamol-exposed fish reveals paracetamol induced liver tissue damage. ALT and AST are two mitochondrial enzymes (Gharaei *et al.,* 2011) and are found in the cell cytoplasm in higher concentrations particularly ALT. Higher levels of these intracellular enzymes in serum might be a result of leakage from cells due to cell membrane damage.

As per histopathology is concern *Gyrocarpus asiaticus* could able to decrease the inflammatory lesions, but not completely restore the normal architecture of liver. As it was an acute study, for reverting back from degenerative state to normal state needs time and treatment for longer period could have restore the architecture of liver to normal state in zebra fish. However, the liver histology was comparatively improved and shown less congestion than paracetamol toxicity group.

Hence in conclusion, zebrafish acts as a physiologically relevant model of paracetamol hepatotoxicity. Here, we use a variety of biochemical, histological, and clinical outcome measures to show the overall relevance of the zebrafish paracetamol toxicity model to human physiology. Further study can be performed with molecular pathway analysis to unravel the potential of *Gyrocarpus asiaticus* in hepatotoxicity model with variable doses.

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