

Original Research Article

## Effect of Flavonoids in Acetaminophen Induced Liver Injury in Danio Rerio

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### ABSTRACT

Liver is the most important organ of living system involved in vital functions like metabolism, storage, detoxification etc. Liver diseases are caused due to excess consumption of alcohol, toxic chemicals as well as certain disorders in the immune system. Only a few liver protective drugs are available for liver disorders. Since ancient time, medicinal plant has been used to treat various disorders. Acetaminophen is the most common analgesic commonly used for the treatment of fever and pain. At therapeutic dose it has no side effect however at higher dosage it alters the enzyme level creating mitochondrial damage, oxidative stress and cell apoptosis. In the present study, we aimed to isolate flavonoids from *Sphaeranthus amaranthoides*, and evaluated its protective role against acetaminophen induced liver injury. Zebra fish embryos development was observed upon acetaminophen injury as well as protection of flavonoids against acetaminophen injury. The acetaminophen induced liver injury decreased the antioxidant defence system thereby significantly increasing the liver marker enzymes in the blood and the DNA from the liver was also fragmented and sheared. Histopathology shows the degeneration of vacuoles in the cell. The treated groups were also observed for the effect of flavonoids against acetaminophen induced liver injury and the result showed the protective nature of flavonoids by restoring the altered enzyme levels and protecting the cells from damage.

**Keywords:** Acetaminophen, Zebra Fish, *Sphaeranthus amaranthoides*, Liver, Antioxidant defence system.

### INTRODUCTION

Liver is the most important organ of living system and performs vital functions in regulations of physiological processes in the body. It is involved in metabolism, secretion and storage. Liver diseases are mainly caused due to excess consumption of alcohol, toxic chemicals, infectious diseases and autoimmune disorders may also be the major cause. Diseases like jaundice, liver cirrhosis, fatty liver disease are commonly occurring worldwide.

In recent years there has been tremendous advancement in the field of

science and hematology; however liver problems are on high rise, the two major liver diseases which has caused high death rate annually account for jaundice and hepatitis. <sup>[1]</sup> Hepatotoxic chemicals damage liver cells by inducing lipid peroxidation and other oxidative damages. <sup>[2]</sup> More than 900 drugs have been found to cause liver diseases and have been stopped from implications. <sup>[3]</sup>

Drug induced liver injury has become a major challenge, various models have been identified for predicting the toxicity, acetaminophen is a common analgesic often used at the time of fever.

At therapeutic doses, it is advised to be safe; however at higher doses it becomes toxic and causes hepatic failure as well as renal failure. A hepatotoxic metabolite, NAPQI (N-acetyl-p-benzoquinone imine that is produced by cytochrome P450 of liver enzymes, remain inactivated at therapeutic doses by Liver GSH. [4,5] at higher doses, these metabolite gets activated due to decrease in level of Gsh, which accounts for the dysfunction of liver, causing mitochondrial DNA damage, oxidative stress and apoptosis of immune cells. [6-8]

In spite of advancement in the recent trends in drug development, presently only a few hepatoprotective drugs are available for the treatment of liver disorders. [9] various natural remedies in from traditional medicinal plants, in Ayurveda have been found to be effective against liver damages and has been recommended for the treatment of liver diseases in the Indian system of medicines. [10] The relative accessibility, low prices, local availability and acceptance by the local communities have made heavy reliability on plant medicine. Since the ancient time, plant medicine is an important part of healthcare system. There are many herbal formulations available which helps in preventing liver damage. [11] There are about 600 formulations made from 55 different plant families which possess hepatoprotective activity however still, very small proportions of hepatoprotective plant have been evaluated for their efficacy and safety. [12] Therapeutic drugs that may support the liver recovery from Apap injury rather than antagonizing its mechanism of action are very rare.

Zebra fish embryos are amenable to chemical screening approaches and helps in the identification of novel compounds. Recently these embryonic screenings can be no further used to adult fish physiology and in therapeutic applications. [13] In the present study, we have characterize a fish model for APAP

liver injury, and to treat the liver injury, flavonoids obtained from *Sphaeranthus amaranthoides* have been studied along with the standard Liver drug Silymarin in order to discover a drug for liver damage.

## **MATERIALS AND METHODS**

### **Animals and chemical treatment**

Zebra fish used in these studies, were obtained from an aquarium shop. The fish were maintained at 27 to 30°C exposed to 12h light and dark periods and fed twice a day.

The fertilized eggs were collected and kept in an egg medium for the clear transparency and the embryos were maintained at 28°.the developmental stages of the embryos were observed and they were transferred into four groups each of 20 per embryos the groups were divided as follows.

- i. Group 1-Control
- ii. Group 2-APAP treated(5mM)
- iii. Group 3-APAP+flavonoid. (5mM+10µM)
- iv. Group 4-APAP+Silymarin (5mM+10µM).

The mortality rates of embryos were observed daily.

Adult zebra fish were also treated the same way as embryos with different concentrations of APAP.

- i. Group 1-Control
- ii. Group 2-APAP treated(10mM)
- iii. Group 3-APAP+flavonoid. (10mM+10µM)
- iv. Group 4-APAP+Silymarin (10mM+10µM).

The mortality rate of embryos was observed daily.

### **Estimation of Anti-oxidant enzyme (Sod, Cat, GSH)**

Zebra fish liver (from 5 to 10 fish of each group) was and subjected to enzyme determination.

### **Estimation of Liver Marker enzymes (ALP, SGPT, SGOT)**

Zebra fish blood (from 5 to 10 fish of each group) was obtained by incision between the anal fin and caudal fin.. serum

was separated by centrifugation (5000 rpm for 10 min) and subjected to enzyme determination in the clinical laboratory.

#### Estimation of Blood Glucose

The serum obtained from blood was given for glucose determination in the clinical laboratory.

#### Estimation of Total Cholesterol

Total cholesterol in the serum was determined in the clinical laboratory.

#### Histopathological studies

Liver from adult zebra fish were fixed in 1% formalin and embedded in agarose and cryosectioned (10 $\mu$ M), and stained with Haematoxylin and eosin using standard technique.

#### Total RNA isolation from Liver tissue

Zebra fish liver tissue was stored at -20C for 24hrs and total RNA was extracted by Trizol reagent according to the standard protocol from liver tissues of each group using TRIZOL, according to the manufacturer's protocol.

#### CDNA Preparation and Real-Time PCR (Quantitative PCR) Assay

Concentration of the extracted RNA was determined and the integrity of RNA was visualized on a 1% agarose gel using a gel documentation system (Bio Rad, Hercules, CA). The first strand of cDNA was synthesized from 2 $\mu$ g of total RNA by reverse transcriptase using M-MLV (Promega, Madison, WI) and oligo (dT) primers (Promega) after which RT-PCR was performed with cDNAs and gene

specific primer pairs and mixed with ABI SYBR Green PCR master mix (Applied Biosystems, Foster City, CA). Real-time PCR cycle parameters included 10 minutes at 95°C followed by 40 cycles involving denaturation at 50°C for 2 minutes, annealing at 95°C for 10 minutes 15 seconds, and elongation at 60°C for 1 minute. The sequences of the specific sets of primer for bax, bcl-2, caspase-3, GAPDH and TNF- $\alpha$  used in this study were taken from literatures. Expressions of selected genes were normalized to the GAPDH gene, which was used as an internal housekeeping control. All the real-time PCR experiments were performed in duplicate, and data were expressed as the mean of at least two independent experiments.

**Statistical analysis:** Data were expressed as mean  $\pm$  S.E.M and analysed by Tukey's test to determine the significance of differences between groups. A p value lower than 0.05, 0.01 or/and 0.001 was considered to be significant.

## RESULTS AND DISCUSSIONS

### Developmental Stages of Zebra Fish Embryos (168hpf)

Upon treatment with acetaminophen and flavonoid the various developmental stages of embryos were observed as shown in table 1.1 and fig.1.1.

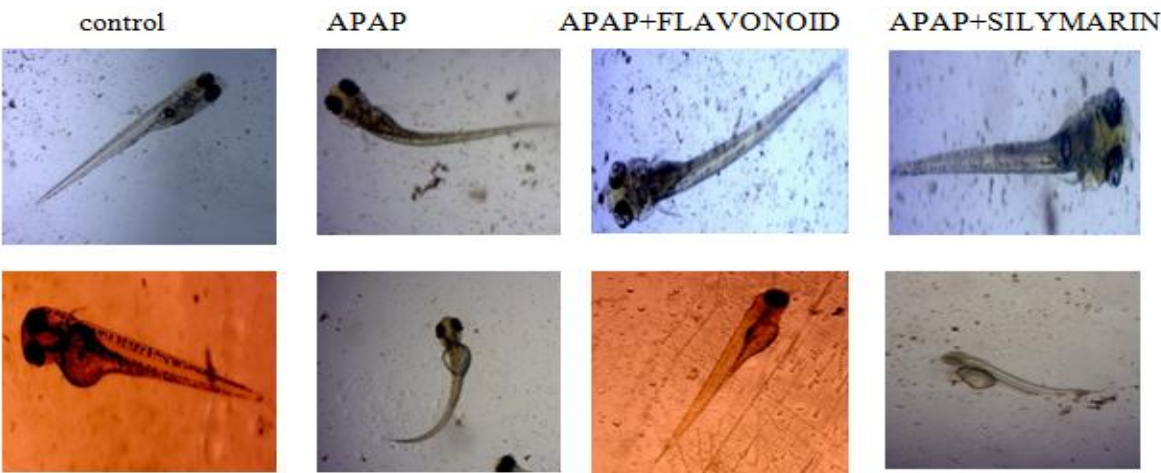
Table 1.1

PARAMETERS	CONTROL	APAP	APAP"+FLAVONOID	APAP+SILYMARIN
Division	Normal	Slow	Slow	Slow
Growth	Normal	Slow	Slow /normal	Slow/normal
Hatching rate	16/20	10/20	14/0	15/20
Swimming behaviour	Normal	Fast	Slow	Slow
Development of liver	Normal	Damage	Improved morphology	Improved morphology.
Body shape	Straight	Curved	Curved/normal	Straight
Tail movement	20/min	12/min	14/min	15/min
Mortality	2/20	3/20	4/20	3/20

Table 1.2 Estimation of CAT, GSH, SOD in the Treated Liver of Zebra Fish

ENZYME SYSTEM	CONTROL	APAP	APAP+ FLAVONOID	APAP+ SILYMARIN
SOD(U/mg protein)	0.029 $\pm$ 0.04	0.012 $\pm$ 0.02	0.058 $\pm$ 0.03	0.11 $\pm$ 0.07
CAT (mmoles of H2O2 decomposed /mg protein/min).	1.033 $\pm$ 0.03	0.87 $\pm$ 0.05	1.141 $\pm$ 0.034	1.302 $\pm$ 0.05
GST( $\mu$ moles CDNB conjugated formed/mg protein/min)	1.66 $\pm$ 0.02	0.56 $\pm$ 0.003	3.21 $\pm$ 0.04	3.3 $\pm$ 0.04

Fig 1.1





### Study on Anti-Oxidant Enzyme System

In the present study, the above graph shows that there is decrease in enzyme in the APAP treated groups, and increase in SOD, CAT and GST activity in the Flavonoid and Silymarin treated groups.

The decrease in antioxidant system due to acetaminophen toxicity is due to depletion of GSH and oxidative stress

causing accumulation of fatty molecules and influences cyt-P450 which forms ROS leading to liver damage and significantly decreasing the amount of catalase, SOD and GST in the liver. The post treatment with flavonoids showed increased defence system which reveals that the flavonoids could be able to protect the liver damage caused due to acetaminophen liver toxicity. [14]

### Study on Liver Marker Enzymes

Table 1.3 Estimation of Liver Marker Enzymes (Alp, Sgpt, Sgot.)

ENZYME SYSTEM	CONTROL	APAP	APAP+ FLAVONOID	APAP+ SILYMARIN
ALP(U/L)	25±5.6	110.3±10.85	58.66±9.34	68±8.6
SGPT(U/L)	75.83±10.5	162.5±27.8	79.5±6.83	80.16±10.02
SGOT(U/L)	38.33±4.3	174.16±18.66	79.5±7.39	80.16±10.02

In the present study, the above graph shows that the acetaminophen treatment markedly increased the levels of SGPT, SGOT, and ALP. The damages in the liver cell is associated with liver necrosis, loss of hepatic architecture and degeneration of cells, which results in increased amount of

liver marker enzymes in the blood. However upon treatment with the flavonoids the level of liver marker enzymes was reduced which confirms for the restoration of liver damage by flavonoid against acetaminophen induced liver injury. [15]

### Estimation of Blood Glucose and Total Cholesterol

Table 1.4. Estimation of blood glucose and total cholesterol

SERUM PARAMETERS	CONTROL	APAP	APAP+ FLAVONOID	APAP+ SILYMARIN
Blood sugar(mg/dL)	42.16±5.3	61.16±7.5	48±8.1	41±8.1
Total cholesterol(mg/dL)	38±6.31	76±11.83	42.83±8.48	40.33±7.39

In the present study, the above graph indicates that the blood glucose and total cholesterol level in the serum of the APAP treated Zebra fish was found to increase and there is decrease in the level of post treated group with flavonoid and Silymarin. The liver damage in the APAP treatment causes increase in level of Glucose and cholesterol which may be due to the depletion of glycogen level enhancing glycolysis and also due to the increased cell proliferation of hippocampus and cancerous tissue.

The decrease in post treatment with flavonoid reduces the glucose and cholesterol level, which accounts for reduced glycolysis and decreased proliferation of cell, which repairs the damaged liver tissue caused due to

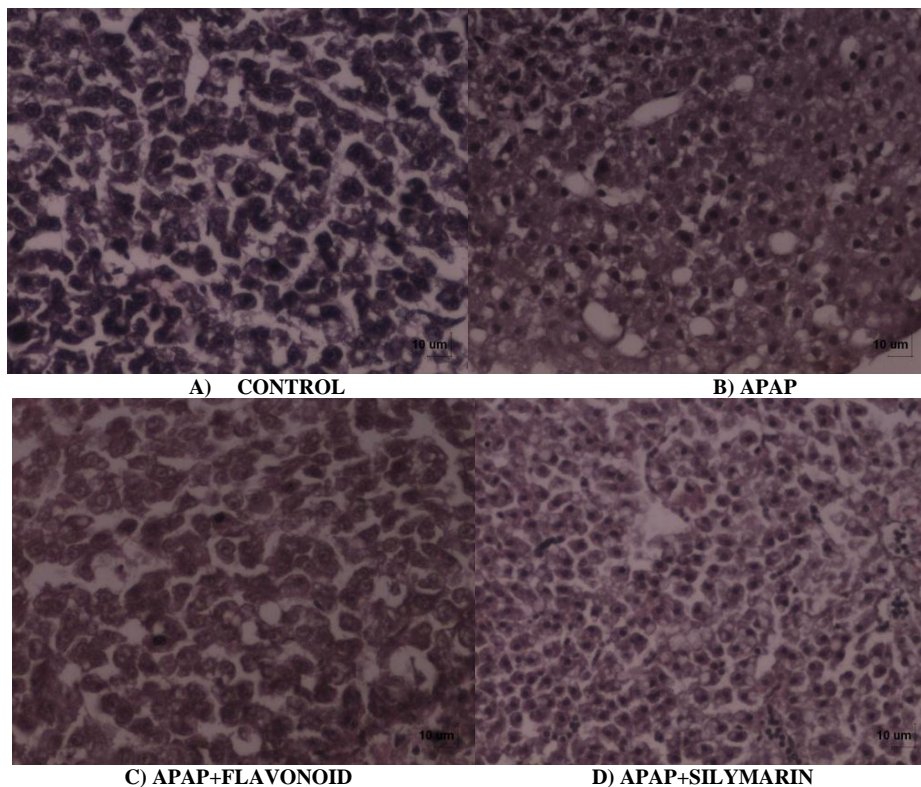
acetaminophen liver injury. Hence, this proves that flavonoid treatment was effective against acetaminophen induced liver injury in restoring the blood glucose and total cholesterol level in the blood. [16]

### Histopathological Studies

In the present study, the histopathological study was carried out to check the effect of acetaminophen on liver damage. The H&E staining of the liver tissue of acetaminophen and post treated group as shown in fig-1.2. In the fig A) shows the control group with normal liver morphology with cells, B) acetaminophen treated group which shows the degeneration of vacuoles in the cell, and necrotic aggregation of cells, C) shows the post treatment with flavonoid indicating

the regeneration of vacuoles, with no necrotic zone, and normal hepatocytes D) The post treatment with Silymarin

showing regeneration of liver tissue with normal hepatocytes and presence of individual cells.



**Fig: 1.2** a) tissues with normal cells. b) Tissue showed degeneration of 2 large vacuoles, c) regeneration of vacuoles, d) showed mild degeneration of vacuoles and regeneration.

The acetaminophen liver injury resulting in degeneration of cells may be due to the accumulation of non-adipose tissue which results in cell necrosis and cell death and Treatment with flavonoid resulted in regeneration of vacuoles and liver cells indicating that the flavonoid treatment decreases the oxidative hepatic injuries such as necrotic aggregations in acetaminophen treated which shows the protective nature of flavonoids against acetaminophen liver injury. <sup>[17]</sup>

### Gene Expression Analysis

**Table.1.5.** Fold enrichment of targets compared to Control tissue sample

Sample	BCL2	BAX	CASPASE	TNF $\alpha$
P(Untreated)	0.42045	0.36699	0.251926	2.230981
T_treated	1.17809	0.232255	0.232727	1.039199
F_treated	0.95797	0.199097	0.213802	1.258545

Quantitative real-time PCR was used to analyse the expression mRNA levels of apoptotic genes (bax, bcl-2, and

caspase-3) in liver of zebra fish exposed to APAP and post treated group. Bax and Bcl-2 are two members of the Bcl2 family that play an important role in the regulation of apoptosis. Bax, is the multidomain pro-apoptotic gene, which was up-regulated after exposure to APAP as compared with the control. Down-regulation of the Bcl-2 gene expression was observed upon exposure to APAP and the Tumor necrosis factor (TNF- $\alpha$ ) gene expression was up-regulated. To assess whether APAP induces apoptosis via the caspase pathway, the gene expression of caspase-3 was examined. The gene expression of caspase-3 was significantly up-regulated upon APAP exposure. The post treated groups Showed Significant down regulation of the apoptotic genes (BAX, TNF- $\alpha$ , CASPASE gene) and up-regulation of BCL2, showing the protective nature of flavonoids in regulation of gene expression.

## SUMMARY AND CONCLUSION

APAP exposure to the fish caused generation of ROS in the liver which activated and transcribes the pro-apoptotic proteins leading to the up-regulation of apoptosis mediator genes. This triggers the transactivation of Bax, inducing cytochrome c release. The cytochrome c then activates the TNF- $\alpha$  leading to apoptosis in DNA damage and mediates the caspase pathway. [18,19]

From the present study, it is shown that the plant *Sphaeranthus amaranthoides* contains various bioactive compounds which are responsible for its physiological and biological properties. The ethanol extract of the plant was evaluated for the presence of phytochemicals, flavonoids, alkaloids, steroids. The flavonoids were isolated from the extract using chloroform and ethyl acetate. The separation of flavonoids was carried out by TLC and column chromatography, Spectroscopic analysis confirmed the compound to be a quercetin derivative. Drug induced liver damage in embryos as well as adult zebra fish was studied, which accounts for the hepatoprotective nature of flavonoids.

The various anti-oxidant enzyme and blood marker enzymes were assayed to check for the liver injury as preliminary study. Total protein, DNA, RNA, cholesterol level was also assayed to check for its alteration. The liver damage in the tissue was further assayed by histopathological studies which showed the degeneration of liver vacuoles and regeneration of the vacuoles upon treatment with flavonoids.

The DNA fragmentation assay showed the changes in DNA. To check for the expression of apoptotic genes, Q-PCR was carried out. The apoptotic genes were up regulated in the injured liver tissue, whereas the genes were down regulated with the flavonoid treatment.

Hence, based on the present study it can be stated that the flavonoids obtained from the plant can be used for the treatment of liver injury.

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