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Original Article

Evaluation of *in vitro* Antioxidant, *in vitro* Cytotoxicity on HepG2 Cell line and Hepatoprotective Activity against Paracetamol Induced Hepatotoxicity in Rats of Piperonylic Acid

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ABSTRACT

Background: Excessive paracetamol use can cause liver damage, which is a major public concern. Pharmacological approaches are under investigation to avert such damage. The principal objective of the present investigation is to look at antioxidant activity (DPPH assay), cytotoxicity (HepG2 cell line) and hepatoprotective effect of piperonylic acid against paracetamol triggered liver damage in a rat model. **Materials and Methods:** Antioxidant activity was assessed for Trolax (Standard control) and piperonylic acid using the DPPH assay model with dosages ranging from 100 to 6.25 µg/mL. The cytotoxicity potential of piperonylic acid was carried out on the selected cancer cell lines HepG2 by MTT assay. Five groups each with 6 rats were given orally every day vehicle (0.25% CMC), paracetamol (640 mg/kg, Hepatotoxic control), 100 mg/kg silymarin (Standard Drug Treated), Piperonylic acid (100 and 200 mg/kg) for 14 days, hepatotoxicity was then triggered using acetaminophen (except normal control group). **Results:** By DPPH assay the Inhibitory Concentration (IC_{50}) of piperonylic acid has been identified to be 78.17±1.29 µg/mL. Trolox exhibited highest antioxidant activity followed by piperonylic acid. Cell viability in HepG2 was considerably dropped in correlation with concentration. The IC_{50} value turned out to be 97.109 µg/mL. Paracetamol efficiently caused liver damage and oxidative stress and pre-treatment with piperonylic acid in rats lowered hepatotoxic indicators considerably and dose-dependently. Piperonylic acid at 200 mg/kg significantly reversed paracetamol triggered changes in liver enzymes (SGOT, SGPT and GGT). **Conclusion:** Our results suggest that pretreatment with piperonlyic acid can attenuate the oxidative stress, significant cytotoxicity potential in HepG2 cell line and protect in defense of paracetamol-induced toxicity to the liver in rats.

Keywords: Acetaminophen induced hepatotoxicity, Antioxidant, MTT Assay (HepG2), Piperonylic acid.

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INTRODUCTION

The hepatocytes of liver are primary involved to executes a range of biological functions, including the metabolism of medicines, proteins, lipids and carbohydrates. Any form of liver disease is a universal issue (Lee *et al.*, 2007) because it can severely impact public health (Ahsan *et al.*, 2009). Medicated- Induced hepatic damage was a significant result of short-term liver Failure around world, with a projected incidence of over one per million (Devarbhavi *et al.*, 2023). Cytochrome P450 enzymes produce the hepatotoxic metabolite NAPQI (N-acetyl-p-benzoquinone-imine) when paracetamol is administered (Sinaga *et al.*, 2021). NAPQI quickly conjugates with Glutathione (GSH) at physiological and therapeutic doses, to creates metabolic waste products excreted in the urine (Yan *et al.*, 2018). In any case, excessive NAPQI production can be followed by a decrease in GSH levels when taking toxic doses of paracetamol. This causes excess NAPQI to bind to proteins in the cell, primarily within the mitochondria, leading to the buildup of reactive oxygen species and stress from oxidation ultimately leading to liver impairment (Coelho *et al.*, 2022). It has been documented that the usage of alternative and complementary therapies has increased in the management of both acute and

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long-term liver illness and dysfunction (Shen *et al*., 2009). Derivatives and natural substances may be among the most beneficial therapy for any sort of liver illness (Al-Asmari *et al*., 2014). For instance, Silymarin, a type of flavonoid derived from milk thistle (*Silybum marianum*), is a common hepatoprotectant medication (Pradhan and Girish, 2006). Furthermore, it is essential to comprehend the impacts on human hepatic mechanisms in order to comprehend a potential medicine, particularly one that will be taken orally (Neto *et al*., 2012). One of the most widely used spices in the world is pepper. Since ancient times, it has also been utilized medicinally, and it is known to possess a variety of biological properties like cancer-preventing, anti-inflammatory, antibacterial capabilities (Deng *et al*., 2016; Raj *et al*., 2011; Tasleem *et al*., 2014; Turner, 2004). Piperonylic acid is isolated from long peppers (*Piper longum* L.) and black peppers (*Piper nigrum* L.) (Bhat and Sivakumar, 2005). Only a few activities like anti-inflammatory, analgesic, antipyretic, inhibitory effects on intestinal α-glucosidase, etc., have been reported (Mujumdar *et al*., 1990; Pradeep and Kuttan, 2002; Rao *et al*., 2009; Takooree *et al*., 2019; Tomey *et al*., 2015). Consequently, our investigation made an effort to evaluate the antioxidant, cytotoxicity effect on HepG2 cell lines and protective effect of piperonylic acid using rats as an experimental animal model for acute hepatotoxicity triggered by a potentially harmful dosage of paracetamol.

MATERIALS AND METHODS

Active Constituent

Piperonylic acid was purchased from Yucca Enterprises, Wadala (E), Mumbai, 400037, India. It is an element of the chemical family of benzodioxoles, has 1,3-benzodioxole replaced with a carboxy group at position 5, weight of 166.13 g/mol. Solid, white powder with a melting point of 229°C (Piperonylic acid PUB CHEM).

Cell line and culture

The National Centre for Cell Science (NCCS), Pune, provided HepG2 cell line. In a humidified atmosphere with 5% CO_2, stock cells were cultivated in DMEM-HG (Dulbecco's Modified Eagle Medium - High Glucose) supplemented with 10% inactivated Fetal Bovine Serum (FBS), 100 IU/mL of Penicillin, 100 g/mL of Streptomycin, and 5 g/mL of Amphotericin B until they reached confluence. TE solution (0.2% Trypsin, 0.02% EDTA) was used to separate the cells. The 96-well micro titer plate was used for cytotoxicity tests, and the stock cultures were cultivated in 25 cm^2 culture flasks. Study site Radiant Research, Bangalore.

Animals

Wistar Albino male rats of 120-180 g were collected from, Pharmacology Department, CESCOP, Kurnool, A.P. Prior to the trial, one week acclimatized and fed a pellet diet with unlimited water. These animals were kept in standard laboratory, whereas the experimental protocol was approved by IAEC/ Institutional Animal Ethics Committee (Approval no: IAEC/ CESCOP/2025-08).

In vitro antioxidant study (DPPH Assay)

96 well microtitre plate was taken and 0.01 mL of different concentrations of piperonylic acid (test) and standard (Trolox) was introduced separately in the test and test blank wells. Instead of piperonylic acid, 0.01 mL of DMSO was taken for control and control blank, 0.2 mL DPPH was placed in the test group as well as control group, whereas 0.2 mL of methanol is added to the test and control blanks instead of DPPH. The approach was carried out for standard by substituting test item with Trolox. The microtiter plate was kept at 37°C approximately 30 min. The absorbance was determined at 490 nm with a microplate reader (Jaishree *et al*., Vijayarajan *et al*., 2016).

In vitro Cytotoxicity MTT Assay

DMEM-HG and 10% FBS were used to trypsinize the cell culture monolayer and adjust it to 100,000 cells/mL. 96-well microtiter plates were seeded with 0.1 mL of diluted cell solution in each well. One day later, a partial monolayer was formed. The monolayer was washed once with DPBS after the supernatant was discarded. Cells were treated at doses ranging from 100 µg/mL to 0.78 µg/mL. Cells which were not treated served as the control for comparison. Microscopic analysis was then performed after the Plates were incubated in a 5% CO_2 atmosphere at 37°C for a full day. After 24 hr, remove the test solutions and add 100 µL of MTT diluted with DPBS in each well. The plate remained 3 hr at 37°C in an atmosphere with 5% CO_2. To dissolve the generated formazan, 100 µL of DMSO was added after the supernatant was discarded. An absorbance measurement was made at 570 nm using a microplate reader (Scudiero *et al*., 1988).

Acute Toxicity report (ProTox-3.0)

The toxicity of piperonylic acid was predicted using Pro Tox 3.0, and the predicted LD/Lethal Dose 50 was found to be 2500 mg/ kg.

Experimental Design

30 Wistar Albino male rats were selected at random into five groups, each with six animals for 14 days experimental trial as follows (Islam *et al*., 2021; Alam *et al*., 2017).

Group I (Normal Control): Animals were treated with vehicle and served as normal control.

Group II (Hepatotoxic Control): Paracetamol was administered alone (640 mg/kg, per oral) dispersed in vehicle.

Group III (Standard Drug Treated): Silymarin and paracetamol at a dose of 100 mg/kg and 640 mg/kg, orally.

Group IV (PA 200 mg/kg): Piperonylic acid and paracetamol at a dose of 200 mg/kg and 640 mg/kg orally using vehicle (0.25% CMC/Carboxymethyl celluloses).

Group V (PA 100 mg/kg): Animals received piperonylic acid 100 mg/kg and paracetamol 640 mg/kg orally using vehicle (0.25% CMC/Carboxymethyl cellulose).

The doses of piperonylic acid were administered based on Protox 3.0, whereas according to Janbaz and Gilani the paracetamol dosing was designated. Following fourteen days of therapy, rats in each group were sacrificed by chloroform inhalation. Afterwards blood samples have been collected via heart puncture followed by centrifugation to separate serum. Hepatic performance indicators such as SGPT, SGOT, and GGT have been evaluated using the typical procedure given in Agapee kits. Liver tissues were collected for histopathological examination.

Statistical analysis

Biochemical estimate data were reported using Graph Pad Prism 10.1.1 version as Mean±SEM. One-way ANOVA to analyse statistical significance, Dunnett's Multiple Comparison Tests, p-value <0.0001.

RESULTS

In vitro antioxidant activity

Antioxidants can provide their electrons to DPPH, a free radical that can scavenge by transforming them into stable diamagnetic molecules. With increasing concentration, the active fraction's ability to scavenge free radicals on DPPH radicals rises. The scavenging activities of piperonylic acid and Trolox (Standard) on DPPH radicals are shown in Table 1. Piperonylic acid reducing capacity reflects the antioxidant's ability to reduce Fe^{3+} to Fe^{2+}.

In vitro Cytotoxicity MTT Assay

It quantifies the conversion of the MTT reagent to formazan, a purple-colored product that reflects metabolic activity in viable cells and is proportional to the number of viable cells in the sample. However, it aids in the identification of substances that cause cell death or impede cell growth. In this study the hepatoprotective potential of piperonylic acid was carried out to determine the cytotoxicity potential on the selected cancer cell lines HepG2.

Cell viability in HepG2 was considerably decreased in a concentration-dependent manner (Table 2, Figure 1). The IC_{50} value was found to be 97.10 µg/mL.

Effect on serum biomarkers

Serum levels of SGOT, SGPT and GGT were considerably higher in the hepatotoxic group than in the control group (all p<0.0001), suggesting that paracetamol was efficacious in inducing liver injury. Silymarin (standard drug) is commonly administered to minimize blood transaminase levels in hepatitis treatment. Notably, piperonylic acid was also effective to lower the SGOT, SGPT and GGT levels on dose dependent manner (Table 3).

Histopathological analysis

The control group exhibited no visible histological abnormalities (Figure 2). The paracetamol/hepatotoxic model's liver pathology exhibited necrosis and ballooning degeneration in the perivenular zone due to severe cell injury, evident large inflammatory cells, and hepatocyte lipid droplet formation (Figure 3). This information confirmed the existence of overdose of paracetamol-induced liver damage. Meanwhile, silymarin and piperonylic acid mitigated the morphological changes by moderating necrosis, reducing inflammatory cell infiltration (Figures 4-6).

DISCUSSION

Paracetamol is an analgesic and antipyretic medication that, when taken in high amounts, becomes a potent hepatotoxic chemical (Goldin *et al*., 1996; Hinson *et al*., 2002; Mitchell *et al*., 1973; Hinson *et al*., 2002; Muriel *et al*., 1992). According to the experimental paradigm of drug-induced rapid hepatocellular

Table 1: Antioxidant activity of piperonylic acid and Trolox by DPPH Assay.

Sample	Concentration (µg/mL)	%Inhibition	IC_{50}
Standard (Trolox)	100	99.06±0.80	9.951±0.48
	50	75.38±0.13	
	25	62.27±0.54	
	12.5	52.23±0.27	
	6.25	43.80±0.67	
Piperonylic Acid	100	57.09±1.40	78.17±1.29
	50	38.26±1.26	
	25	28.18±1.4	
	12.5	17.21±1.28	
	6.25	8.02±1.07	

destruction using paracetamol is highly renowned. Long-term usage of paracetamol in high doses produces NAPQI a harmful reactive metabolites and free radicals via the CYP450 enzyme's biotransformation pathway (Tejo, 2021). Among the mechanisms underlying acute liver failure is oxidative stress, which disrupts cellular homeostasis and leads to hepatocytes death (Chidiac *et al*., 2023). The free radicals produced will cause necrosis, which is a secondary problem caused by lipid peroxidation or damage conditions in cells and can result in the premature death of cells and living tissues (Alshehri *et al*., 2020). Cell necrosis damages the permeability of the liver cell membrane, allowing enzymes contained in cells, such as SGOT (Serum Glutamic-Oxaloacetic Transaminase), SGPT (Serum Glutamic-Pyruvic Transaminase) and GGT (Gamma-Glutamyl Transferase) to depart readily and enter the extracellular space and blood vessels; this raises enzyme activity in the blood over normal levels (Amirabagya *et al*., 2021).

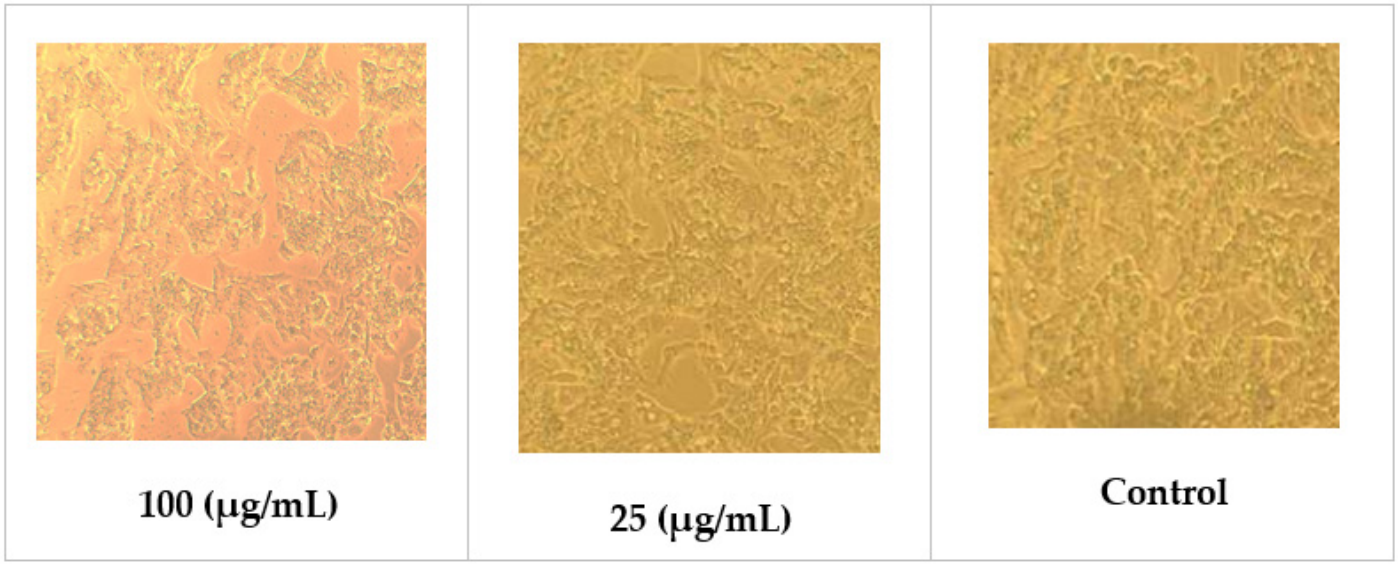
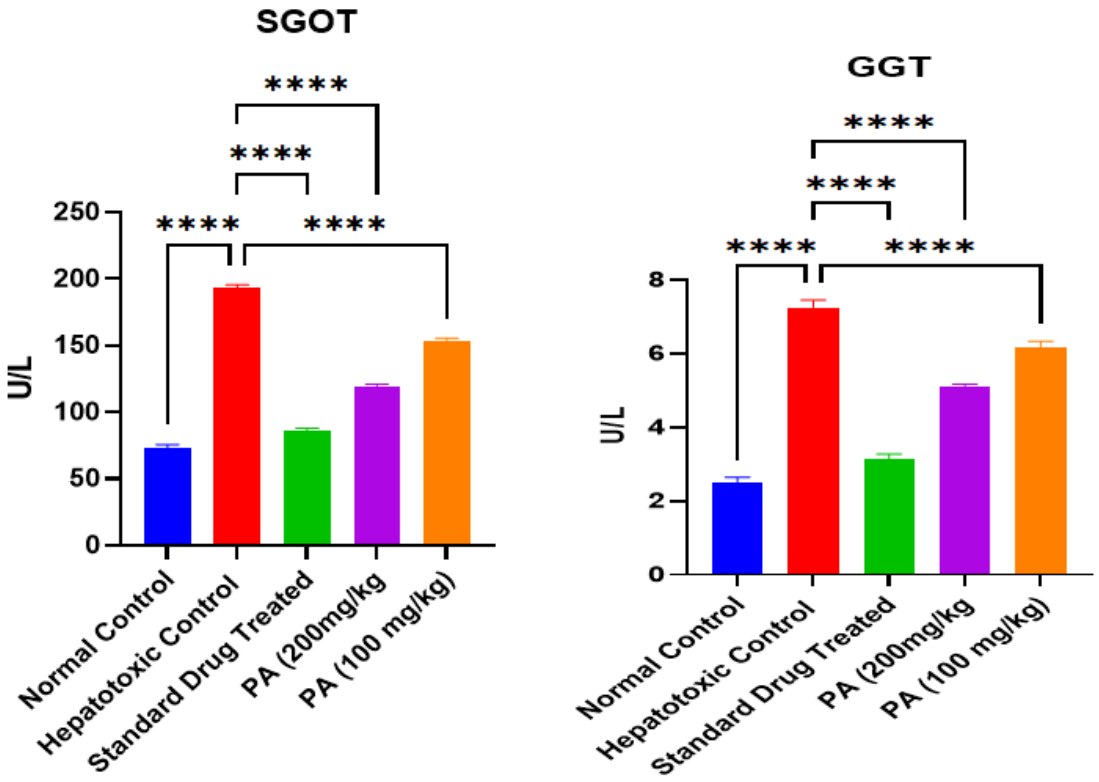
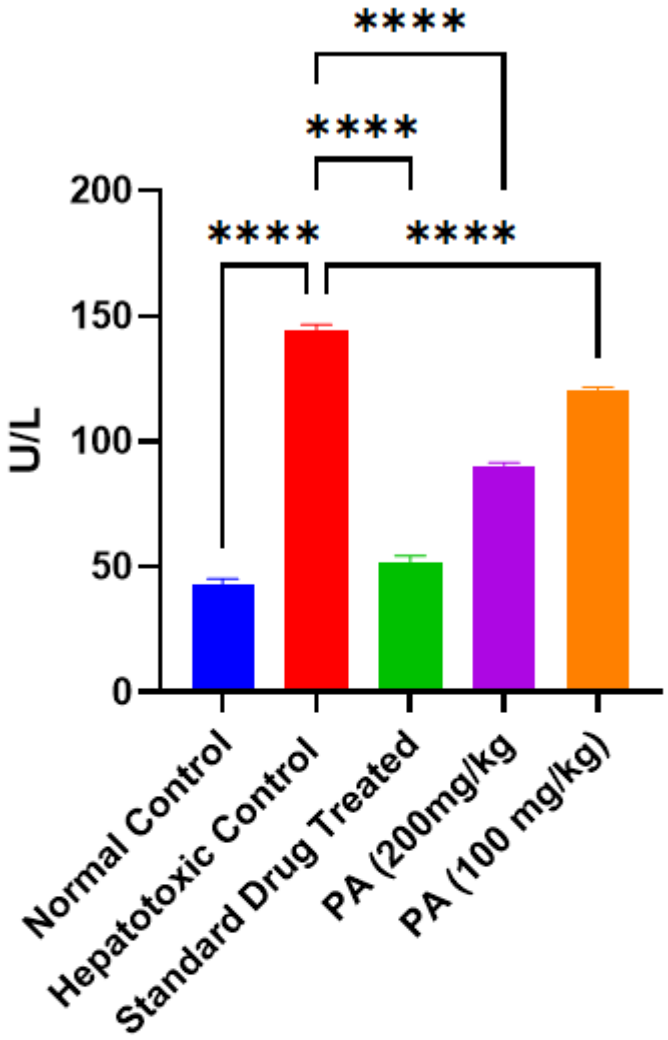


Figure 1: Cytotoxicity of piperonylic acid on HepG2 cell line by MTT Assay.





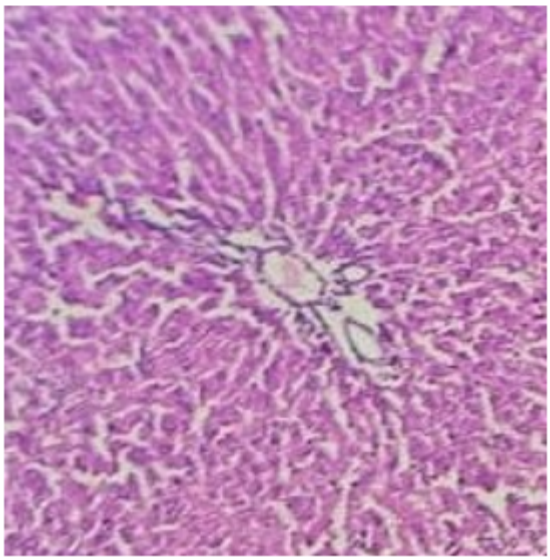


Figure 2: Histopathological findings of Group I with normal central vein and parenchymal cells.

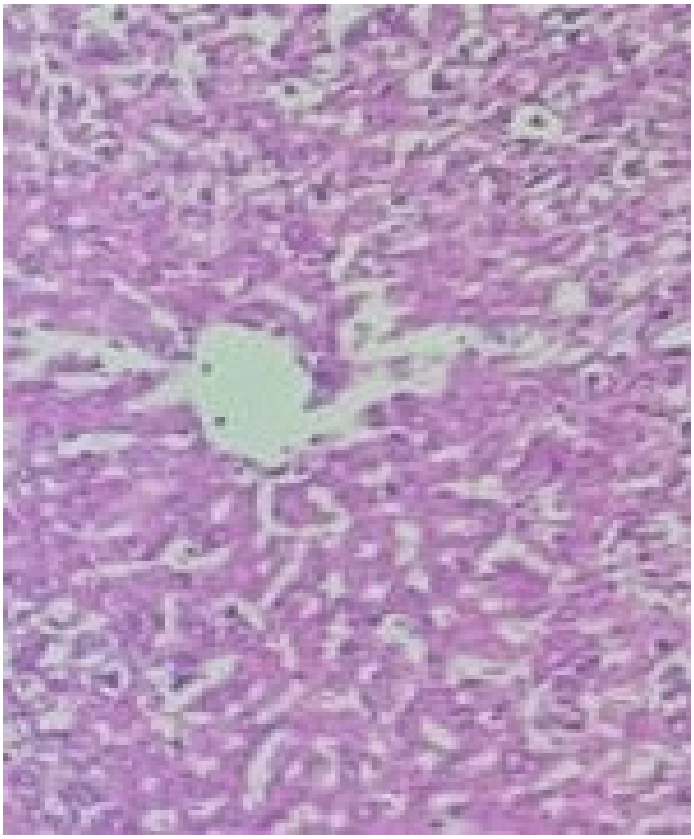


Figure 3: Histopathological findings of Group II ballooning degeneration, congestion in of hepatocytes, necrosis sinusoids indicating liver damage.

Table 2: ***In vitro*** **cytotoxicity of piperonylic acid in terms of percentage cell viability against HepG2 cell line by MTT assay.**

Concentration (µg/mL)	% of Cell Viability (Mean±S.D)	IC_{50} (µg/mL)
100	57.33±1.31	97.109
50	63.81±0.55	
25	82.22±3.27	
12.5	86.39±2.11	
6.25	90.40±3.05	
3.125	94.67±3.10	
1.56	96.09±2.73	
0.78	98.89±0.48	

In the currently ongoing study, it became obvious that the repeated use of acetaminophen notably enhanced serum hepatic biomarkers, confirming a presence of hepatic damage (Table 3). The administration of piperonylic acid in a dose-related approach alongside silymarin resulted in considerable restoration these enzyme levels.

The most common disease that has no effective treatment is Human Hepatocellular Carcinoma (HCC), a malignant tumour that arises from hepatocyte (Vakili Zahir *et al.*, 2018). A prior investigation revealed that HepG2 cells, a cell line derived

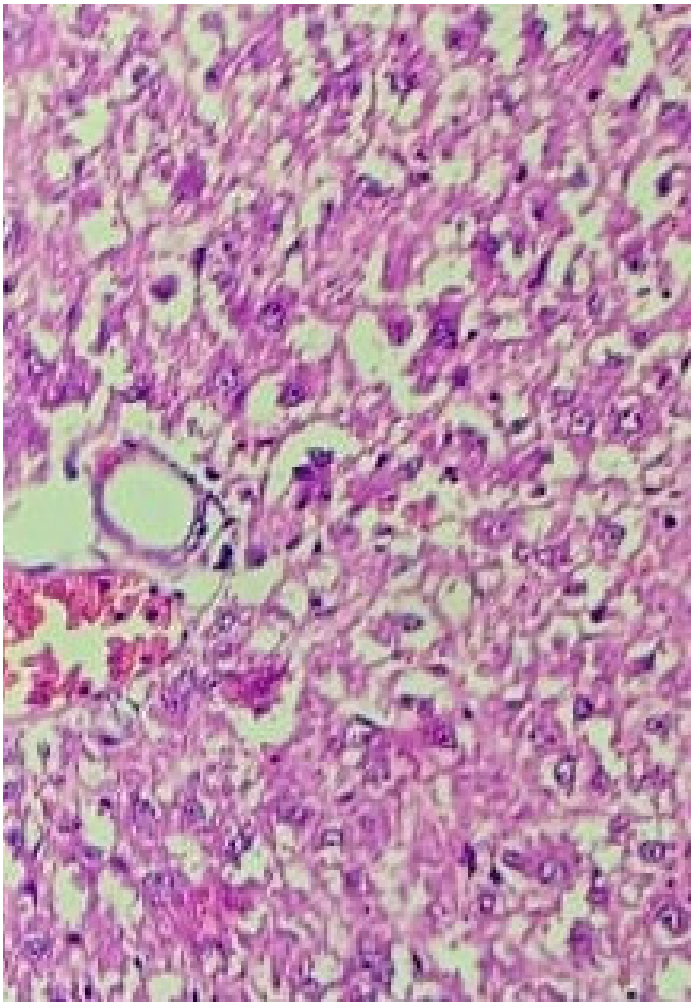


Figure 4: Histopathological findings of Group III with mild necrosis and less damage.

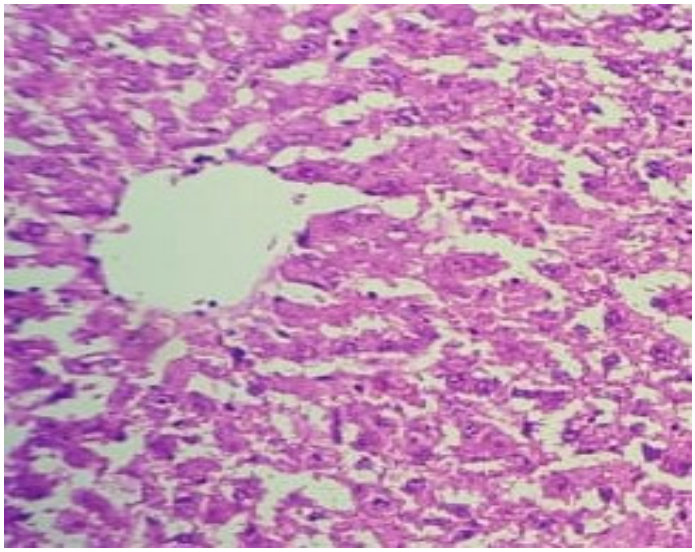


Figure 5: Histopathological findings of Group IV less peripheral necrosis, less ballooning damage.

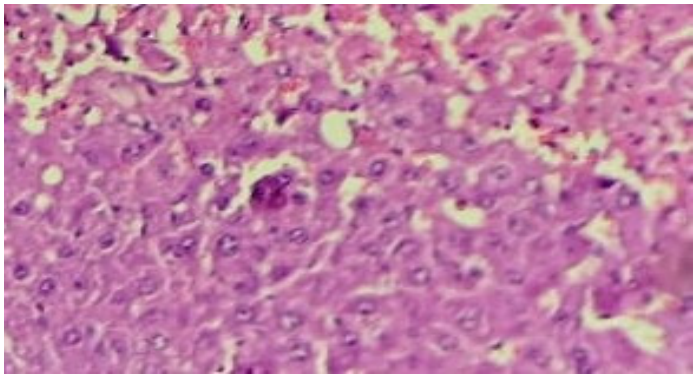


Figure 6: Histopathological findings of Group V with necrosis, ballooning,Congestion indicating moderate liver damage.

Table 3: Effect of Piperonylic acid on SGPT, GGT and SGOT levels in rats treated with paracetamol.

	Normal Control	Hepatotoxic Control	Standard Drug treated	PA (200 mg/kg	PA (100 mg/kg)
SGOT	73.33±2.431	193.2±2.301	85.83±2.088	118.8±2.182	153.8±1.701
SGPT	42.83±2.386	144.5±2.094	52±2.366	90.33±1.282	120.2±1.470
GGT	2.5±0.1414	7.250±0.2012	3.150±0.1232	5.08±0.08724	6.18±0.1515

Mean±SEM, n= 6, p<0.0001 with respect to Group II.

from Hepatocellular Carcinoma, were halted in the S phase of the cell cycle (Venkatachalapathy *et al.*, 2021). The findings of cytotoxicity/MTT assay revealed piperonylic acid exhibited significant potency in HepG2 cell line.

The present study also revealed DPPH inhibition assay of different concertation of piperonylic acid and the Inhibitory Concentration (IC_{50}) of piperonylic acid has been identified as 78.17±1.29. The piperonylic acid's potential to restore raised blood enzymes in acetaminophen-induced liver damage could be attributed to its antioxidant action.

CONCLUSION

Based on both *in vitro* and *in vivo* results, the current study showed that piperonylic acid had substantial hepatoprotective potential. The molecule demonstrated antioxidant activity, suggesting the potential to neutralize free radicals and minimize oxidative stress. It efficiently had cytoprotective activity against HepG2 cell lines. Furthermore, in the paracetamol-induced hepatotoxicity model, treatment with piperonylic acid markedly improved liver function markers and reduced histopathological damage. Overall considered piperonylic acid exhibits encouraging promise as a natural hepatoprotective agent. Additional research is necessary to support its development for therapeutic application.

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ABBREVIATIONS

CMC: Carboxymethyl cellulose; **CYP450:** Cytochrome P450; **DMEM-HG:** Dulbecco's Modified Eagle Medium - High Glucose; **DMSO:** Dimethyl Sulfoxide; **DPBS:** Dulbecco's Phosphate Buffer Saline; **DPPH:** 2,2-diphenyl-1-picrylhydrazyl assay; **EDTA:** Ethylene diamine tetra acetic acid; **FBS:** Fetal Bovine Serum; **GSH:** Glutathione; **GGT:** Gamma-Glutamyl Transferase; **HCC:** Hepatocellular carcinoma; **HepG2:** Human Liver Carcinoma; **IAEC:** Institutional Animal Ethics Committee; **LD_{50}:** Lethal Dose 50; **MTT:** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **NAPQI:** N-acetyl-p-benzoquinone-imine; **PA:** Piperonylic acid; **SGOT:** Serum Glutamic-Oxaloacetic Transaminase; **SGPT:** Serum Glutamic-Pyruvic Transaminase; **TE:** Trypsin-EDTA solution.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

FINANCIAL SUPPORT AND SPONSORSHIP

The authors declare that no external funding was received for this study.

AUTHOR CONTRIBUTIONS

Venumadhuri and Mastanaiah contributed to the conceptualization and design of the study. Venumadhurin performed the experiments and data collection. Venumadhuri and Mastanaiah analyzed the data and drafted the manuscript.

SUMMARY

Using both *in vitro* and *in vivo* models, piperonylic acid was studied for its hepatoprotective, cytotoxic, and antioxidant properties. The DPPH free radical scavenging assay was used to assess antioxidant capacity and demonstrated a dose-dependent decrease in free radicals. HepG2 human liver cancer cells were used to test the *in vitro* cytotoxicity of piperonylic acid, which showed moderate cytotoxic effects with tolerable cell survival at lower concentrations. In a paracetamol-induced liver injury model in rats, the hepatoprotective efficacy was further investigated; therapy considerably decreased liver damage and enhanced hepatic function. Overall, the study emphasizes piperonylic acid as a potentially useful phytochemical with antioxidant and hepatoprotective qualities.

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