SYNERGISTIC HEPATOPROTECTIVE ACTIVITY OF CHRYSIN AND CURCUMIN AGAINST LIVER FIBROSIS

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Abstract

Curcumin and chrysin Oxidative stress has been considered a key causing factor of liver damage induced by a variety of agents, including alcohol, drugs, viral infections, environmental pollutants, and dietary components, which in turn results in the progression of liver injury, non-alcoholic steatohepatitis, non-alcoholic liver disease, liver fibrosis, and cirrhosis. Curcumin is one of the most commonly used indigenous molecules endowed with various shielding functionalities that protect the liver. The aim of the present study is to curcumin and chrysene against synergistic pharmacological effects and molecular mechanisms, as well as clinical evidence, of curcumin as a lead compound in the prevention and treatment of oxidative associated liver diseases. "hepatoprotective or hepatotoxicity or liver" along with "oxidative or oxidant." Results showed that curcumin exerts remarkable protective and therapeutic effects on oxidative-associated liver diseases through various cellular and molecular mechanisms. Those mechanisms include suppressing the proinflammatory cytokines, lipid peroxidation products, PI3K/Akt, and hepatic stellate cells activation, as well as ameliorating cellular responses to oxidative stress such as the expression of Nrf2, SOD, CAT, GSH, GPx, and GR. Taking together, curcumin itself acts as a free radical scavenger over the activity of different kinds of ROS via its phenolic, β-diketone, and methoxy group. Further clinical studies are still needed to recognize curcumin's structure-activity relationships and molecular mechanisms in oxidative-associated liver diseases.

Keywords: curcumin, chrysene, hepatotoxicity, liver fibrosis, natural product

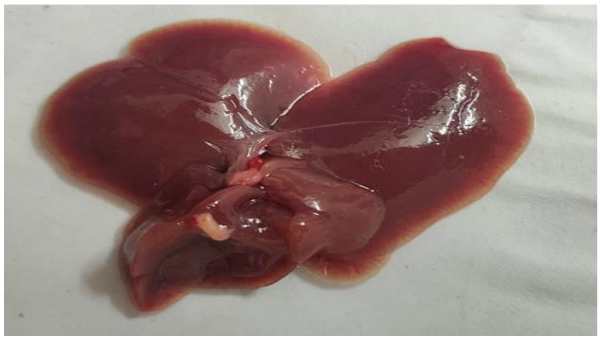
1. Introduction

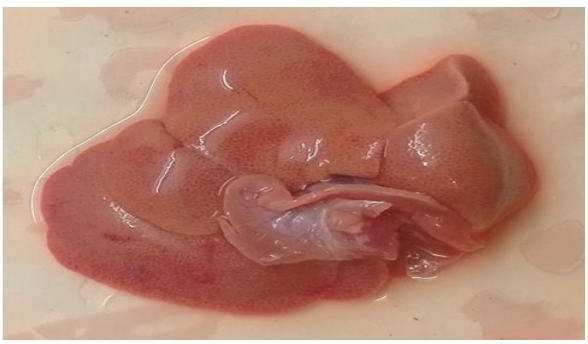
Hepatic fibrosis is a reversible wound-healing response characterized by the accumulation of extracellular matrix (ECM) or "scar" tissues that follow the chronic but not self-limited liver disease. The ECM components in the fibrotic liver are similar regardless of the underlying cause. Hepatic fibrosis has evolved in the past 20 years from a pure laboratory discipline to an area of great bedside relevance to practicing hepatologists. This evolution reflects a growing awareness of the molecular underpinnings of fibrosis and its natural history and methods of detection in chronic liver disease. These advances have culminated in clear evidence that cirrhosis can be reversible, and in realistic expectations that effective antifibrotic therapy will significantly alter the management and prognosis of patients with liver disease. In view of this remarkable progress, clinicians must now view liver fibrosis in a new light as a clinical problem in its own right amenableto specific diagnostic tests and therapies that are independent of the etiology. In that spirit, it is necessary to integrate current knowledge about the nature and prognosis of fibrosis in different forms of chronic liver disease with recent advances in elucidating its pathophysiology. These advances form the basis for the rational treatment of hepatic fibrosis **(Friedman, 2001; Gressner *et at.*,2002; Mann and Smart. *et al,* 2002).**

The clinical manifestations of cirrhosis vary widely, from no symptoms at all to liver failure, and are determined by both the nature and severity of the underlying liver disease as well as the extent of hepatic fibrosis. Up to 40% of patients with cirrhosis are asymptomatic and may remain so for more than a decade, but progressive deterioration is inevitable once complications develop including ascites, variceal hemorrhage, or encephalopathy. In such patients, there is 50% 5-year mortality, with approximately70% of these deaths directly attributable to liver disease **(Fattovich *et al.*, 1997).**

The overall prevalence of cirrhosis in the United States is estimated at 360 per 100,00 population, or 900,000 total patients, the large majority of whom have chronic viral hepatitis or alcoholic liver disease. Cirrhosis affects hundreds of millions of patients worldwide. In the US, it is the most common non-neoplastic cause of death among hepatobiliary and digestive diseases, accounting forapproximately 30,000 deaths per year. In addition, 10,000 deaths occur due to liver cancer, the majority of which arise in fibrotic livers, with the mortality rate steadily rising **(El-Serag and Mason. *et al,* 2000; Befeler and Di Bisceglie. *et al,* 2002).**

Figure 1. Gross anatomy of mice liver (A) Normal liver; (B) Fibrotic liver





The most commonly associated pharmacological groups are antibiotics, nonsteroidal anti-inflammatory analgesics (NSAIDs), antidepressants, and anticonvulsants. Drug-induced liverinjury has been an adverse event, hard to identify, prevent and treat; thereby, pharmacist intervention can contribute to the diminution of the deleterious effects on patient health. **(Chronic hepatitis. Annu. Rev. Med. *et al,* 2007)**

The natural history of liver fibrosis is influenced by both genetic and environmental factors. These genetic factors may explain the broad spectrum of responses to the same etiological agent found in patients with chronic liver diseases. However, some studies have yielded contradictory results due to poor study design and further research is required to clarify the actual role of genetic variants in liver fibrosis. **(Bataller Ramon, Brenner A David, *et al,* March 2005)**

Table 1.1 Effect of different Inflammatory factors on the effect of fibrosis

	Factors	Cell Sources	Effect On Fibrosis
		Many cell types	Decreases
		Lymphocytes	Decreases
		Leucocytes, including Kupffer cells	Increases
		Leucocytes, including Kupffer cells, And myofibroblasts	Decreases
		Leucocytes, including Kupffer cells	Decreases
		Megakaryocytes and platelets in the blood. Expressed by many cell types, however , including myofibroblasts	Increases
		Expressed by many cell types, includingmyofibroblasts	Increases
		Leucocytes, including Kupffer cells	Increases

2. Mechanisms of Hepatotoxicity

The hepatocytes, cholangiocytes, Kupffer cells, ductal and endothelial cells are involved in themechanisms by which drugs cause hepatotoxicity, having direct effects on cellular organelles such as mitochondria, endoplasmic reticulum, cytoskeleton, microtubules or nucleus. The drugmetabolites generated in the liver through biotransformation can cause hepatic damage of toxic or reactive substances such as electrophilic chemicals or free radicals, and thus an unchain a variety of chemical reactions may happen. **(Amariles Pedro, *et al,* 2018)**

Mitochondrial dysfunction: may be generated by the disruption of β-oxidation of lipids and oxidative energy production within the hepatocytes. Mitochondrial membrane permeabilizationcan lead to apoptosis, a rupture in the mitochondrial membrane can lead to ATP depletion and subsequent necrosis, and an abnormal function can also lead to fat accumulation, so steatosis can be present.

Immune response: is attributed to the formation of new antigens, this gives origin to the idiosyncratic hepatotoxicity. Moreover, it can be accompanied by the presence of inflammatory cells such as neutrophils and lymphocytes.

Oxidative stress: is produced by ATP depletion accompanied by an increase in intracellular calciumconcentration, it can generate necrosis. **(Amariles Pedro, *et al,* 2018)**

3. DRUG IDENTIFICATION AND CHARACTERISATION

A drug sample of Curcumin was procured as a gift sample from SRL Limited, India, and Chrysin was ordered from TCI Chemical (India) Pvt Ltd. The characterization studies like physical properties, melting point, solubility studies, UV calibration curve, Differential Scanning Calorimetry, and Nuclear Magnetic Resonance were performed and observations taken are compiled in the tables below.

3.1 PHYSICAL PROPERTIES OF CURCUMIN

A small quantity of the drug was taken on a clean glass slide. The sample was observed for color, state, and hygroscopicity by visually inspecting it under white light.

Table 3.1: General appearance

S.no.	Parameter	Reported	Observed
1.	Color	bright yellow-orange powder	yellow-orange powder

2.	**State**	crystalline powder	crystalline powder
3.	**Hygroscopicity**	Hygroscopic	Hygroscopic

3.2 PHYSICAL PROPERTIES OF CHRYSIN

A small quantity of the drug was taken on a clean glass slide. The sample was observed for color, state, and hygroscopicity by visually inspecting it under white light.

Table 3.2: General appearance

S.no.	Parameter	Reported	Observed
1.	**Color**	Cream color powder	Cream color powder
2.	**State**	crystalline powder	crystalline powder
3.	**Hygroscopicity**	Non-Hygroscopic	Non-Hygroscopic

3.3 MELTING POINT

The melting point of Curcumin was determined by using melting point apparatus. Curcumin was filled intoa capillary tube with one end sealed and inserted into the instrument. Melting of curcumin was observed for the point where curcumin starts to liquefy by the glass in the front of the instrument and temperature was noted with the help of a thermometer.

Table 3.3.1: Melting point determination

Parameter	Reported	Observed
Melting Point	180°C	178-183°C 177-182°C 175-180°C

The melting point of Chrysin was determined by using melting point apparatus. Chrysin was filled into a capillary tube with one end sealed and inserted into the instrument. Melting of Chrysin was observed forthe point where Chrysin starts to liquefy by the glass in the front of the instrument and temperature was noted with the help of a thermometer.

Table 3.3.2: Melting point determination

Parameter	Reported	Observed
Melting Point	285°C	283-287°C 282- 286°C 282-287°C

3.4 SOLUBILITY STUDIES

To determine curcumin's solubility in different solvents, 5mg/ml solutions of different solvents will be used during formulation (Chloroform, Ethanol, Methanol, Acetone, Hexane, PBS-7.4, PBS-6.8) were prepared, and then centrifuged to sediment the undissolved drug particles. 0.1 ml of supernatant was collected and diluted up to 10 ml. Absorbance was taken at 480nm by using Shimadzu 1240 UV-VIS spectrometer Japan.

S.No.	Reagent	Curcumin	chrysine
1.	Chloroform	Not-soluble	Not-soluble
2.	Ethanol	Soluble	soluble

3.	Methanol	soluble	soluble
4.	Acetone	Soluble	Not-soluble
5.	Hexane	Not-soluble	soluble
6.	Pbs-7.4	Not-soluble	Not-soluble
7.	Pbs-6.8	Not-soluble	Not-soluble

3.4 UV DETERMINATION OF CURCUMIN AND CHRYSIN

TABLE 3.5.1: UV OF CURCUMIN AND CHRYSINE

Drug	Reported	Observed
Curcumin	**421**	**420**
Chrysin	**264**	**267**

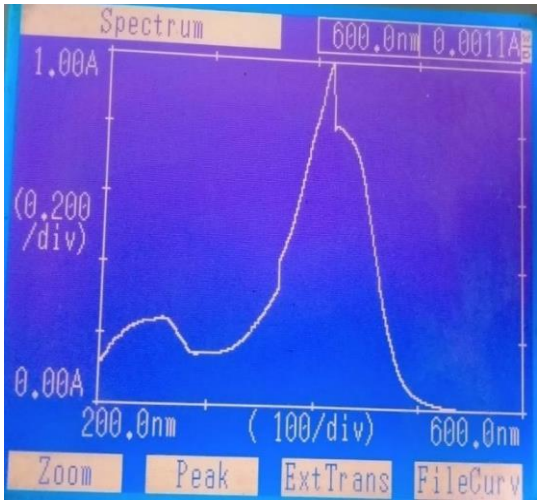


Figure 2 UV scan of CURCUMIN

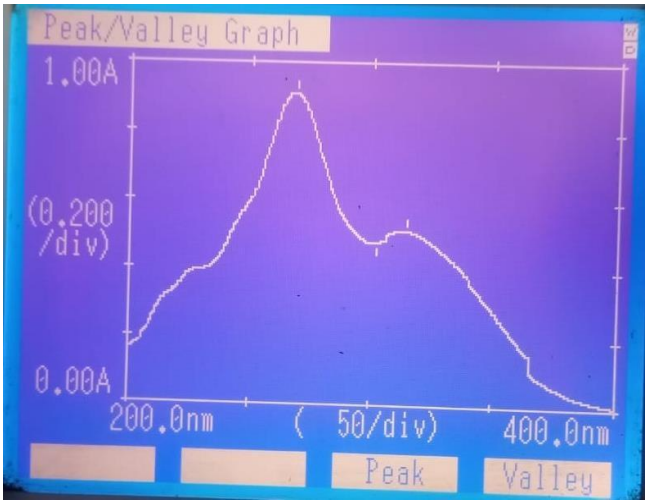


Figure 3 UV scan of Chrysin

3.6 **Nuclear Magnetic Resonance**

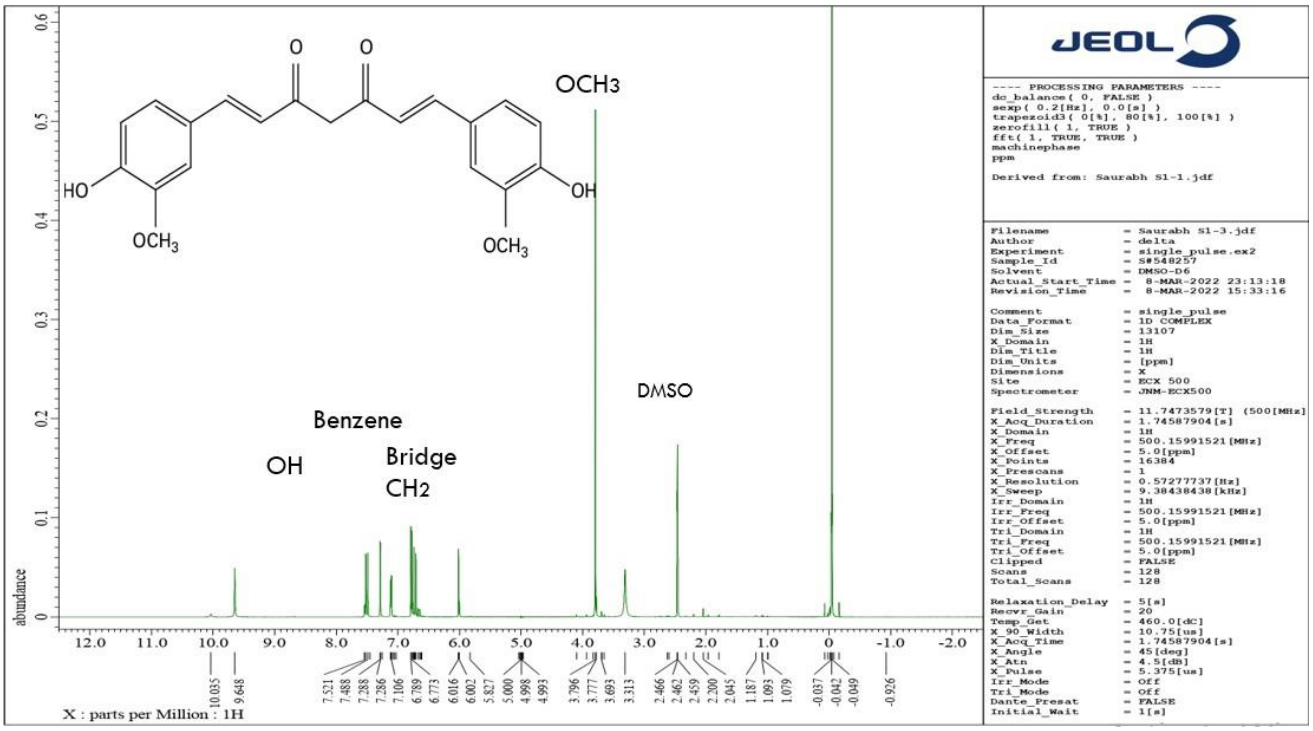


Figure.4 NMR spectra of curcumin

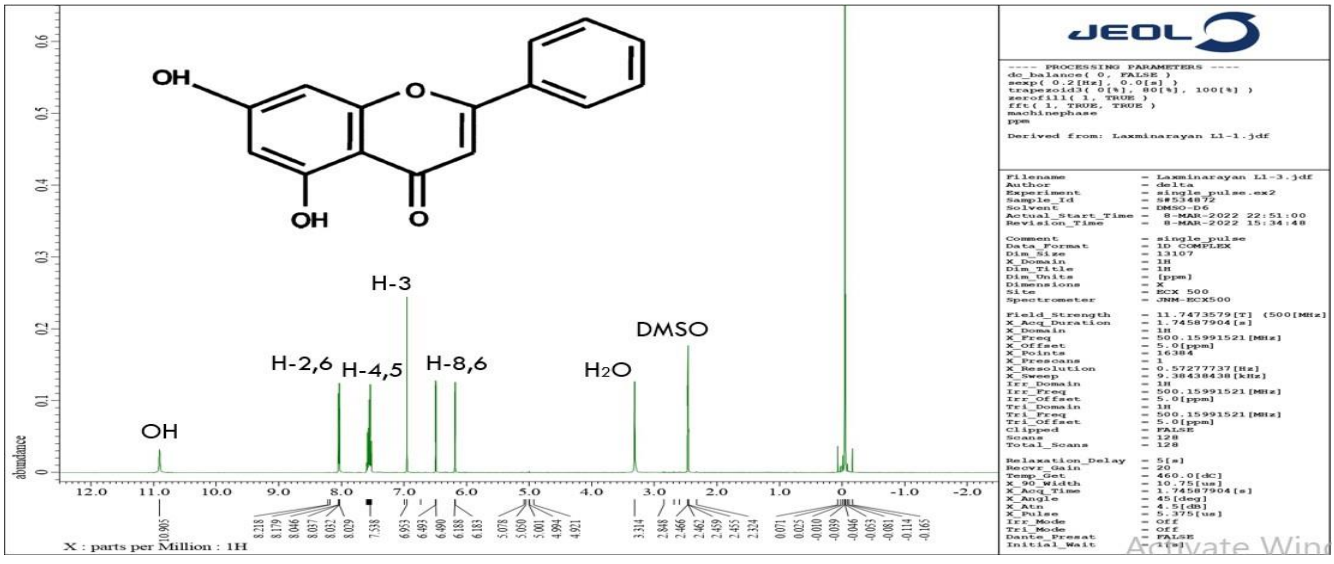
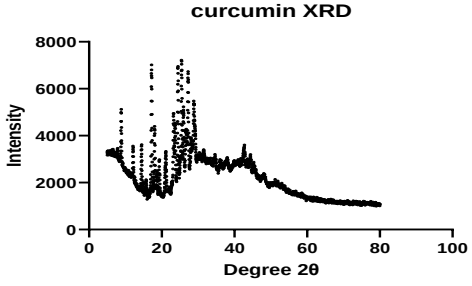
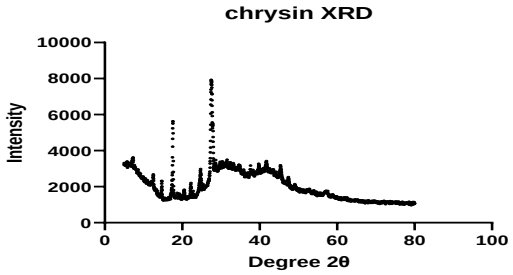


Figure.6 NMR spectra of chrysin

3.7 XRD





3.8 Differential Scanning Calorimetry (DSC)

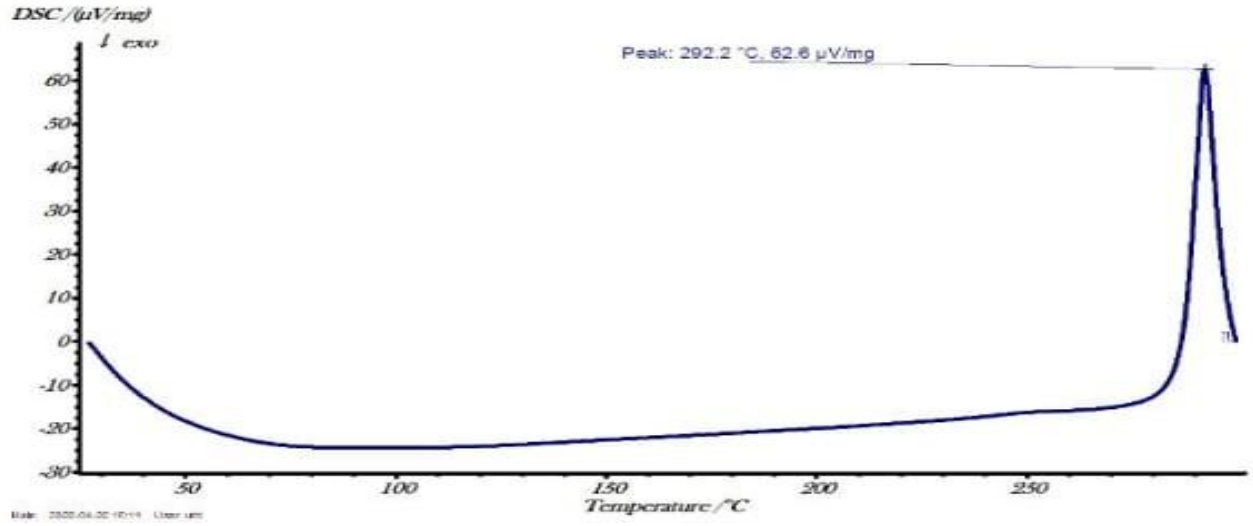


Figure: 6.7.1 DSC of Chrysin

Chemicals

2-Nitro propane was purchased from Sigma Aldrich, Curcumin was a gift sample from SRL limited, Chrysin was from TCI Chemicals (India) Pvt Ltd., and all other chemicals used were of technical grade.

Animal Ethical Clearance

The local Institutional Animal Ethical Committee of our University obtained ethical clearance forconducting experiments on animals from the committee for the purpose of control and supervision of experiments on Animals (CPCSEA) No.371/CPCSEA/IAEC/2022/23

Procurement of Animals and maintenance

Adult male/female ball mice weighing 22±5 obtained from the Institute of Pharmacy Vikram University Ujjain M.P. K.A. All the animals were apparently healthy without any infection. All animal experiments were performed in accordance with local Institutional guidelines for the care and use of laboratory animals.

Maintenance of the animals:

The animal room was cleaned daily with antiseptic lotion. Animals were maintained at a constant temperature (22-25°C) with 70-75% relative humidity and 12 hr photoperiod. The animals were placedin small groups (5 animals/cage) having a paddy husk bed. The cages along with the bed were changedevery alternate day. Animals were provided standard laboratory chow and water ad libitum. Before starting any experiment, the animals were acclimatized at least 7 days in the laboratory environment.

5. Methodology

Selection of dose

The dose of 2-NP (200µg /kg wt) was obtained from the previous work of Me Lean E.K. et al, 1991 andChanchal et al., 2006. The dose of curcumin l00mg/ kg wt. based on previous work of Sreepriya and Bali, 2006 with minor modifications. The dose of chrysin 50mg/kg is based on the previous work of Karima
M. Moawad, 2007 with minor modifications

Experimental Design

In group-1 vehicle control (0.5%CMC), In group-2 disease control–toxicity (2-NP + Ethanol 10 %), group-3 standard treatment (silymarin 50mg/kg), group-4 treatment group (curcumin 100mg/kg), group-5 treatment group (chrysin 50mg/kg), group-6 treatment group (curcumin 50mg/kg + chrysin 25mg/kg), group 7 treatment group (curcumin 100mg/kg + chrysin 50 mg/kg), group 8 treatment group (curcumin 50mg/kg + chrysin 25 mg/kg), group 9 treatment group (curcumin 50mg/kg + chrysin 50 mg/kg)

- Group-1: Vehicle control (0.5%CMC)
- Group-2: Disease control–toxicity (2-NP + Ethanol 10 %)
- Group-3: Standard treatment (silymarin 50mg/kg)
- Group-4: Treatment group (curcumin 100mg/kg)
- Group-5: Treatment group (chrysin 50mg/kg)
- Group-6: Treatment group (curcumin 50mg/kg + chrysin 25 mg/kg)
- Group 7: Treatment group (curcumin 100mg/kg + chrysin 50 mg/kg)
- Group 8: Treatment group (curcumin 50mg/kg + chrysin 25 mg/kg)
- Group 9: Treatment group (curcumin 50mg/kg + chrysin 50 mg/kg)

Blood collection, Plasma, and Serum separation

The animals were sacrificed 24hr after the last treatment. Blood was collected from the heart (cardiac puncture) of each animal in an Eppendorf tube without anticoagulant allowed to clot for 30 min at room temperature serum was separated by centrifugation. Serum was used for the estimation of Glutamate Pyruvate Transaminase (SGPT), Glutamate Oxaloacetate Transaminase (SGOT), and Alkaline Phosphatase (ALP). The blood collected with an anticoagulant containing EDTA (1mg/ml) wasused for the estimation of Lipid profile, total protein, albumin, globulins, urea, and creatinine.

Sacrifice of animals and organ collection

The mice were sacrificed at the end of 8 weeks and 24 h after the last dose of 2-NP. Mice were sacrificed by cervical dislocation and immediately liver, kidney, brain, and thigh muscles were removedand washed thoroughly with ice-cold 0.9% sodium chloride solution (saline). Each organ of every animal was suspended in 0.15 M potassium chloride in polypropylene containers, sealed with parafilm,labeled carefully, and stored at -20°C until assays were carried out.

Histopathology

The histological sections of the liver and kidney of mice were taken by adopting the procedure as described by Humason (1972). The tissues were isolated and gently rinsed with physiological saline solution (0.9% NaCl) to remove mucus and other debris adhering to them. They were fixed in Bourn's fluid (75 ml saturated aqueous picric acid, 25 ml 40% formaldehyde, and glacial acetic acid) for 24 hours.

The fixative was removed by washing through running tap water overnight. Then the tissues wereprocessed for dehydration. Ethyl alcohol was used as the dehydrating agent, as it is the most suitable and economical besides its hardening effect. The alcoholic transfer schedules were arranged to utilize dehydration and hardening effect. The tissues were passed through successive series containing 30%, 50%, 70%, 80%, 90%, 95%, and absolute alcohols. Then the tissues were cleaned in methyl benzoate and embedded in paraffin wax. Sections of 5u thickness were cut using "SIPCON" rotatory microtome. The sections were stained with Harris hematoxylin (Harris, 1900) and counter-stained with eosin, dissolved in 95% alcohol. After dehydration and cleaning, the sections were mounted in Canadian balsam. Photomicrographs of the section preparations were taken using Magnus photomicrography equipment.

Grouping of animals

Group	Dose	Route	Vehicle
Vehicle control	50mg/kg	Oral	(0.5%CMC)
Disease control–toxicity (2-NP + Ethanol 10%)	200µg/kg	I.P	(0.5%CMC)
Standard treatment	(Silymarin 50mg/kg)	Oral	(0.5%CMC)

Treatment group	curcumin 100mg/kg)	Oral	(0.5%CMC)
Treatment group	(Chrysin 50mg/kg)	Oral	(0.5%CMC)
Treatment group	(Curcumin 50mg/kg + chrysin 25 mg/kg)	Oral	(0.5%CMC)
Treatment group	curcumin 100mg/kg + chrysin 50 mg/kg)	Oral	(0.5%CMC)
Treatment group	(Curcumin 50mg/kg + chrysin 25 mg/kg)	Oral	(0.5%CMC)
Treatment group	(Curcumin 50mg/kg + Chrysin 50 mg/kg)	Oral	(0.5%CMC)

Table 8.2 Effect of Curcumin and Chrysin on liver enzymes of mice with 2-NP (4th week)

TITLE	ALT	ALP	AST	BILIRUBIN
Control	34.98 ± 1.20	20.88 ± 1.46	38.82 ± 2.04	0.1200 ± 0.013
2NP	76.82 ± 2.36	45.00 ± 1.86	83.32 ± 2.11	0.2960 ± 0.023
G1 Silymarin	38.04 ± 1.09	18.58 ± 0.73	37.52 ± 2.03	0.1960 ± 0.019
G2 curcumin 100	41.98 ± 1.75	25.50 ± 0.92	46.02 ± 1.86	0.1580 ± 0.015
G3 chrysin 50	41.16 ± 1.72	24.24 ± 0.74	39.70 ± 1.56	0.1700 ± 0.018
G4(cur100+chr25)	35.06 ± 1.45	20.82 ± 0.532	42.80 ± 1.74	0.1400 ± 0.007
G5(cur100+chr50)	41.70 ± 1.39	18.90 ± 0.52	38.76 ± 1.44	0.1340 ± 0.010
G6 (cur50+chr.25)	44.58 ± 1.85	24.46 ± 0.95	35.71 ± 1.51	0.1440 ± 0.006
G7 (cur50+chr50)	32.44 ± 1.61	20.66 ± 1.27	33.30 ± 1.792	0.1560 ± 0.013

The Enzymes showed that ALT, ALP, AST, and Bilirubin indicators of liver enzyme activity, and bilirubin were significantly elevated in 2-NP administered mice compared to controls, but the treatment ofmice with Curcumin 50mg/kg Chrysin 50mg/kg were and 2-NP caused those markers to significantlydecrease compared to 2-NP mice

Effect of Curcumin and Chrysin on liver enzymes of mice with 2-NP (8th week)

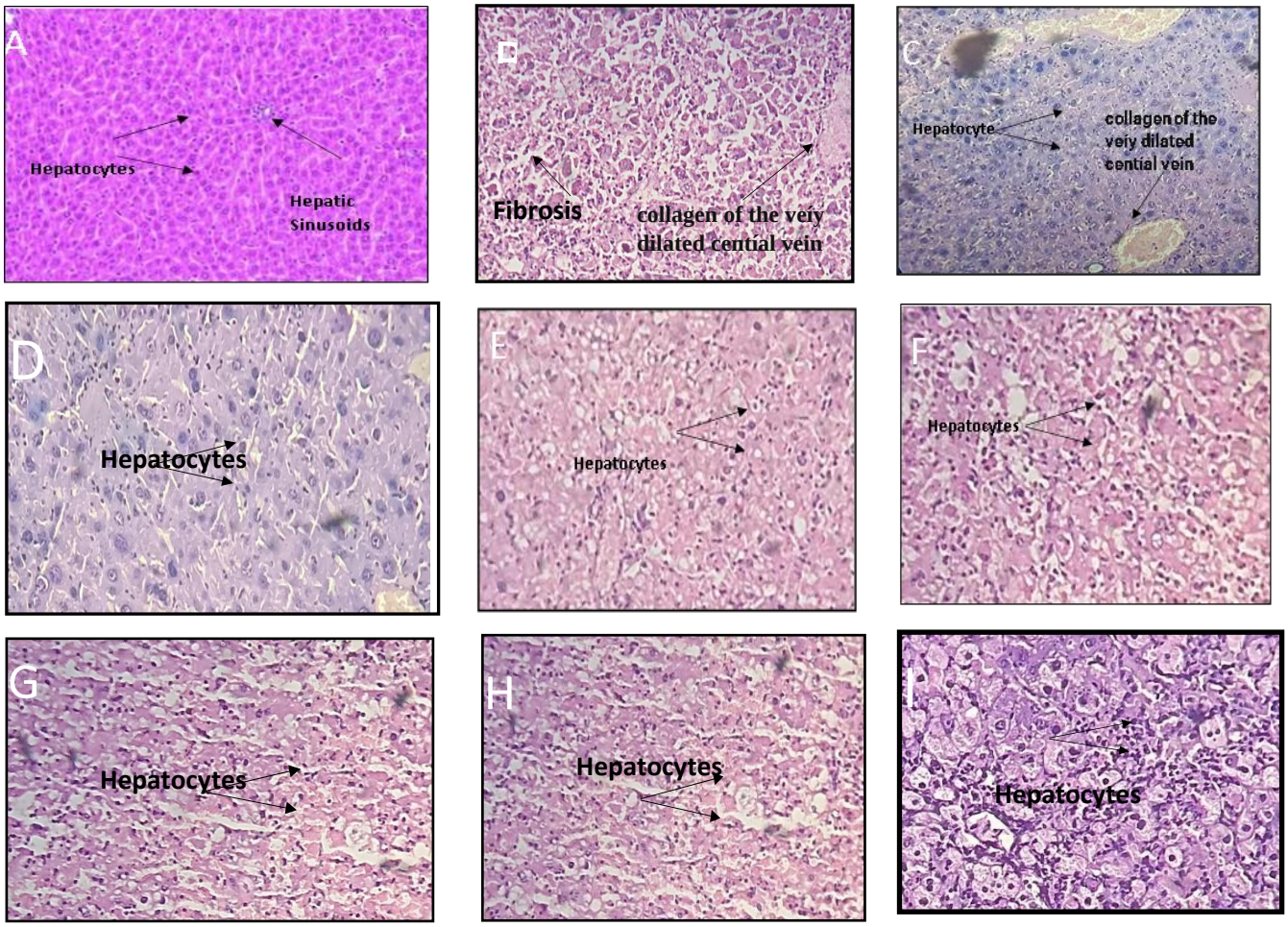
TITLE	ALT	ALP	AST	BILIRUBIN
Control	34.98 ± 1.20	20.88 ± 1.46	39.42 ± 1.19	0.1200 ± 0.013
2NP	146.8 ± 2.36	83.00 ± 1.97	157.3 ± 2.97	0.4960 ± 0.023
G1 curcumin 100	52.38 ± 1.16	35.50 ± 0.92	53.84 ± 1.24	0.2240 ± 0.015

G2 Silymarin	48.04 ± 1.09	28.58 ± 0.73	46.52 ± 1.05	0.1980 ± 0.007
G3 chrysin 50	51.16 ± 1.76	34.24 ± 0.74	57.22 ± 0.67	0.2120 ± 0.019
G4(cur100+chr25)	46.46 ± 0.81	30.82 ± 0.53	49.30 ± 1.50	0.1860 ± 0.005
G5(cur100+chr50)	36.44 ± 1.49	30.66 ± 1.27	52.80 ± 1.74	0.1940 ± 0.010
G6 (cur50+chr.25)	56.58 ± 0.81	34.46 ± 0.95	49.56 ± 0.94	0.1920 ± 0.011
G7 (cur50+chr50)	36.44 ± 1.49	20.50 ± 0.62	35.68 ± 1.17	0.1280 ± 0.012

The Enzymes showed that ALT, ALP, AST, and Bilirubin indicators of liver enzyme activity, and bilirubin were significantly elevated in 2-NP administered mice compared to controls, but the treatment ofmice with Curcumin 50mg/kg Chrysin 50mg/kg were and 2-NP caused those markers to significantlydecrease compared to 2-NP mice

In the 4th week and 8th weeks showed that ALT, ALP, AST, and Bilirubin indicators of liver enzymeactivity, and bilirubin were significantly elevated in 2-NP administered mice compared to controls, but the treatment of mice with Curcumin 50mg/kg Chrysin 50mg/kg were and 2-NP caused those markers to significantly decrease compared to 2-NP mice

This shows that this significant increase in the deposition of extracellular matrix in the 2NP group simultaneously result in Curcumin 50mg/kg Chrysin 50mg/kg group showing similar to the control group



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