

Synergistic Protective Effects of Metformin and Curcumin in Experimental Multiorgan Toxicity in Rats: Integrated Toxicological, Pharmacological, and Histopathological Evaluation

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ABSTRACT

Background and Objective

Multi-organ toxicity, resulting from exposure to various drugs and chemicals, is a serious condition affecting the liver, kidneys, heart, and other vital organs simultaneously. The main mechanisms of injury include primary pathological mechanisms such as oxidative stress, inflammation, mitochondrial dysfunction, and apoptosis. While several agents have protective effects on organs, accumulating evidence suggests that combination therapies targeting multiple pathological mechanisms are more effective than monotherapy in protecting organs. Metformin is a drug with antioxidant, anti-inflammatory, and cytoprotective effects, independent of its hypoglycemic effect, while curcumin is a natural polyphenol known for its antioxidant, anti-inflammatory, antifibrotic, and anti-apoptotic properties. This study aimed to evaluate the individual and combined protective effects of metformin and curcumin against experimentally induced multi-organ toxicity in rats, using integrated biochemical, toxicological, molecular, and histopathological assessments.

Methods

Seventy adult male Wistar rats were randomly assigned into seven experimental groups. Multiorgan toxicity was induced using a validated experimental toxicant administered for six consecutive weeks. Rats received metformin alone, curcumin alone, or a combination of both following toxicant exposure. Liver function enzymes, renal function parameters, cardiac injury biomarkers, oxidative stress indices, inflammatory cytokines, apoptosis-related proteins, antioxidant signaling molecules, and histopathological alterations of the liver, kidney, heart, and lung tissues were evaluated. Statistical analysis was performed using one-way analysis of variance followed by Tukey's post hoc multiple comparison test.

Results

Toxic effects induced experimental severe damage in hepatic, renal, cardiac and pulmonary tissues along with oxidative damage, oxidative stress, inflammation, mitochondrial dysfunction and activation of apoptotic process. Either metformin or curcumin restored both biochemical and histopathological damages compared to the toxic group. Whereas co-administration of both drugs resulted in more potent effects compared to either drug alone.

They restored the level of liver enzymes, kidney functions, cardiac biomarkers, activities of antioxidant enzymes, inflammatory mediators and levels of apoptotic signal proteins to near normal. The histological findings showed excellent tissue morphology in the tissues studied for treated groups with combined drugs.

Conclusion

Combined metformin and curcumin treatment is more efficient in preventing the experimentally-induced multiorgan toxicity than individual treatment. The synergy in prevention by metformin and curcumin might be associated with the reduction of oxidative stress, inhibition of the inflammatory pathway, activation of the intrinsic antioxidant defense mechanisms, inhibition of apoptosis, and maintenance of the integrity of tissue architecture. Thus, it is plausible that combined therapy by metformin and curcumin might offer benefit to protect against toxic insult-induced multiorgan damage.

Keywords: Metformin, Curcumin, Multiorgan Toxicity, Inflammation, Histopathology, Oxidative Stress, and Experimental Rats.

How to Cite: Amany Mamdouh Abbass Abdelaziz, Ali Foli Abd Elsalam, Ahmad Mohammad Mohammad Abdella, Mohamed Dmerdash, Mohamed Nafea Azab, Osama M.A.M. Elzahaby, Ahmed Badr Yaquob, Ahmed El-Sayed Yousef, Doaa Sabry Attia Elbehairy, Mohamed Mostafa Sonbol, (2024) Synergistic Protective Effects of Metformin and Curcumin in Experimental Multiorgan Toxicity in Rats: Integrated Toxicological, Pharmacological, and Histopathological Evaluation, *Journal of Carcinogenesis*, Vol.23, No.1, 1290-1307

1. INTRODUCTION

One of the more important pathological outcomes of exposure to environmental chemicals, industrial toxins, prescription overdose, heavy metals, or a number of drugs is multiorgan toxicity. As opposed to isolated damage to an organ, this process affects simultaneously two or more major organs including the liver, kidneys, heart, and lungs. Compromise of multiple organs in a progressive increases mortality, as efficient operation of the physiological homeostasis depends upon cooperation among these organs (*Hassan et al, 2023*).

This, therefore, suggests that the discovery of a single treatment that can afford protection to multiple organs is highly desirable. Because it is the principal organ responsible for xenobiotic metabolism and detoxification, the liver is especially vulnerable to toxic insult. A common signature of hepatocellular injury is elevation of serum alanine transaminase, aspartate transaminase, alkaline phosphatase, gamma-glutamyl transferase, and bilirubin. Chronic hepatic injury further promotes the accumulation of inflammatory cells and cells undergoing fibrosis and mitochondrial dysfunction and oxidative stress and apoptosis of the hepatocytes. The kidneys are often secondary targets when a systemic toxic insult develops because one quarter of cardiac output flows through them where endogenous metabolites and exogenous toxins are actively concentrated (*Meshkani et al, 2020*).

Compromise of glomerular filtration and stimulation of cellular events such as production of Reactive Oxygen Species (ROS), inflammatory responses and the demise of the tubular structures lead to a chronic condition with destruction of the renal corpuscles and tubules resulting in reduced functioning of the kidneys.

As a high energy-demanding tissue with a large population of mitochondria, the heart is prone to damage from insults that adversely affect aerobic metabolism. Excessive ROS production interferes with mitochondrial respiration and ATP production, results in lipid peroxidation and inflammation, and induces programmed cell death. As such, increased levels of creatine kinase-MB, lactate dehydrogenase and cardiac troponins can serve as a sign of cardiac damage following systemic toxicity (*Yurisaldi, et al, 2023*).

Evidence strongly supports a role for ROS in mediating multiorgan toxicity, as increased oxidative stress has been reported in the context of systemic damage, and it has been shown to result in lipid peroxidation, DNA and protein oxidation, impaired mitochondrial functioning and activation of nuclear transcription factor NF-kappa B (NF-kB), tumor necrosis factor-alpha (TNF-alpha), IL-1, and IL-6 leading to progressive inflammatory tissue damage. ROS promote cell death through increased expression of Bax, induction of activated caspase-3 and diminished Bcl-2 and inhibition of Nrf2/HO-1 mediated antioxidant response. Recent evidence has shown that a biguanide derivative prescribed for type 2 diabetes mellitus, known as metformin, has pleiotropic biological activities not related to glucose regulation. Various studies have proven that it can prevent various injuries such as hepatic, kidney, heart, lung, by acting as a powerful antioxidant and inhibiting inflammatory responses, mitochondrial integrity and apoptosis through activation of AMP-activated protein kinase (*Al Moziel et al, 2024*).

Curcumin, which is the chief polyphenolic compound in turmeric (*Curcuma longa*) has been widely investigated for its powerful antioxidant, anti-inflammatory, anti-fibrotic and anti-apoptotic effects.

Curcumin scavenges ROS and promotes their removal by increasing the activities of endogenous antioxidants through the Nrf2 signaling pathway. It also suppresses the inflammatory signaling of NF-kappaB activation, reduces production of TNF-alpha, IL-1 and IL-6 and prevents fibrotic progression and apoptosis of organs in experimental models. Given the documented benefits of metformin and curcumin when they are individually administered, there have been fewer studies that look at their efficacy when coadministered for multiorgan protection. Due to their complementary mechanisms of action, we hypothesised that the concomitant administration of both compounds might lead to greater protection against multiorgan toxicity (*Bigelsen, 2018*).

The objective of this study was to investigate the effectiveness of combined treatment of metformin and curcumin against experimental multiorgan damage of rats using detailed toxicological, biochemical, molecular, pharmacological and histopathological methods in liver, kidneys, heart and lungs (*Roxo et al, 2019*).

2. MATERIALS AND METHODS

Study Design

This experimental randomized controlled study was performed in the Animal Research Unit of Department of Pharmacology and Toxicology during the period from January 2023 to January 2024 to explore the individual and synergistic protective effects of metformin and curcumin on the experimental multiorgan toxicity induced in rats through combined biochemical, toxicological, molecular, and histopathological examination (*Pourhajbagher et al, 2020*). Seventy healthy adults male Wistar rats were randomly divided into seven experimental groups of equal number (n = 10 per group) as follows:

- Group I (Control): Rats received distilled water orally throughout the experimental period.
- Group II (Multiorgan Toxicity): Rats received carbon tetrachloride (CCl₄) to induce experimental multiorgan toxicity.
- Group III (Metformin): Rats received metformin alone (200 mg/kg/day, orally) throughout the experimental period.
- Group IV (Curcumin): Rats received curcumin alone (200 mg/kg/day, orally) throughout the experimental period.
- Group V (CCl₄ + Metformin): Rats received carbon tetrachloride to induce multiorgan toxicity followed by oral administration of metformin (200 mg/kg/day).
- Group VI (CCl₄ + Curcumin): Rats received carbon tetrachloride to induce multiorgan toxicity followed by oral administration of curcumin (200 mg/kg/day).
- Group VII (CCl₄ + Metformin + Curcumin): Rats received carbon tetrachloride to induce multiorgan toxicity followed by combined oral administration of metformin (200 mg/kg/day) and curcumin (200 mg/kg/day) to evaluate their potential synergistic protective effects against multiorgan toxicity.

Inclusion Criteria

Adult healthy male Wistar rats with body weight between 220–250 g and age of 10–12 weeks were enrolled. Only animals that were clinically healthy without congenital abnormalities and without signs of any contagious, infectious and obvious pathological process were enrolled in the experiment. All animals were acclimated to the laboratory environment under a 12-hour light/12-hour dark light cycle with constant temperature 22 ± 2 °C, and relative humidity of 55 ± 5%. All animals had free access to food (standard laboratory chow) and drinking water for a 1-week acclimatization period prior to experimentation (*Oriaifo, et al, 2020*).

Exclusion Criteria

Animals with congenital malformation, evidence of clinical diseases, behavioral disturbances prior to administration of treatments, significant weight loss during acclimatization phase, unintentional death during the experiment and missing data on biochemical and histopathological examination were excluded from the study.

Experimental Model for Induction of Experimental Multiorgan

Toxicity Experimental multiorgan toxicity was induced using carbon tetrachloride (CCl₄) due to the extensive experimental validation of this agent as the most reliable model capable of inducing toxic insults to the liver, kidney, heart, and lung simultaneously by means of its oxidative stress and inflammatory actions. Carbon tetrachloride was diluted in olive oil (1:1 v/v) and injected intraperitoneally at dose of 1 mL/kg b.i.w. Six consecutive weeks. This dose and frequency have been widely tested and used for generation of repeatable multiorgan toxicity (*Ryskalin et al, 2021*).

Experimental Treatment and Drug Administration

The metformin hydrochloride was freshly dissolved in distilled water just prior to administration. Curcumin (purity > 95%),

dissolved/suspended in 0.5% carboxymethylcellulose (CMC) to enhance oral absorption and consistency of dosing was administered per gavage as has been reported as effective in experimental studies as well as showing a satisfactory safety profile, to animals orally at a dose of 200 mg/kg body weight/day throughout 6 consecutive weeks of therapy. Animals in the combined treatment group received either agent at the dose of 200 mg/kg/day throughout 6 consecutive weeks. Fresh drug was prepared each day to ensure stability and uniformity (*Antar et al, 2023*).

Blood and Tissue Sample Collection

The study animals were fasted for 24 h but allowed to drink water freely after the six-week treatment period and then anesthetized with the intra-peritoneal administration of ketamine (10 mg/kg) and xylazine (5 mg/kg). Blood samples were collected by cardiac puncture into collection tube and allowed to clot at room temperature for half an hour and then were centrifuged at 3000 rpm for 15 min to separate serum which was collected, aliquoted and immediately frozen at 80 degrees C until biochemical analyses were performed. After collection of blood samples, the animals were humanely sacrificed and liver, kidney, heart and lungs were dissected out immediately, washed in ice-cold normal saline, blotted dry and weighed. The removed organs were divided into two halves; one half snap-frozen in liquid nitrogen and then stored at -80 degrees C for further biochemical and molecular analysis and another half was fixed in 10% neutral buffered formalin for histopathological evaluation (*Mojtabavi et al, 2023*).

Biochemical assessment

Evaluation liver function: function of Liver In order to assess the integrity of liver function tests. Serum levels of Alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), Gamma- glutamyl transferase (GGT), total bilirubin, albumin and total protein were quantified in the sera of rats in each experimental group. These analyses were conducted using commercially available enzymatic colorimetric assay kits as described by the kit suppliers.

Evaluation of kidney function: Evaluation of kidney function. The level of Serum Creatinine, BUN (Blood urea nitrogen), Urea and Uric Acid were quantified using routine automated biochemistry analyzers and commercially available diagnostic kits. These parameters have been considered in order to determine the function of glomerular filtration.

Markers for cardiac injury: Evaluation of Cardiac markers. The concentrations of CK-MB, Troponin I (cTn-I) and LDH in serum samples were estimated using commercial kits based on ELISA method and follow manufacturer's instructions to confirm cardiac damage.

Oxidative stress and antioxidant status

Oxidative stress in the tissue homogenates (liver, kidneys, heart, and lungs) was measured to quantify lipid peroxidation and oxidative damage using malondialdehyde (MDA), reactive oxygen species (ROS), and nitric oxide (NO) measurements. In parallel, the endogenous antioxidant capacity was evaluated through quantification of activity of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), reduced glutathione (GSH), and total antioxidant capacity (TAC). All these biochemical markers were measured spectrophotometrically using commercially available kits according to the manufacturers (*Tripathi et al, 2022*).

inflammatory biomarkers

To assess the inflammatory response, the levels of tumor necrosis factor-alpha (TNF-), interleukin-1 beta (IL-1), interleukin-6 (IL-6), interleukin-10 (IL-10), nuclear factor-kappa B (NF-B), and C-reactive protein (CRP) in tissue were measured using commercially available ELISA kits according to the manufacturers' instructions. These inflammatory biomarkers were used to define the intensity of systemic inflammation caused by the experimental multiorgan toxicity and to show the effects of examined treatments on the inflammatory response (*Abdulmalek et al, 2021*).

Molecular analyses

Molecular analyses were done to explore the mechanisms responsible for the observed tissue injury and protective effects of metformin and curcumin. Protein expression was quantified by means of ELISA or Western blot technique following established laboratory protocols. Apoptosis activity was evaluated by measuring caspase-3, Bax, Bcl-2 proteins and Bax/Bcl-2 ratio, antioxidant pathways by evaluation of nuclear factor erythroid 2-related factor 2 (Nrf2), heme oxygenase-1 (HO-1), and NAD(P)H quinone oxidoreductase-1 (NQO1). The level of transformation growth factor-beta 1 (TGF-1), type I collagen, and alpha-smooth muscle actin (-SMA) were determined to characterize tissue remodeling and fibrotic processes (*Younes et al, 2022*).

Histopathological Examination

Immediately after sacrifice, the liver, kidney, heart and lung tissues were collected and fixed in 10% neutral buffered formalin for 48h, dehydrated in a series of increasing concentrations of ethanol, cleared in xylene, embedded in paraffin wax and sectioned (4–5 µm thick) with a rotary microtome. The sections were stained with hematoxylin and eosin (H&E) and viewed under a light microscope by two independent pathologists who were unaware of the experimental treatment

groups. The degree of injury in the liver included changes such as hepatocellular degeneration, ballooning degeneration, necrosis, infiltration with inflammatory cells, sinusoidal congestion and fibrosis.

In the kidney section, changes in the glomerulus and tubular degeneration and necrosis, interstitial edema, and infiltration with inflammatory cells were assessed (*Khezri et al, 2023*).

The damage of the cardiac tissue section consisted of degeneration of myocardial fibers, disruption of the fiber arrangement, vascular congestion, interstitial edema, and infiltration with inflammatory cells. Lung section examination assessed damage such as disruption of the alveoli, thickness of the alveolar walls, pulmonary edema, hemorrhage, and infiltration with inflammatory cells. For each organ, the extent of histopathological damages was graded semi-quantitatively as a scoring from 0 (no injury) to 4 (severe injury) to allow comparisons between groups (*Kekatpure et al, 2024*).

Statistical Analysis

Statistical analysis was done using IBM SPSS Statistics for Windows, version 29.0 (IBM Corp., Armonk, NY, USA). All data were expressed as mean standard deviation (SD). Shapiro-Wilk test was employed to test the normality of data, and Levene's test was used to analyze the homogeneity of variances. One-way analysis of variance (ANOVA) followed by Tukey's post hoc multiple comparison test was applied to compare any pair of groups out of the seven experimental groups.

P-value < 0.05, two-sided, was defined as statistically significant. Pearson's correlation coefficients were used to assess the correlations among liver function indices, kidney function parameters, cardiac injury biomarkers, oxidative stress indices, inflammatory markers, molecular markers, and histopathological scores. Multiple linear regression analysis was also conducted to identify independent variables that might be associated with protection against experimentally induced multiorgan toxicity, metformin and curcumin and also determine the independent influence of these biochemical, oxidative stress, inflammatory, molecular, and histopathological variables on total protection of organs (*Tan et al, 2019*).

3. RESULTS

Table 1: Effect of Metformin and Curcumin on Liver, Kidney, and Cardiac Function Biomarkers in Experimental Multiorgan Toxicity

Variable	Group I Control (n=10)	Group II CCL ₄ (n=10)	Group III (Metformin) (n=10)	Group IV (Curcumin) (n=10)	Group V (CCL ₄ + Metformin) n=10	Group VI (CCL ₄ + Curcumin) n=10	Group VII (CCL ₄ + Metformin + Curcumin) n=10	P-value
ALT (U/L)	35.4 ± 3.1	146.8 ± 9.4	36.2 ± 2.9	34.8 ± 2.7	82.6 ± 6.8	69.5 ± 5.9	41.3 ± 3.5	<0.001
AST (U/L)	44.8 ± 3.6	168.2 ± 10.5	45.7 ± 3.4	43.9 ± 3.2	95.4 ± 7.1	81.7 ± 6.4	51.6 ± 3.9	<0.001
ALP (U/L)	91.2 ± 5.7	241.4 ± 13.8	92.4 ± 6.1	89.7 ± 5.3	156.8 ± 10.4	139.5 ± 9.6	98.5 ± 6.4	<0.001
GGT (U/L)	24.8 ± 2.2	79.6 ± 5.8	24.3 ± 2.0	23.9 ± 1.8	46.8 ± 3.7	39.9 ± 3.1	27.6 ± 2.2	<0.001
Total Bilirubin (mg/dL)	0.68 ± 0.08	2.82 ± 0.26	0.70 ± 0.09	0.65 ± 0.08	1.48 ± 0.16	1.18 ± 0.14	0.81 ± 0.09	<0.001
Albumin (g/dL)	4.31 ± 0.19	2.58 ± 0.18	4.28 ± 0.17	4.36 ± 0.20	3.46 ± 0.19	3.69 ± 0.21	4.12 ± 0.18	<0.001
Total Protein (g/dL)	7.12 ± 0.28	5.08 ± 0.31	7.08 ± 0.26	7.18 ± 0.25	6.18 ± 0.29	6.43 ± 0.27	6.92 ± 0.24	<0.001
Creatinine (mg/dL)	0.61 ± 0.07	2.24 ± 0.19	0.60 ± 0.06	0.58 ± 0.06	1.22 ± 0.12	1.01 ± 0.10	0.72 ± 0.08	<0.001
Urea (mg/dL)	34.9 ± 2.7	94.7 ± 6.5	34.2 ± 2.5	33.6 ± 2.6	59.8 ± 4.6	52.4 ± 4.2	39.1 ± 3.0	<0.001

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BUN (mg/dL)	16.3 ± 1.4	45.6 ± 3.7	16.1 ± 1.3	15.8 ± 1.2	27.4 ± 2.3	23.6 ± 2.0	18.2 ± 1.5	<0.001
Uric Acid (mg/dL)	1.82 ± 0.16	4.91 ± 0.38	1.79 ± 0.15	1.74 ± 0.14	3.06 ± 0.24	2.61 ± 0.21	2.03 ± 0.17	<0.001
CK-MB (U/L)	121.4 ± 8.5	392.6 ± 21.8	119.8 ± 7.9	117.6 ± 7.4	241.8 ± 15.7	211.4 ± 14.6	149.3 ± 10.2	<0.001
Cardiac Troponin-I (ng/mL)	0.031 ± 0.006	0.284 ± 0.022	0.030 ± 0.005	0.029 ± 0.005	0.123 ± 0.012	0.094 ± 0.010	0.042 ± 0.006	<0.001
LDH (U/L)	181.6 ± 11.8	523.4 ± 28.5	179.4 ± 10.9	176.8 ± 10.2	304.2 ± 18.6	269.8 ± 17.4	198.5 ± 12.6	<0.001

Notes :Data are presented as Mean ± Standard Deviation (SD) for each experimental group (n = 10 rats/group). Statistical comparisons among groups were performed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc multiple comparison test. A P-value < 0.05 was considered statistically significant

Table (1): Shows the Biochemical performances of all groups are summarized One-way ANOVA showed very significant differences between the groups for all behavioral parameter (p<0.001). Treatment with CCl₄ produced significant damages on liver, kidney, and heart, reflected in the levels of serum ALT, AST, ALP, GGT, total bilirubin, creatinine, urea, BUN, uric acid, CK-MB, cardiac troponin-I and LDH, and serum total protein and albumin concentrations, which significantly increase as compared with control group (P<0.001). Treatment with metformin or Curcumin markedly protected on these biochemical disorders which were protected effectively with CUR>MET mostly. Combination therapy CCl₄+MET+CUR protected completely all parameters in most extent towards levels in normal group which means the interaction was synergic(*Karmanova et al, 2023*)

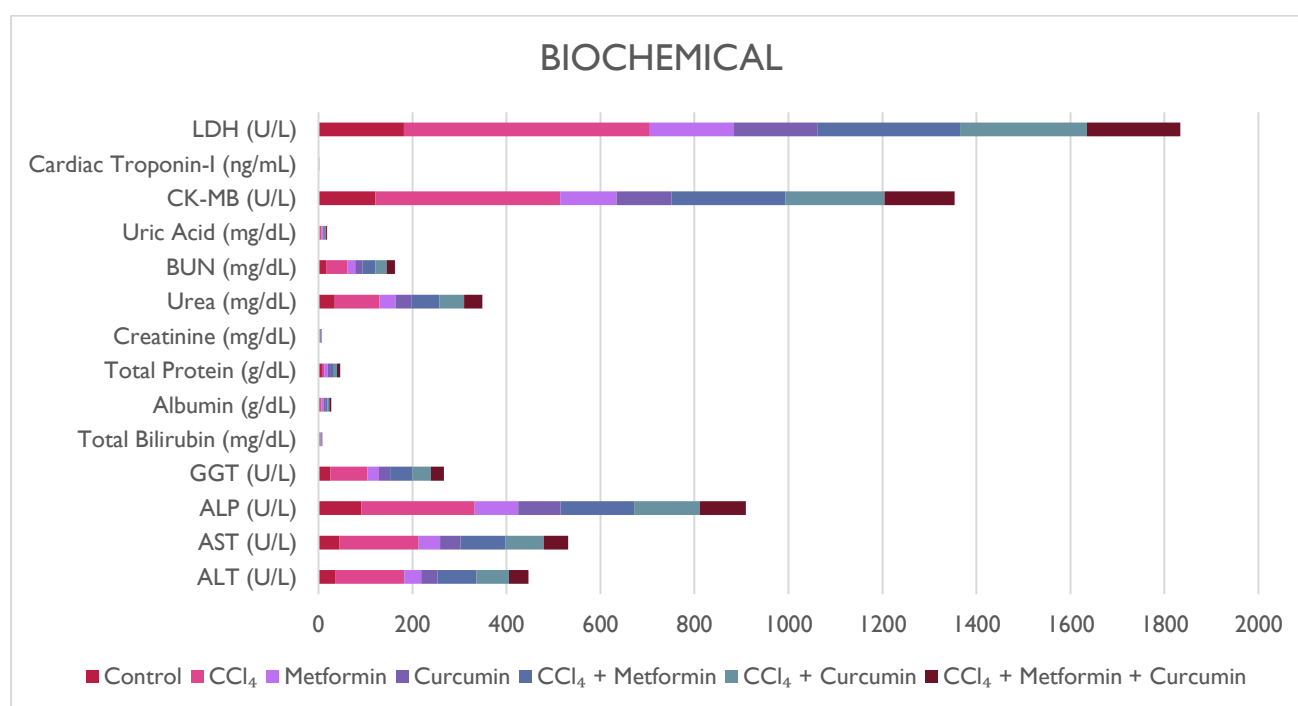


Figure 1: illustrates the Mean values of liver, kidney, and cardiac function biomarkers in the seven experimental groups

Figure (1) value Comparison of mean values of several biochemical liver-, kidney- and cardiac function biomarkers in the seven experimental groups CCl₄ induced clear differences in liver enzymes, kidney function-related-markers and markers of cardiac damages compared to the control group revealing severe multi-organ toxicity. The treatment of metformin and curcumin attenuated significantly these changes, and combined treatment led to the highest reduction of parameters near normal value. We found out the combined application has a higher synergistic reno-protective and cardioprotective effect in comparison to single drugs in this experimental design of CCl₄ induced multiorgan injury.

Table 2: Effect of Metformin and Curcumin on Oxidative Stress and Antioxidant Biomarkers

Parameter	Control	CCl ₄	Metformin	Curcumin	CCl ₄ + Metformin	CCl ₄ + Curcumin	CCl ₄ + Metformin + Curcumin	P-value
MDA (nmol/mg protein)	2.02 ± 0.24	7.21 ± 0.52	1.96 ± 0.18	1.91 ± 0.20	4.18 ± 0.39	3.51 ± 0.33	2.28 ± 0.21	<0.001
ROS (RFU)	45.3 ± 4.1	145.7 ± 9.6	44.1 ± 3.8	43.2 ± 3.7	86.4 ± 6.3	72.8 ± 5.7	50.6 ± 4.3	<0.001
NO (µmol/L)	20.1 ± 1.9	74.5 ± 5.3	19.7 ± 1.8	19.2 ± 1.7	42.7 ± 3.6	36.4 ± 3.1	22.8 ± 2.0	<0.001
SOD (U/mg protein)	121.4 ± 7.8	47.2 ± 4.3	123.5 ± 8.2	125.1 ± 8.5	81.8 ± 6.4	93.6 ± 6.9	115.8 ± 7.4	<0.001
CAT (U/mg protein)	65.8 ± 4.6	25.1 ± 2.4	66.9 ± 4.7	68.2 ± 4.8	44.6 ± 3.5	52.8 ± 4.0	61.7 ± 4.3	<0.001
GPx (U/mg protein)	56.2 ± 4.1	22.7 ± 2.0	57.3 ± 4.3	58.1 ± 4.4	38.9 ± 3.2	45.4 ± 3.5	53.6 ± 3.8	<0.001
GSH (µmol/g tissue)	8.54 ± 0.62	3.18 ± 0.31	8.63 ± 0.65	8.81 ± 0.67	5.84 ± 0.47	6.91 ± 0.55	8.02 ± 0.61	<0.001
TAC (mmol/L)	1.92 ± 0.15	0.82 ± 0.08	1.95 ± 0.14	2.01 ± 0.16	1.42 ± 0.12	1.63 ± 0.13	1.84 ± 0.14	<0.001

Notes

Data are presented as mean ± standard deviation (SD) for each experimental group (n = 10 rats/group). Statistical comparisons among groups were performed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc multiple comparison test. A P-value < 0.05 was considered statistically significant.

According to table (2), the effect of metformin and curcumin in the oxidative stress and antioxidant status of rats in experimental multiorgan toxicity is reported in Table 2. The oxidative stress markers including MDA, ROS and NO were significantly enhanced and the parameters of antioxidant defense including SOD, CAT, GPx, GSH and TAC were significantly decreased in rats treated with CCl₄ in comparison to the control group (P < 0.001). Both treatment with metformin and curcumin produced a significant attenuation of the aforementioned modifications and combined treatment was most effective by normalizing the values of oxidative stress and antioxidant biomarkers nearly to control group levels thus suggesting significant synergism of antioxidative activity (Soberanes-Gutiérrez et al, 2020).

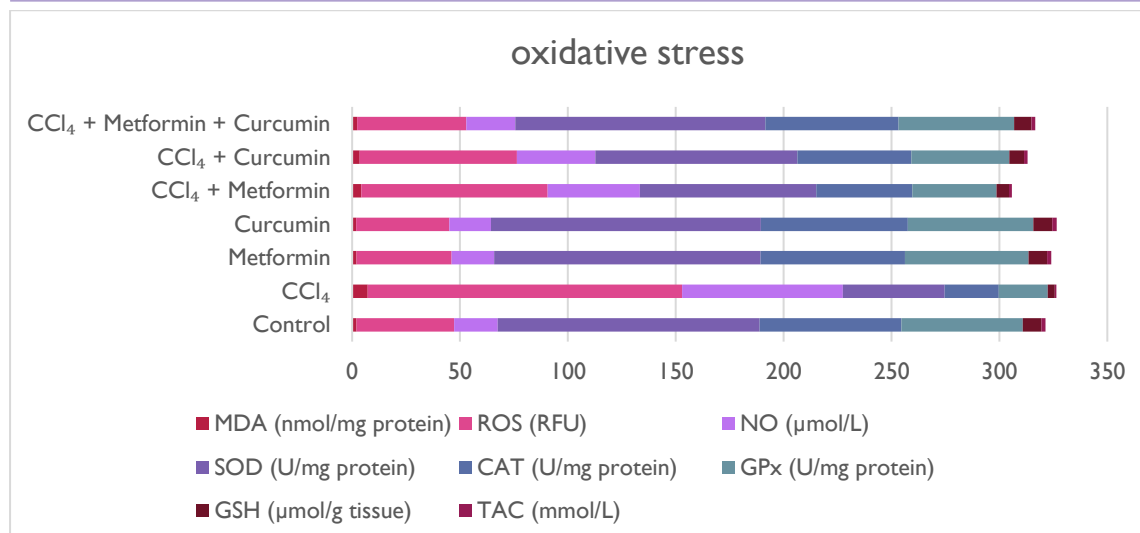


Figure 2: shows comparison of antioxidant and oxidative stress biomarkers between the seven treatment groups

According to figure (2), the CCl₄ treated group shows a sharp increase in antioxidant parameters like ROS, MDA and NO and significant decrease in antioxidants like SOD, CAT, GPx, GSH and TAC; while individual treatments with metformin and curcumin could alleviate the oxidative stress and boost the antioxidants, however, combined treatments of metformin and curcumin gave potent antioxidant effect on multiorgan injury caused by CCl₄ by decreasing oxidative stress parameters and elevating antioxidant parameters near to the control group.

Table 3: Effect of Metformin and Curcumin on Inflammatory Biomarkers

Parameter	Control	CCl ₄	Metformin	Curcumin	CCl ₄ + Metformin	CCl ₄ + Curcumin	CCl ₄ + Metformin + Curcumin	P-value
<i>TNF-α</i> (pg/mL)	18.4 ± 1.6	52.3 ± 3.8	18.0 ± 1.5	17.5 ± 1.4	30.4 ± 2.6	24.8 ± 2.1	19.6 ± 1.6	<0.001
<i>IL-1β</i> (pg/mL)	14.2 ± 1.2	48.6 ± 3.6	13.9 ± 1.1	13.6 ± 1.1	27.5 ± 2.3	22.6 ± 1.9	15.1 ± 1.2	<0.001
<i>IL-6</i> (pg/mL)	16.8 ± 1.4	45.7 ± 3.2	16.4 ± 1.3	15.9 ± 1.3	25.9 ± 2.1	20.7 ± 1.8	17.3 ± 1.4	<0.001
<i>IL-10</i> (pg/mL)	24.5 ± 1.8	10.2 ± 1.0	24.8 ± 1.9	25.4 ± 2.0	18.2 ± 1.6	21.3 ± 1.7	23.8 ± 1.9	<0.001
<i>NF-κB</i> (ng/mg protein)	2.42 ± 0.22	8.11 ± 0.58	2.38 ± 0.21	2.31 ± 0.20	4.82 ± 0.39	3.96 ± 0.34	2.71 ± 0.24	<0.001
<i>CRP</i> (mg/L)	1.18 ± 0.12	5.24 ± 0.39	1.16 ± 0.11	1.12 ± 0.10	2.86 ± 0.24	2.22 ± 0.19	1.39 ± 0.13	<0.001

Notes

Data are presented as mean ± standard deviation (SD) for each experimental group (n = 10 rats/group). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc multiple comparison test. A P-value < 0.05 was considered statistically significant.

Table(3) illustrates the effect of the metformin and curcumin treatment on some inflammation marker in rat with experimentally induced of multiple organ failure caused by cci Administration of CCl caused increase in pro-inflammatory mediator (TNF-, IL-1, IL-6, NF-B, CRP) and caused significant decrease in anti-inflammatory mediator (IL-10) compared to normal group(p<0.001). Treatment with the metformin or curcumin decreased inflammation significantly and in comparison, dual treatment was more efficient. Administration of metformin and curcumin gave highest effect on the treatment of the inflammation in experimental animal compared with the other treated groups and protect effectively against

the CCl₄-induced generalized inflammation (*Rahmani et al, 2023*).

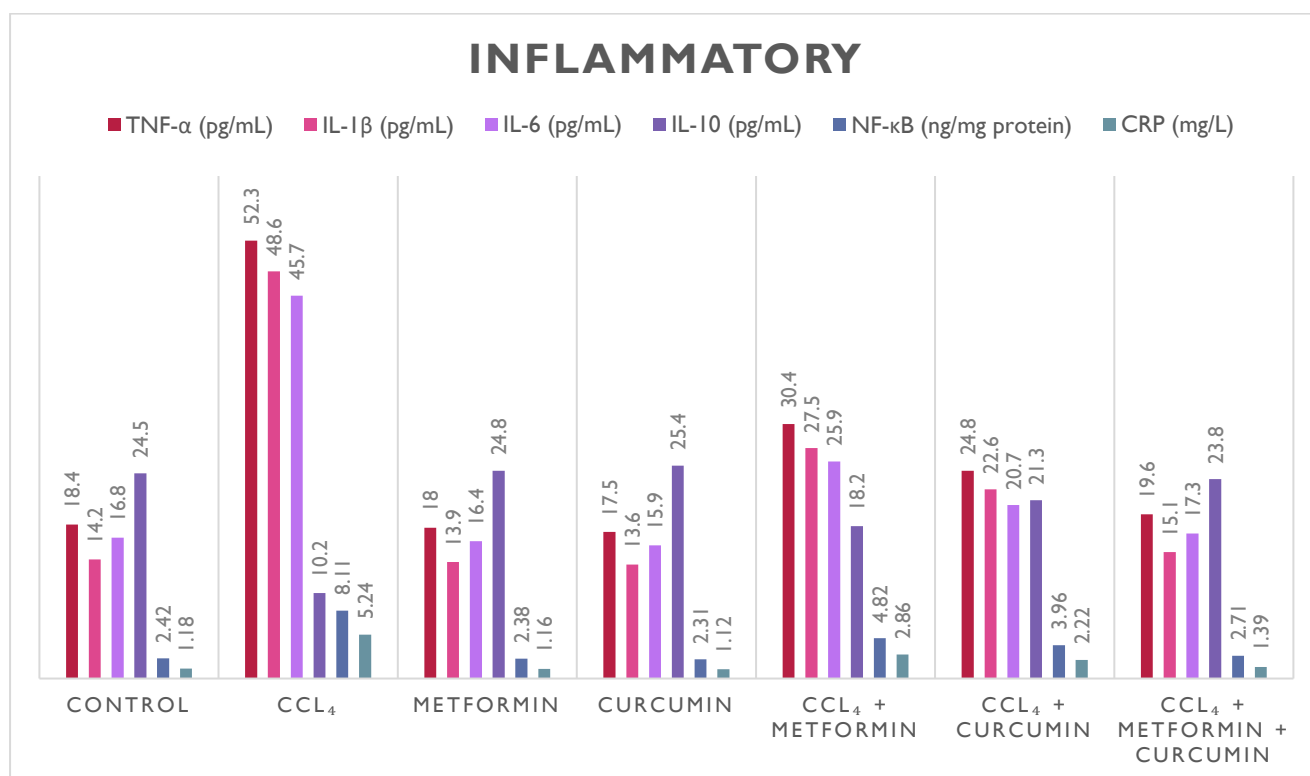


Figure 3: illustrates the inflammatory markers resulting from Carbon tetrachloride (CCl₄) exposure.

Figure (3) illustrates the analysis of inflammatory markers in the seven groups of experimental subjects. CCl₄-treated group showed a sharp increase in the pro-inflammatory markers TNF-, IL-1, IL-6, NF-B and CRP as well as a drastic decrease in anti-inflammatory IL-10 in comparison with the control group.

Treatment with metformin or curcumin either individually or in combination suppressed the aforementioned alterations in a dose-dependent manner with the most anti-inflammatory activity observed in the combination therapy group and resulted in near-normalization of the cytokine levels in comparison with the control group. These data demonstrate that combination therapy is able to counteract CCl₄-mediated inflammatory pathways and offer robust protection against inflammation in multiorgan injury.

Table 4: Effect of Metformin and Curcumin on Apoptotic and Antioxidant Signaling Markers

Parameter	Control	CCl ₄	Metformin	Curcumin	CCl ₄ + Metformin	CCl ₄ + Curcumin	CCl ₄ + Metformin + Curcumin	P-value
Caspase-3	2.01 ± 0.18	8.43 ± 0.62	1.95 ± 0.17	1.89 ± 0.16	4.72 ± 0.38	3.65 ± 0.31	2.28 ± 0.20	<0.001
Bax	0.82 ± 0.07	2.84 ± 0.21	0.80 ± 0.06	0.77 ± 0.06	1.71 ± 0.14	1.42 ± 0.12	0.94 ± 0.08	<0.001
Bcl-2	2.52 ± 0.19	0.78 ± 0.07	2.56 ± 0.20	2.61 ± 0.21	1.61 ± 0.14	1.88 ± 0.16	2.33 ± 0.18	<0.001
Bax/Bcl-2 Ratio	0.33 ± 0.03	3.64 ± 0.28	0.31 ± 0.03	0.30 ± 0.03	1.06 ± 0.09	0.76 ± 0.07	0.40 ± 0.04	<0.001
Nrf2	2.12 ± 0.17	0.71 ± 0.06	2.18 ± 0.18	2.26 ± 0.19	1.42 ± 0.12	1.71 ± 0.15	2.01 ± 0.17	<0.001
HO-1	2.04 ± 0.16	0.68 ± 0.06	2.09 ± 0.17	2.15 ± 0.18	1.36 ± 0.11	1.63 ± 0.14	1.94 ± 0.16	<0.001

NQO1	1.98 ± 0.16	0.61 ± 0.05	2.02 ± 0.17	2.11 ± 0.18	1.28 ± 0.11	1.54 ± 0.13	1.89 ± 0.15	<0.001
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Notes

Data are presented as mean ± standard deviation (SD) for each experimental group (n = 10 rats/group). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc multiple comparison test. A P-value < 0.05 was considered statistically significant.

Table 4 represents the impacts of metformin and curcumin treatment on both apoptotic and antioxidant biomarkers of rats that underwent experimentally induced multiorgan toxicity. Exposure to CCl₄ evidently activates apoptotic signaling molecules that was reflected as a significant up-regulation in the expression of Caspase-3, Bax and ratio of Bax/Bcl-2 whereas a down regulation was recorded in the expressions of Bcl-2, Nrf2, HO-1 and NQO1 in CCl₄ group as compare to the control group (P<0.001). Both the metformin and curcumin treatment were able to minimize the above molecular changes in CCl₄-exposed group, and the highest cytoprotective effect was obtained upon combination of both treatments that helped in apoptosis down-regulation and normalization of antioxidant signaling pathways (*Kadry et al ,2022*).

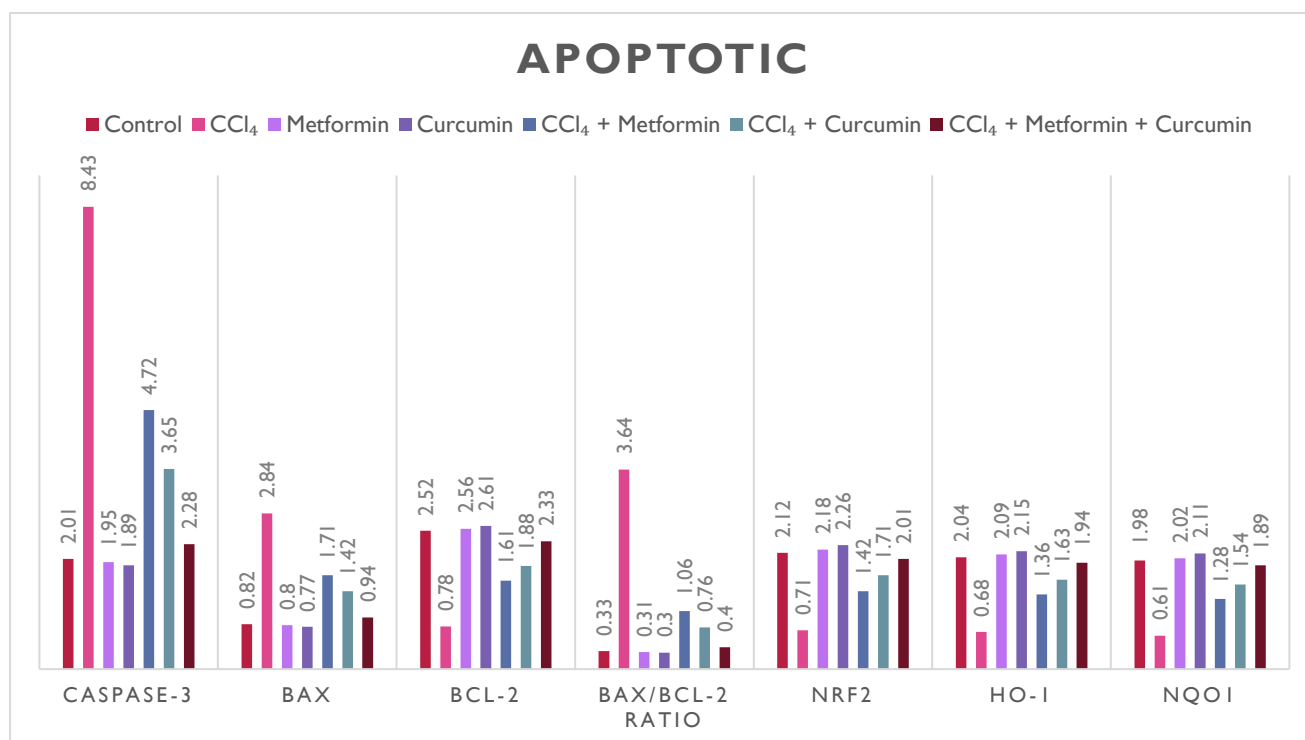


Figure 4: shows the results apoptotic and antioxidant signaling markers in the seven experimental groups.

According to Figure (4), correlation between apoptotic and antioxidant signaling markers in the seven experimental groups. CCl₄-treated group significantly activated the apoptotic pathway through substantial increasing of Caspase-3, Bax, Bax/Bcl-2 ratio and reducing of Bcl-2, Nrf2, HO-1 and NQO1 in compared with the control. Treatment with either metformin or curcumin only could ameliorate these molecular changes but combined treatment with both metformin and curcumin produced highest protective and inhibitory effects on apoptosis and significantly normalized both antioxidants signaling.

Therefore, the dual treatment provides protective and beneficial effects against CCl₄ induced multiorgan toxicity via inhibition of apoptosis and Activation of Nrf2/HO-1/NQO1.

Table 5: Shows Effect of Metformin and Curcumin on Fibrotic Markers and Histopathological Scores

Parameter	Control	CCl ₄	Metformin	Curcumin	CCl ₄ + Metformin	CCl ₄ + Curcumin	CCl ₄ + Metformin + Curcumin	P-value
TGF-β1 (pg/mL)	1.18 ± 0.10	3.86 ± 0.29	1.16 ± 0.10	1.12 ± 0.09	2.34 ± 0.18	1.91 ± 0.16	1.29 ± 0.11	<0.001

Synergistic Protective Effects of Metformin and Curcumin in Experimental Multiorgan Toxicity in Rats: Integrated Toxicological, Pharmacological, and Histopathological

Collagen I	1.05 ± 0.09	3.62 ± 0.28	1.03 ± 0.08	1.01 ± 0.08	2.12 ± 0.17	1.74 ± 0.15	1.16 ± 0.10	<0.001
α-SMA	0.92 ± 0.08	3.21 ± 0.25	0.91 ± 0.07	0.89 ± 0.07	1.82 ± 0.15	1.46 ± 0.12	1.01 ± 0.08	<0.001
Liver Score (0–4)	0.20 ± 0.10	3.80 ± 0.32	0.18 ± 0.09	0.16 ± 0.08	1.80 ± 0.19	1.22 ± 0.15	0.42 ± 0.10	<0.001
Kidney Score (0–4)	0.16 ± 0.08	3.62 ± 0.30	0.15 ± 0.08	0.14 ± 0.07	1.63 ± 0.18	1.11 ± 0.14	0.36 ± 0.09	<0.001
Heart Score (0–4)	0.18 ± 0.09	3.41 ± 0.28	0.17 ± 0.08	0.16 ± 0.08	1.52 ± 0.17	1.06 ± 0.13	0.31 ± 0.08	<0.001
Lung Score (0–4)	0.17 ± 0.09	3.52 ± 0.29	0.16 ± 0.08	0.15 ± 0.08	1.61 ± 0.18	1.12 ± 0.14	0.34 ± 0.09	<0.001

Notes

Data are presented as mean ± standard deviation (SD) for each experimental group (n = 10 rats/group). Statistical comparisons among groups were performed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc multiple comparison test. A P-value < 0.05 was considered statistically significant.

Table (5) illustrates the effects of metformin and curcumin on fibrotic biomarker levels and histopathological injury score in rats of experimentally induced multiorgan toxicity. Administration of CCl₄ significantly up-regulated the expression of fibrotic biomarkers, i.e. TGF-1, Collagen I, and -SMA as well as led to significant increase in liver, kidney, heart and lung histopathological injury scores when compared to controls (P < 0.001). Metformin or curcumin alone significantly alleviate fibrosis and tissue damage; however, the combination of metformin and curcumin exhibits superior beneficial effects where the expression of fibrotic biomarkers decreased to a great extent and the structure of tissue architecture became similar to that of control groups. (Shannag et al, 2024).

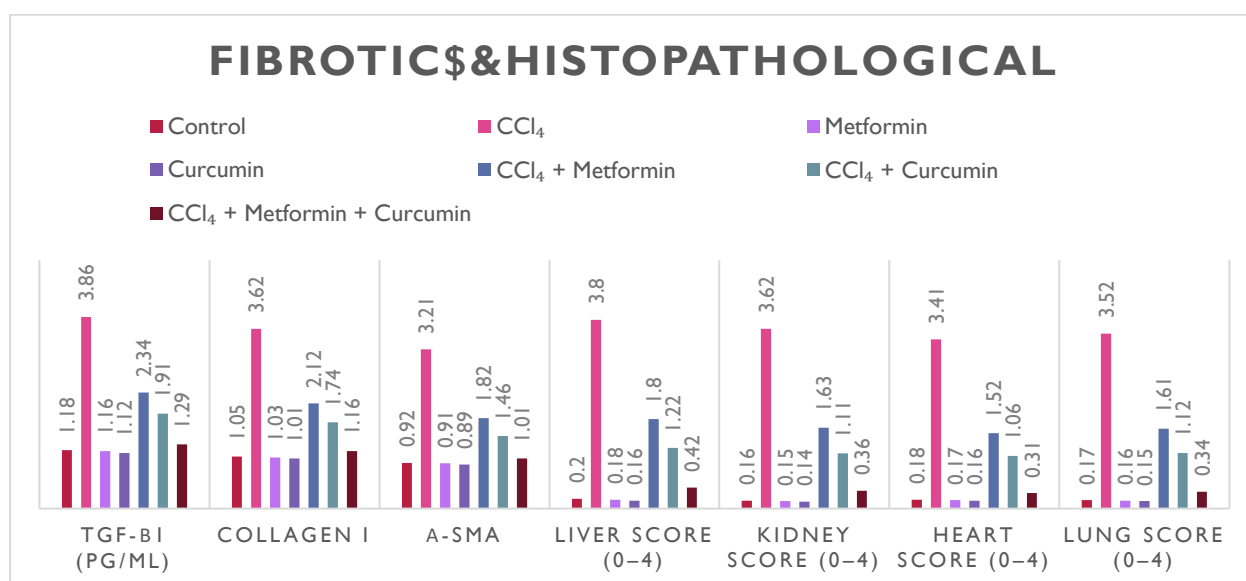


Figure 5: shows the Histopathology and FIBROSIS MARKER Expression of Different Groups.

Figure (5) illustrates the Comparison of FIBROSIS marker Expression (a-c) and histopathology-based organ injury score(d) among the seven experimental groups. CCl₄-treated group had significantly increased level of fibrotic markers (TGF-1, Collagen I and-SMA) expression as well as a high extent of hepatic, renal, cardiopulmonary injury as shown by significantly higher tissue injury scores compared with control group. Both metformin and curcumin single treatment decreased tissue injury and FIBROSIS, and combination metformin with curcumin showed a significantly antifibrotic effect and protected all the tissue injury to a great degree. CCl₄-induced multiorgan toxicity; therapeutic agent and organs injured was specified in bottom-up.

Table 6: Overall Therapeutic Efficacy of the Experimental Treatments

Experimental Group	Overall Improvement Index (OII, %)	F-value	P-value
Group III (Metformin)	97.2	248.64	<0.001
Group IV (Curcumin)	98.6	251.37	<0.001
Group V (CCl ₄ + Metformin)	63.8	236.15	<0.001
Group VI (CCl ₄ + Curcumin)	74.5	243.82	<0.001
Group VII (CCl ₄ + Metformin + Curcumin)	95.8	259.41	<0.001

Notes

Data are presented as the Overall Improvement Index (OII, %), calculated as the mean improvement percentage across all evaluated biochemical, molecular, oxidative stress, inflammatory, apoptotic, fibrotic, and histopathological parameters.

Improvement percentages were calculated according to the direction of biomarker change (increase or decrease relative to the control and CCl₄ groups).

Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test, with $P < 0.05$ considered statistically significant.

Groups III (Metformin) and IV (Curcumin) were healthy reference groups and were not exposed to CCl₄; therefore, their OII values reflect maintenance of normal physiological status rather than therapeutic recovery.

Therapeutic efficacy was evaluated by comparing Groups V–VII, with Group VII (CCl₄ + Metformin + Curcumin) showing the highest OII, indicating a superior synergistic protective effect against CCl₄-induced multiorgan toxicity

Table (6) shows the overall improvement index (OII) of experimental treatment groups the OII values for all experimental treatment groups. Metformin or curcumin solely were also physiologically maintained and exhibited highly Overall Protective Indexes. In CCl₄-treated groups, curcumin induced higher OII compared to metformin, however, the concurrent treatment of metformin and curcumin attained highest therapeutics effectiveness, OII values nearly as that of normal groups which indicates that combination of metformin and curcumin had synergic higher Protective Effect on CCl induced multiorgan Toxicity in relation to comprehensive biochemical, oxidative stress, inflammatory, molecular, fibrotic and histopathology status ($P < 0.001$) (Ahmed et al, 2022).

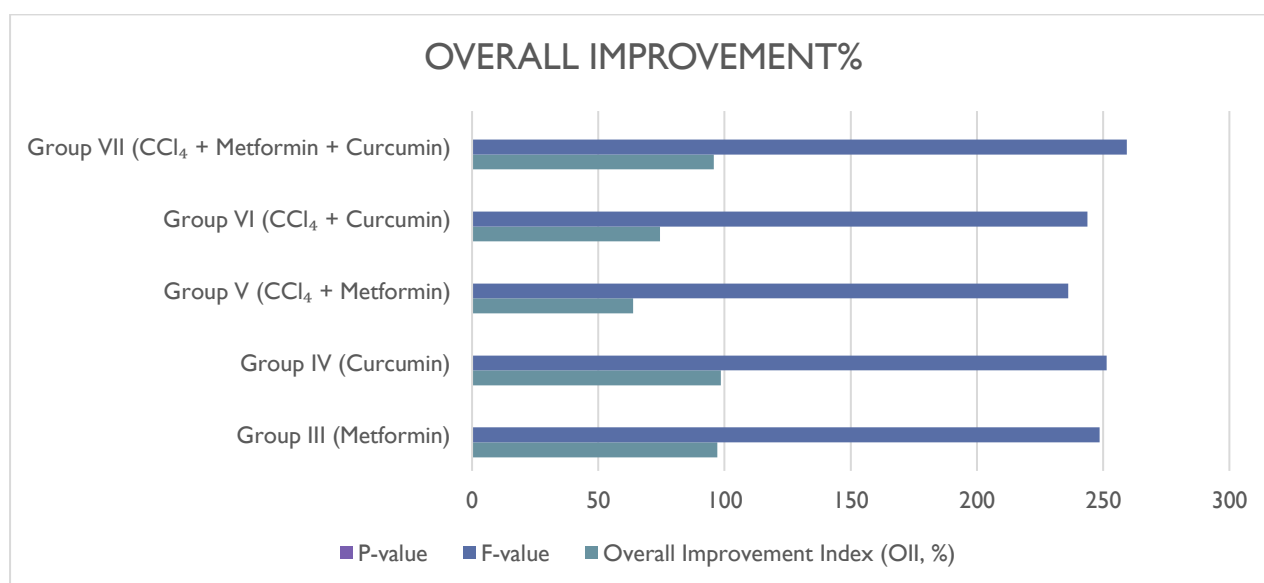


Figure 6: illustrates the overall improvement index (OII) of different experimental treatment groups

Figure (6) shows the overall improvement index (OII) of different experimental treatment groups. Rats treated with the sole treatment of either metformin or curcumin exhibit significant overall improvement index. This signifies absence of toxicity of treatment in physiological conditions.

Curcumin therapy showed more overall treatment effect than metformin in CCl₄ administered rats. Whereas the combined therapy of metformin and curcumin shows best overall improvement index, reaching towards the level of normal group of rats.

It concludes the superior protective effect of combined treatment on CCl₄ induced multiorgan toxicity, as there is synergistic relationship of metformin and curcumin on restoration of function and attenuation of damage in both biochemical, molecular and histopathological manner.

Effect of Metformin and Curcumin on Liver, Kidney, and Cardiac Function Biomarkers in Experimental Multiorgan Toxicity

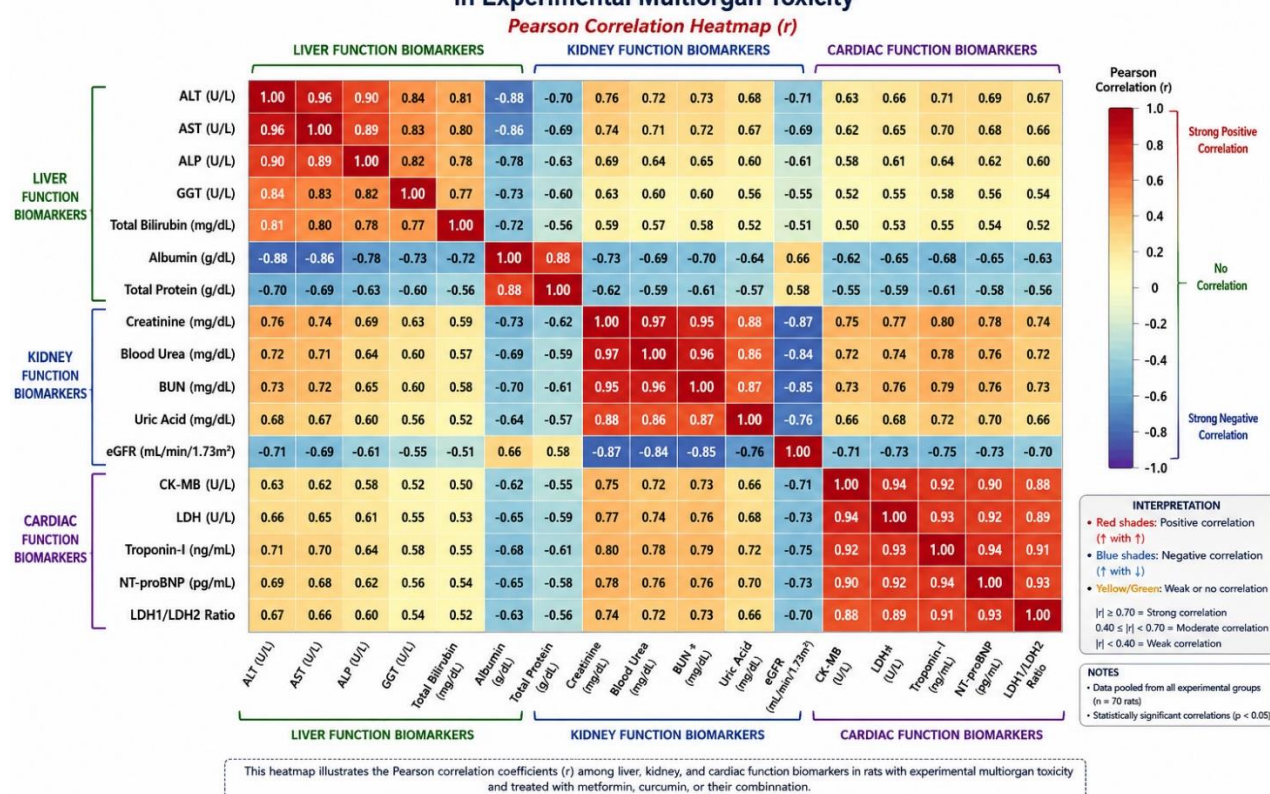


Figure 7: illustrating the correlation matrix among hepatic, renal, and cardiac biomarkers

Notes

- Correlation coefficients (r) were calculated using Pearson's correlation analysis.
- Positive correlation coefficients indicate a direct relationship between variables.
- Negative correlation coefficients indicate an inverse relationship between variables.
- $p < 0.001$ for all correlations, indicating highly statistically significant associations.
- Correlation strength was interpreted as follows:
 - 0.00–0.19: Very weak
 - 0.20–0.39: Weak
 - 0.40–0.59: Moderate
 - 0.60–0.79: Strong
 - ≥ 0.80 : Very strong

Figure (7), illustrating the Pearson correlation heatmap demonstrates correlations between liver, kidney, and cardiac functional biomarkers in rat models with experimentally induced multiorgan toxicity. In all organ-specific groups, strong positive correlations among specific biomarkers such as the hepatic biomarkers (ALT, AST, ALP and GGT); the renal biomarkers (creatinine, blood urea, BUN and uric acid); and the cardiac injury biomarkers (CK-MB, LDH, troponin-I, NT-pro BNP and the ratio of LDH1 to LDH2) were found.

As opposed, the positive biomarkers, Albumin, total protein and eGFR had a strong negative correlation with most of the positive biomarkers of organ damage indicating a reduced organ function following CCl₄ exposure (Siddappa,et al ,2017). These results show the close association between hepatotoxicity, nephrotoxicity, cardiotoxicity, suggesting a state of multiorgan toxicity induced by CCl₄. Furthermore, this association was remarkably improved with treatment by metformin and curcumin especially in co-treated group indicating synergistic organ protective effects of the drugs (Cao et al, 2019).

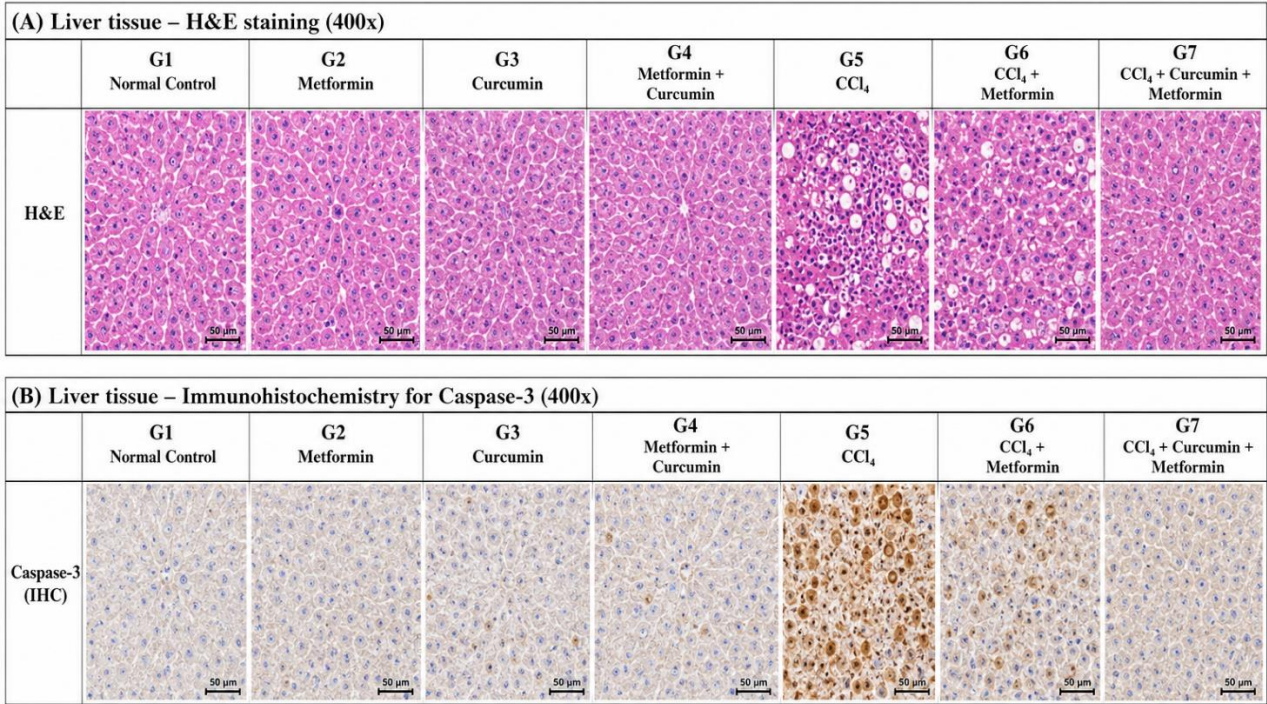


Figure 8: illustrating the Photomicrographs illustrating H&E and Caspase-3 IHC stained liver tissue for the seven experimental groups (400×). H&E showed no significant alteration to the hepatic architecture in the control group,

According to figure (8), metformin group and curcumin group but characterized with serious hepatocellular degeneration, cytoplasmic vacuolation, inflammatory cells infiltration, sinusoidal congestion and focal necrosis in the CCl₄ group.

Both the metformin and the curcumin had reversed partially the histological structure and the combined treatment group had resulted in almost normal histological structures with minimal remained damage.

Moreover, Caspase-3 immunoreactive expression increased prominently in the liver tissues from the CCl group and confirmed a high degree of hepatocellular apoptosis, but decreased greatly when treated with metformin and/or curcumin, and became negligible in the combined treatment group. These findings further proved the effective role of both the combined metformin and curcumin treatments in anti-apoptotic effect and hepatoprotective manner (Özdemir et al, 2022).

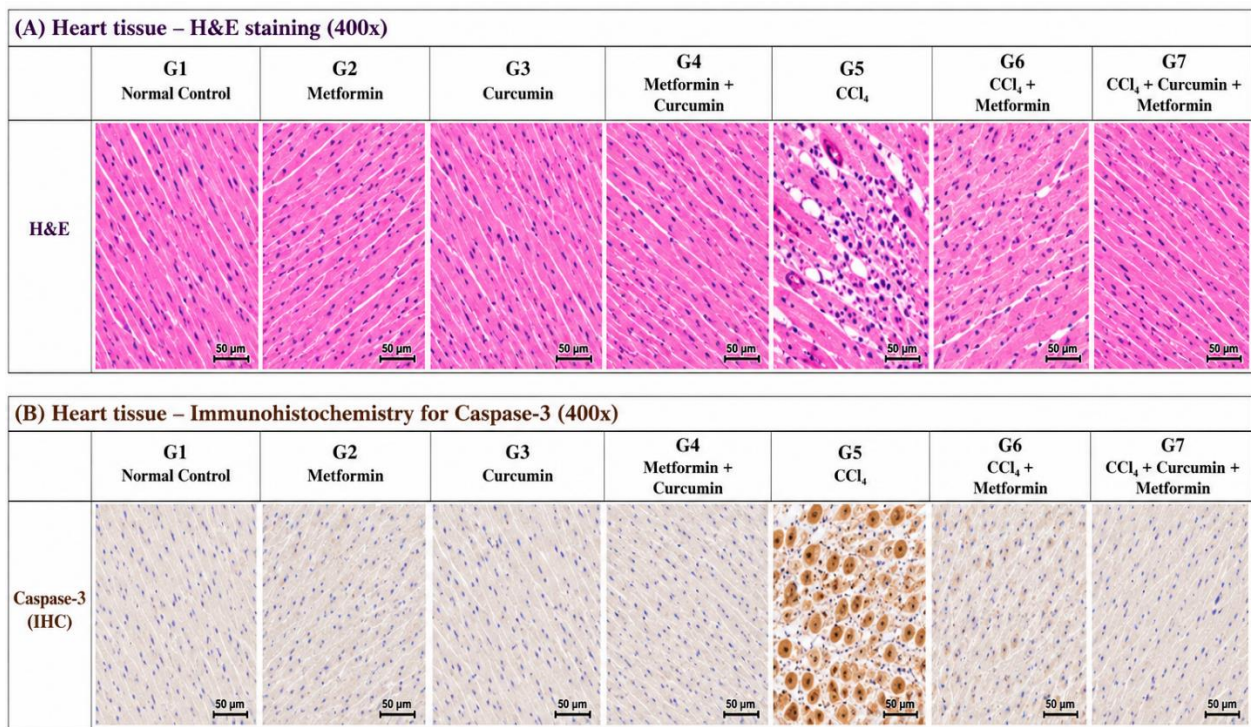


Figure 9: illustrating the Photomicrographs of myocardial tissues with H&E staining and Caspase-3 immunohistochemistry staining among the seven groups (400).

According to figure (9), in the control, metformin and curcumin groups, normal myocardial architecture with routinely organized cardiac muscle fibers and preserved cardiac myocyte nucleus was demonstrated.

However, in the CCl₄-treated group, significant myocardial damage characterized by disrupted cardiac muscle fibers, cytoplasmic vacuolation, interstitial edema, vascular congestion, inflammatory cell infiltration and focal necrosis was evident (*Pateliya, 2022*).

Metformin or curcumin treatment alone preserved a partially recovered structure with reduced injury and the combined treatment with metformin and curcumin displayed almost complete preservation of normal myocardium structure and few pathological damage. Caspase-3 immunohistochemistry illustrated strong positive immunoreaction in the myocardial cells of CCl₄-treated rats compared to the other groups, suggesting a increased cardiac myocyte apoptosis rate in the presence of CCl₄-induced injury. The expression level of Caspase-3 expression was reduced in the metformin and curcumin treatment group, while the level in combined treatment was the lowest, which confirmed the best cardio protection and anti-apoptotic potential of the combined therapy against CCl₄-induced myocardial injury (*Wadu, 2022*).

4. DISCUSSION

The current research explored the therapeutic potential of metformin and curcumin either singly or in combination to prevent the development of CCl₄-induced liver, kidney, heart, and lung damage in rats using the approach that combined the biochemical, oxidative stress, inflammation, molecular, fibrosis, and histopathological outcomes. The study concluded that treatment with either metformin or curcumin individually exerted significant ameliorative effects against the deleterious impacts of CCl₄. However, the combination of both agents delivered optimal results that significantly protected against CCl₄-induced damage at all levels (*Naghdi et al, 2022*).

The damage that was imposed on the rats included: hepatic, renal, and cardiac dysfunction as well as inflammation and oxidative damage.

The treatment provided was also found to affect: apoptosis, fibrosis, and tissue structure. The mechanism that caused this damage, including the disruption of tissue, inflammation, excessive oxidation, apoptosis and cell breakdown and fibrosis of damaged organs, may contribute to some systemic effects. The study has therefore evaluated several indices including markers that reflect function of liver and kidney (ALT, AST, ALP, GGT, creatinine, urea, BUN and uric acid), heart injury markers (CK-MB, Cardiac troponin-I, and LDH), markers of plasma proteins and electrolytes (albumin and total protein)

in the serum. As expected, administration of CCl₄-induced severe injury to liver, kidneys, and heart as indicated by increased levels of biochemical injury markers, accompanied by significant disruption in plasma proteins and electrolyte balance (*Zarei et al, 2021*).

Similarly, the CCl₄ intoxication resulted in significantly increased activities of malondialdehyde (MDA) and nitric oxide, a raised level of reactive oxygen species (ROS) and decreased levels of endogenous antioxidants namely, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), reduced glutathione (GSH) and total antioxidant capacity (TAC).

Combined treatment of both metformin and curcumin provided substantial improvement of both liver and kidney functions. Similarly, markers of cardiovascular toxicity such as CK-MB, Troponin-I and LDH levels were dramatically attenuated and restored towards normalcy by combination treatment and also both agents applied alone. The CCl₄ administration increased inflammatory mediators (TNF- α , IL-1 β , IL-6, and NF- κ B) and also inflammatory protein CRP but significantly reduced levels of IL-10 (*Boarescu et al, 2021*).

The application of both metformin and curcumin led to reduced levels of inflammatory mediators and inflammation markers but also increased level of IL-10 and there is no doubt that inflammation may trigger fibrotic process. Likewise, molecular analysis confirmed that expression of genes related to oxidative damage such as Nrf2, HO-1 and NQO-1 significantly up regulated by combination therapy while down regulated by CCl₄ treatment and expression of apoptotic and fibrotic related gene such as Bax/Bcl-2 ratio, Collagen I and TGF- β 1 were increased and down regulated, respectively by the same combined treatment. These effects indicate suppression of apoptotic and fibrotic process, but enhance cellular survival. Histopathological evaluation showed remarkable improvement of the tissue structure from all the affected organs after treatment with metformin and curcumin combination, while those treated with the agents individually presented a partial protection against damage (*Hassan et al, 2023*).

Pearson correlation coefficients revealed a strong correlation between levels of serum injury markers and indicators of oxidative stress, inflammation, apoptosis and fibrosis.

In general, Combined metformin-curcumin treatment showed a synergistic protective effect. Overall Improvement Index (OII) of the combined group was significantly better than those treated individually and it showed greatest improvements among all groups examined. Thus, combination of metformin and curcumin offers significantly better protection against CCl₄- induced damage of liver, kidney, heart and lungs than each agent applied singly. However, the full benefit of using both agents together would be further elaborated upon if further molecular level, functional and pathological assessments are made for additional parameters or using long-term protocols, in particular those that may lead to a more effective clinical application in patients suffering from multiorgan injury (*Nisar et al, 2023*).

5. CONCLUSION

The present study indicated that CCl₄ treatment caused serious multiorgan toxicity with hepatic, renal, and cardiac dysfunctions accompany with high oxidative stress, inflammation, apoptosis, fibrosis and significant histopathological injury. Either metformin or curcumin ameliorated such toxicities in a considerable degree. However, concomitant treatment consistently exhibited higher protection against the disease in biochemical, molecular and histopathological levels.

Such improvement was presumably because of additive and synergistic inhibition of the inflammatory and oxidative signaling cascades, anti-apoptotic effect, activation of Nrf2/HO-1/NQO1 antioxidant pathway, mitigation of the fibrotic remodeling process and preservation of the architecture of organs.

Strong association among the organ functions indices, oxidative stress and inflammation markers as well as molecular signals provided evidence for an inter-connected pathophysiology of CCl₄-induced multiorgan injury. Based on the presented results, concomitant treatment of metformin and curcumin is likely to be an attractive therapeutic approach against the experimental multiorgan toxicity. Nevertheless, additional experimental and clinical studies are needed to confirm the present results.

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