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Original Article

# Combinatory Effects of Resveratrol and Curcumin in a Murine Model of Acuteon Chronic Liver Failure

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## ABSTRACT

**Objectives:** Acute-on-Chronic Liver Failure (ACLF) in cirrhosis patients involves rapid health decline, organ failure, and high mortality. This study aimed to assess the therapeutic effects of curcumin and resveratrol in treating ACLF in mice. **Materials and Methods:** ACLF was induced in 6-8-week-old C57BL6 mice (20-24 g) by chronic liver damage and carbon tetrachloride (CCl4) injections for 10 weeks, followed by acute injury via Acetaminophen (APAP) and Lipopolysaccharide (LPS). Mice were divided into four groups and received intraperitoneal injections three times a week for three weeks: 1) control (ACLF); 2) resveratrol (40 mg/kg); 3) curcumin (25 mg/kg); 4) curcumin + resveratrol (15 mg/kg + 26 mg/kg). Blood and tissues were collected for histological, cell death, and cytokine analysis. **Results:** Hepatoprotective effects were evident as treatments reduced serum levels of ALT, IL-18, TNF-α, IFN-γ, and hepatocyte apoptosis, as shown by TUNEL analysis. IL-10 levels increased in treatment groups. Mechanistically, the treatment reduced pro-apoptotic and pro-inflammatory markers, while enhancing anti-inflammatory and cell-survival pathways. **Conclusion:** The results suggest that curcumin and resveratrol mitigate liver damage by modulating apoptotic pathways and inflammatory responses in mice with ACLF.

**Keywords:** Resveratrol, Curcumin, Acute-on-chronic Liver Failure, Combination Therapy.

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## INTRODUCTION

Liver cirrhosis is associated with high rates of morbidity and mortality, representing a major public health burden worldwide.[1] A recent study by Gu and colleagues examined the incidence of cirrhosis in Germany concluding that 0.94% of hospitalized individuals had been diagnosed with cirrhosis. Notably, over half of these patients (54.8%) developed cirrhosis or related complications as a secondary health problem, despite being admitted for alternative reasons. Alcoholic liver cirrhosis was responsible for the majority (52%) of admissions related to cirrhosis, making it the primary factor behind these cases.[1] When individuals with liver cirrhosis experience Acute Decompensation (AD), they are in danger of experiencing cirrhosis-related symptoms that require hospitalization. This can lead to a higher risk of death.[2] Over time, the occurrence of various complications related to cirrhosis has been altered, with a decrease in the prevalence of bleeding while an increase in incidents of infections, hepatocellular carcinoma, Portal Vein Thrombosis (PVT), hepatorenal syndrome, ascites, and Hepatic Encephalopathy (HE) has been observed.[1] Recent research has closely analyzed the development of AD and uncovered the presence of diverse clinical presentations, including SDC, UDC, and pre-ACLF among those with cirrhosis.[2,3] The second group advances to a state of ACLF within three months, mainly caused by intense inflammation throughout the body. As per the standards set by the European Association for the Study of the Liver (EASL), ACLF is marked by the inability of both liver and non-liver organs to function properly in patients with sudden deterioration of liver cirrhosis, and is linked to a mortality rate of about 30% within 28 days.[4-6] A noteworthy prevalence of ACLF has been revealed through recent research in patients who have been hospitalized due to Acute Decompensation (AD) worldwide. Diverse causes of AD have been recognized, such as bacterial infections, severe alcoholic hepatitis, blood loss accompanied by shock, and the toxic effects of drugs leading to encephalopathy.[7] The primary goal in caring for these patients is to identify and address the causes that led to their condition, while also addressing any dysfunction in the organs within and outside of the liver. Unfortunately, there is currently no targeted treatment option for this particular group of individuals. Liver Transplantation (LT) continues to be the sole effective remedy for these patients. There is a severe shortage of donor organs caused by intense competition among individuals

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on the waiting list, and patients with ACLF may occasionally have disqualifying factors for undergoing a liver transplant, such as uncontrollable bacterial infections. The study and evaluation of different types of extracorporeal liver support, including albumin dialysis, are ongoing, with a continuous exploration of novel techniques and experimental treatment approaches.[8] Turmeric contains a primary curcuminoid, referred to as curcumin, which is recognized as the key element responsible for its biological effects. The impacts of this substance include strong anti-inflammatory and antioxidant properties, in addition to the ability to regulate various signaling pathways.[9-11] Multiple incurable diseases and the growth of cancer are intricately correlated to the presence of oxidative stress.[12,13] Numerous investigations have conclusively demonstrated that curcuminoids possess the ability to serve as scavengers of free radicals, resulting in a reduction of lipid peroxidation stemming from the actions of said free radicals.[14] Curcumin has demonstrated antioxidant and anti-inflammatory properties through modulation of pathways such as NF-κB. Similarly, resveratrol shows anti-fibrotic and hepatoprotective effects in liver injury models.[14] Scientists have become intrigued with resveratrol, which is a type of phenolic compound derived from resveratrol, because of its wide array of capabilities.[15,16] There is increasing proof that resveratrol could potentially provide advantageous results for liver fibrosis.[17,18] Resveratrol has the ability to significantly hinder chronic liver injury by utilizing various mechanisms such as its antioxidant and anti-inflammatory traits, as well as its ability to suppress bacterial translocation.[19-21] Qiao and colleagues' research suggests that resveratrol significantly exacerbates the disruption of gut microbiota in mice.[22] Critically, Chen and colleagues have confirmed that administering resveratrol improves the outcomes of liver diseases by reducing the permeability of the intestine and reducing the incidence of bacterial translocation in a specific rat model of Non-Alcoholic Steatohepatitis (NASH).[23] While the individual benefits of curcumin and resveratrol on liver health are documented, their synergistic potential in a clinically relevant model of ACLF, particularly their combined impact on apoptosis, mitochondrial health via PINK1, and immune cell trafficking via the MAdCAM-1/CCL25 axis, remains unexplored. Therefore, this study aimed to investigate the efficacy and underlying protective mechanisms of this combination therapy in a murine model of ACLF.

## MATERIALS AND METHODS

### Animal

Male C57Bl6 mice aged six to eight weeks were included in the study. Upon arrival, their weights were recorded, ranging from 20 to 24 g. Mice were housed under standard laboratory conditions (22±2°C; 12-hr light/dark cycle) at the Institutional Animal Facility. Mice had *ad libitum* access to standard laboratory chow and water. The Ethics Committee of "Xi'an Zhongkai Animal Experiments Medical Research Ethics, China (Code: 13885)" has approved the research. The mice were categorized into four groups ($n$=20 per group) and were injected intraperitoneally three times a week for three weeks, with the following doses: 1) control (ACLF); 2) resveratrol (40 mg/kg); 3) curcumin (25 mg/kg); 4) curcumin + resveratrol (15 mg/kg + 26 mg/kg).

All procedures were performed in compliance with the ARRIVE guidelines and the institutional ethics committee regulations. Throughout the chronic injury phase and the subsequent acute injury and treatment phase, all mice were monitored daily for clinical signs of distress. This monitoring included assessment of general appearance (e.g., piloerection, hunched posture), activity levels (e.g., lethargy, unresponsiveness), and signs of pain or sickness (e.g., labored breathing, abdominal swelling). Body weight was recorded three times per week.

To minimize animal suffering, humane endpoints were established prior to the study. Animals were to be immediately euthanized by $CO_2$ asphyxiation followed by cervical dislocation if they met any of the following criteria:

- Loss of >20% of their initial body weight.
- Inability to ambulate or access food and water.
- Exhibition of severe clinical signs, such as persistent hypothermia, labored breathing, or unresponsiveness to gentle stimuli.

### Sample collection

Mice were euthanized via intraperitoneal injection of a combination of xylazine (20 mg/mL), ketamine (50 mg/mL) and saline (0.9% V/V). Serum was obtained by extracting blood from a retro-orbital puncture and storing it in a blood collection tube. The serum was isolated through centrifugation, with the rotation set at 3000 rotations per minute and a duration of 15 min. The amounts of four cytokines (IL-18, IL-10, TNF-α, and IFN-γ) were measured through the use of a mouse-specific ELISA Kit obtained from Wuhan EIAab Science Co., Ltd., in China, as well as other ELISA kits commercially available from Jingmei Biotech Co., Ltd., in China. The methods instructed by the producers were diligently adhered to. The level of Alanine Transaminase (ALT) was measured using typical biochemical techniques on a Hitachi Automatic Analyzer created by Hitachi, Inc., based in Japan.

### Histology assessment

The liver tissue samples were placed in a chemical solution made of 4% paraformaldehyde from Solarbio, located in Beijing, China, and were left to soak for a period of 48 hr. Afterward, the samples were preserved by coating them with paraffin. Afterwards, tissue samples were sliced to create 4 μm thick sections. The specific sections underwent subsequent treatment using Hematoxylin and Eosin (H&E) or Masson stain, in compliance with the provided guidelines from Solarbio. A comprehensive review was

performed using an optical microscope to study all deviations from the normal condition.

### Immunofluorescence Microscopy

IF microscopy was carried out on liver cryosections exactly as outlined in previous studies. The Axioskop 2 plus microscope (Zeiss, Jena, Germany) was utilized to visualize the IF images. The antibody MAdCAM1 (catalog number 16-5997-85; ThermoFisher Scientific, Waltham, MA, USA) was used in mouse tissue. ImageJ was used to perform a quantitative analysis on various random fields.

### Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assays

To gain a deeper understanding of the progression of apoptosis following post-acute injury, the process of cell death was thoroughly evaluated by identifying DNA strand damage associated with apoptosis using the TUNEL assay. This procedure was carried out utilizing the *in situ* Cell Death Detection Kit from Roche (Catalog No. 11684795910) by the provided instructions.

### Quantitative reverse transcriptase-PCR

To measure the amounts of inflammatory substances (IL-6 and IL-1β) and substances that reduce inflammation (IL-4 and IL-1ra receptor antagonist), the process of retrieving all RNA from liver tissue was executed. The frozen liver samples were obtained through the TRIzol technique by Invitrogen (located in Shanghai, China), and the suggested protocol was followed. The concentration of RNA was evaluated by utilizing a NanoDrop ND-100 spectrophotometer provided by NanoDrop Technologies Thermo Scientific. An ABI kit from Invitrogen was utilized to perform PCR on 2 μg of RNA for cDNA synthesis. The resulting cDNA was then combined with SYBR Green from Kappa and analyzed using Biorad's Real-Time PCR Detection System. GAPDH was utilized as the control, and the experiment was conducted in triplicate. The ΔΔCt method, also known as the two-cycle threshold method, was utilized to examine and interpret the data.

### Western Blot

To fully examine the process of apoptosis in different subjects, the proteins were extracted from the liver tissue of mice belonging to both the control and treatment groups. The proteins were extracted using the SDS lysis method according to the provided protocol from snap-frozen liver samples. Bradford's method was used to determine protein concentration. A final concentration of 50 μg/well was loaded onto the wells, with GAPDH serving as the internal control. The primary antibodies, including Caspase3 (#9662; Cell Signaling Technology), Bax, and Bcl-2, were diluted to 1:1000 and incubated overnight at 4°C. An alternative antibody targeted against rabbits (111-035-003; ImmunoJackson) was utilized with a dilution rate of 1:20,000. The reactions were replicated three times and the data was evaluated using a luminol-based chemiluminescent compound called ECL (Thermo Scientific) to identify Horseradish Peroxidase (HRP) on the immunoblots.

### Enzyme Linked Immunosorbent Assays (ELISA)

To ascertain the levels of ALT, CCL25, IL-10, IL-18, IFN-γ, and TNF-α in the bloodstream, a specialized DuoSet ELISA kit from R&D Systems (Cat. No. DY334, R&D Systems, Minnesota, USA) was employed. Within the duration of 1 hr, citrate-containing Vacutainers were utilized to gather whole blood, which was then centrifuged at a rate of 2000 rotations per minute for 10 min in order to acquire the serum. After storing at -80°C, the serum was held until it was examined. The ELISA test was conducted following the manufacturer's instructions. Before analysis, the samples were diluted with sample buffer at a ratio of 1:4 and each sample were tested twice. Additionally, TNF-α, IL-10, IL-18, ALT, IFN-γ, and CCL25 protein extracts from liver tissue were evaluated using an ELISA kit provided by Cusabio. To determine the concentrations of these substances, a standard curve was created using known amounts of recombinant chemokines. The values for the samples were then measured by using this curve to estimate the concentrations.

### Statistical Analysis

All data are expressed as Mean±Standard Deviation (SD). Comparisons between multiple groups were analyzed using one-way Analysis of Variance (ANOVA) followed by Tukey's *post hoc* test. A two-tailed unpaired Student's *t*-test was applied where only two groups were compared. A $p$-value <0.05 was considered statistically significant. Statistical analyses were performed using GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA). Each experimental group included 6 mice ($n$=6), unless otherwise specified. All assays were conducted in triplicate.

## RESULTS

### Effect of ALT and inflammatory cytokines

As shown in Figure 1, serum levels of TNF-α, ALT, IFN-γ, and IL-18 were significantly higher in the ACLF model group (TNF-α: 190.77±12.34 ng/L, ALT: 8415.87±3567.54 IU/L, IFN-γ: 715.38±86.03 ng/L, IL-18: 85.19±3.49 ng/L; $n$=20 per group) compared to the treatment groups (TNF-α: 124.40±4.12 ng/L, ALT: 38.64±8.82 IU/L, IFN-γ: 398.66±32.91 ng/L, IL-18: 55.38±1.25 ng/L; $p$<0.001 for all comparisons, one-way ANOVA with Tukey's post hoc test). Conversely, IL-10 levels were significantly lower in the ACLF model group (IL-10: 3.49±0.24 ng/L) compared to the treated groups (IL-10: 6.85±0.64 ng/L; $p$<0.001). These findings demonstrate that resveratrol, curcumin, and their combination significantly attenuate systemic inflammatory responses and hepatocellular injury in the ACLF model.

## Liver Histopathology

To assess the precision of the ACLF models and examine the physical changes occurring in the early stages after induction, tissue samples from the liver were examined using staining methods including Masson and hematoxylin-eosin. The ACLF models were used for comparison purposes.

Upon examination, a firm consistency and bumpy surface were noted in the livers of patients with ACLF. Signs of cell swelling, cell death, and the presence of immune cells were observed in the tissue samples, along with an increase in binucleated liver cells and the formation of several false lobules. (Figures 2a, 2e). In contrast, the livers from treatment groups displayed nearly smooth surfaces; hepatic cells appeared intact, and the structure of hepatic lobules was well-preserved. Minimal fibroplasia was observed under the light microscope (Figures 2b, 2c, 2d, 2f, 2g, 2h).

## Curcumin plus resveratrol is able to reduce the expression levels of MAdCAM1

Figure 3 demonstrated the variations in MAdCAM1 levels, which were identified through the use of both immunohistochemistry and immunofluorescence staining techniques.

## Curcumin plus resveratrol reduces apoptosis

TUNEL staining revealed a marked reduction in hepatocyte apoptosis in all treatment groups compared to the ACLF group (Figure 4A). Quantification of TUNEL-positive cells (Figure 4B) showed that the percentage of apoptotic cells in the curcumin-treated and resveratrol-treated groups was significantly decreased compared to the untreated ACLF model (Control: 100.0±10.2%; Curcumin: 48.7±7.9%; Resveratrol: 52.1±8.3%; $p<0.001$ vs. Control for both). The combination of curcumin and resveratrol resulted in the greatest reduction in apoptosis (Cur + Res: 35.4±6.7%), which was also statistically significant compared to either monotherapy group ($p<0.05$, one-way ANOVA, Tukey's post hoc test, $n=20$ per group).

These results indicate that both agents-especially when combined-have a protective anti-apoptotic effect in ACLF-induced liver injury.

Western blot analysis (Figure 4C and 4D) showed that all treatments significantly ($p<0.001$) altered protein expression, with the combined curcumin and resveratrol (Cur+Res) group showing the strongest effects. This combination treatment promoted cell survival by decreasing pro-apoptotic Bax (to ~0.4-fold) and Caspase-3 (to ~0.2-fold) while increasing anti-apoptotic Bcl-2 (to ~4.1-fold). Additionally, the Cur+Res treatment strongly upregulated the mitochondrial protective protein PINK1 (~5.2-fold) and downregulated the immune adhesion molecule MAdCAM-1 (~0.3-fold).

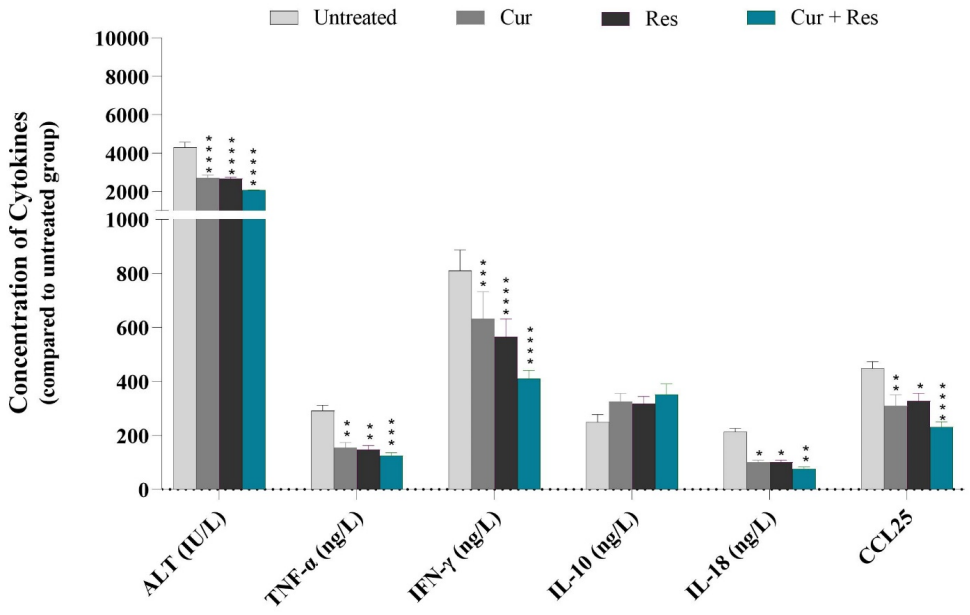


**Figure 1:** Curcumin and resveratrol treatment alleviates serum markers of liver injury and inflammation in a murine model of ACLF. Serum concentrations of Alanine Transaminase (ALT), Tumor Necrosis Factor-alpha (TNF-α), Interferon-gamma (IFN-γ), interleukin-10 (IL-10), interleukin-18 (IL-18), and chemokine ligand 25 (CCL25) were quantified by ELISA. Data are presented as the Mean±Standard Deviation (SD) for n=20 mice per group. Statistical significance was determined by a one-way Analysis of Variance (ANOVA) with Tukey's *post hoc* test. Asterisks denote a significant difference compared to the untreated group (*$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$).

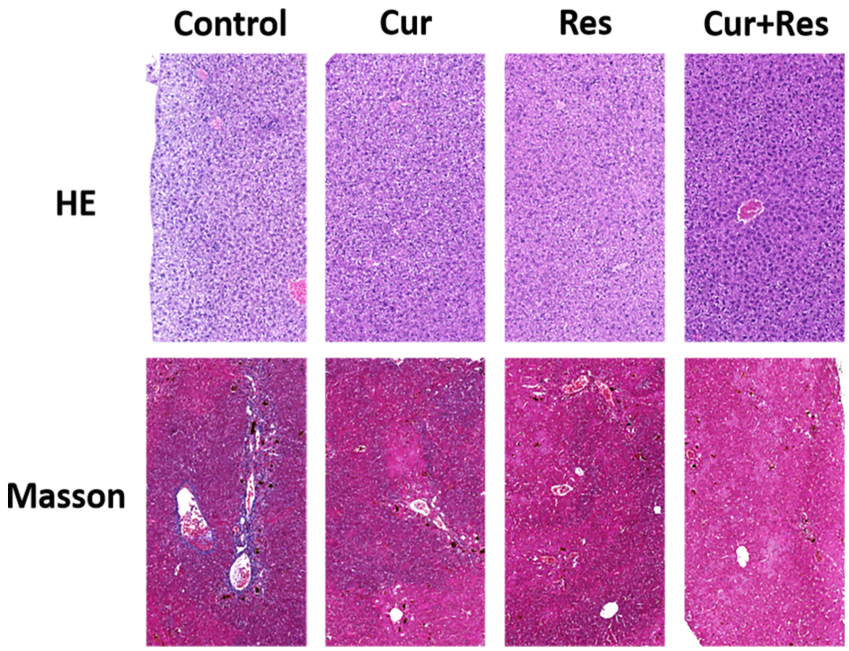


**Figure 2:** Pathological features of liver tissue under light microscope by HE (magnification 400x) and Masson staining (magnification 200x).

### Curcumin plus resveratrol is able modulation cytokine

Based on the RT-PCR tests, the treatment groups showed a decrease in levels of proinflammatory cytokines (IL-6 and IL-1β) and an increase in levels of anti-inflammatory cytokines (IL-1ra and IL-4) (Figure 5).

## DISCUSSION

The principal finding of this study is that a combination of resveratrol and curcumin exerts a potent synergistic protective effect in a murine model of ACLF, surpassing the efficacy of either monotherapy. Our work provides novel insight by demonstrating that this synergy likely involves at least two distinct mechanisms: the direct mitigation of hepatocyte apoptosis coupled with enhanced mitochondrial quality control, and the suppression of inflammatory cell recruitment into the liver. Notably, the mechanisms contributing to liver damage and overall inflammation may differ depending on the underlying factors of ACLF, suggesting the possibility of distinct treatment approaches based on the cause of ACLF. ACLF induced by bacterial infection is linked to marked elevations in IL-6, IL-1ra, and TNF-α, whereas alcohol-induced ACLF is associated with a particular rise in IL-8.[24] Different stimuli and primary liver conditions cause various types of immune reactions to occur. For instance, bacterial infections cause inflammation through specific detection by PRRs, yet also by means of virulence components leading to heightened levels of IL-6, TNF-α and acute phase proteins.[25]

Based on our findings, MAdCAM-1 and MAdCAM1 levels were down-regulated among the treated groups. The main location of this protein is on the outer layer of high endothelial venules found in the digestive tract and Peyer's patches. Its essential role is to draw lymphocytes towards mucosal tissues, playing a vital role in the protection of these areas. Additionally, this molecule is vital in promoting communication between white blood cells and the inner lining of blood vessels.[26] MAdCAM-1 has been identified in cells that coat the blood vessels within the lymph nodes situated in the mesentery, the layer of tissue underlying the internal lining of the small and large intestines, and the central nervous system. Additionally, it has been detected in the endothelial cells of the mammary gland during lactation. The fact that it is found in large quantities indicates its significant contribution to various bodily processes, including its involvement in immune defense and facilitating the movement of white blood cells to targeted areas.[27,28] Along with its traditional function of directing immune cells to mucosal lymphoid tissue, the production of MAdCAM-1 is notably elevated during prolonged periods of inflammation and illness, such as diabetes, cirrhosis, sclerosing cholangitis, and Inflammatory Bowel Disease (IBD). This heightened level of MAdCAM-1 indicates that it may have a distinct role in the development and worsening of these ailments, as it enables immune cells to adhere and move to affected areas.[29-32] In the context of IBD, particularly Crohn's disease, MAdCAM-1 plays a crucial role by serving as the primary binding factor for lymphocytes that possess the α4β7 receptor. This helps these lymphocytes to enter the intestinal tract. The process of recruiting is incredibly important in the onset and continuation of long-term inflammation. AA vast number of research studies involving animals and clinical trials involving humans emphasize the crucial functions of both α4β7 and MAdCAM-1 in

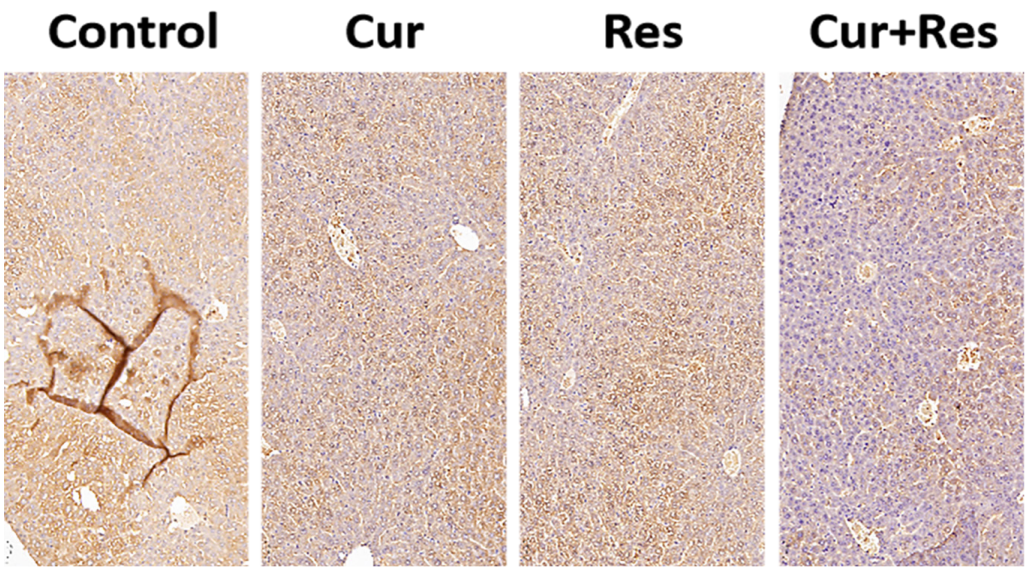


**Figure 3:** The levels of MAdCAM1 in the studied groups. DAPI (blue) and MAdCAM1 (green). Scale bar=50 μm.

creating models of immune-mediated colitis, emphasizing their significance in understanding the fundamental mechanisms of inflammatory bowel disease.[33]

The activation of MAdCAM-1 in lymphoid and brain endothelial cells is triggered by various cytokines, such as TNF-α and IL-1β. Yet, the means by which this occurs in High Endothelial Cells (HEC) still remains to be thoroughly investigated. Since MAdCAM-1 is activated by Th1 cytokines like IL-1β, it is likely that the process of inducing it is similar to that of other adhesion molecules, including VCAM-1 and ICAM-1. These molecules also aid in adhesion and are stimulated by Th1 cytokines, but their activation specifically relies on the NF-κB/PARP complex.[34]

Our findings align perfectly with this mechanism. The significant reduction in MAdCAM-1 expression and pro-inflammatory cytokines like TNF-α and IL-1β strongly suggests an inhibition of the NF-κB signaling pathway. NF-κB is a master transcriptional regulator for these molecules, and its inhibition by both curcumin and resveratrol is well-documented in other models of inflammation.[35] Therefore, while we did not directly measure NF-κB activation, our results are highly consistent with a mechanism where the combination treatment suppresses this key pro-inflammatory cascade to reduce immune cell trafficking and liver damage.

During our examination, we observed a decrease in the amount of CCL25 detected in the group that underwent treatment. This particular chemokine is naturally produced in the small intestine's epithelium and mucosal vessels. The molecule attaches to immune cells, specifically B and T cells, that carry its receptor, CCR9, and are responsible for traveling to the gut. This connection is crucial for directing these lymphocytes to the lining of the intestine, aiding in immune monitoring and reactions in the gut.[36] CCR9 and α4β7 are both present on lymphocytes that are bound for the gut. This allows for better attachment to MAdCAM-1 on the surface of mucosal vessels, which is essential for their movement towards the gut. This process is vital in enabling efficient immune responses in the intestinal mucosa.[37,38] Extensive research has confirmed that the pairing of α4β7 and CCR9 molecules is a key factor in promoting the migration of lymphocytes to the gastrointestinal tract. The vast majority of lymphocytes in the small intestine's epithelial lining and underlying tissue have been identified as possessing both CCR9 and α4β7. Furthermore, the small number of CCR9+ lymphocytes present in the bloodstream, especially those with increased α4β7, are primarily memory cells. The amount of these cells rises considerably during intestinal inflammation, emphasizing their crucial role in the immune reaction of gut-related diseases.[39,40] B cells that produce IgA in the small intestine possess the CCR9 protein and their distribution within the gut is determined by CCL25. The migration of B cells to the intestinal mucosa is facilitated by this connection, thereby playing a significant role in preserving mucosal immunity and allowing for the production of IgA, a critical intestinal defense antibody.[41]

This research found a noticeable decrease in the levels of Bax and caspase and an increase in the levels of Bcl-2 and PINK1 in the groups that received treatment. Our findings confirm the critical role of apoptotic regulation in ACLF and suggest that curcumin and resveratrol may preserve hepatocyte viability by modulating Bax/Bcl-2 and PINK1 pathways. The striking upregulation of PINK1 is a particularly novel finding, pointing towards the activation of mitochondrial quality control pathways, such as mitophagy. The interplay between apoptosis and mitophagy is often regulated by the mTOR signaling pathway, another known target of resveratrol and curcumin.[42,43] The observed decrease

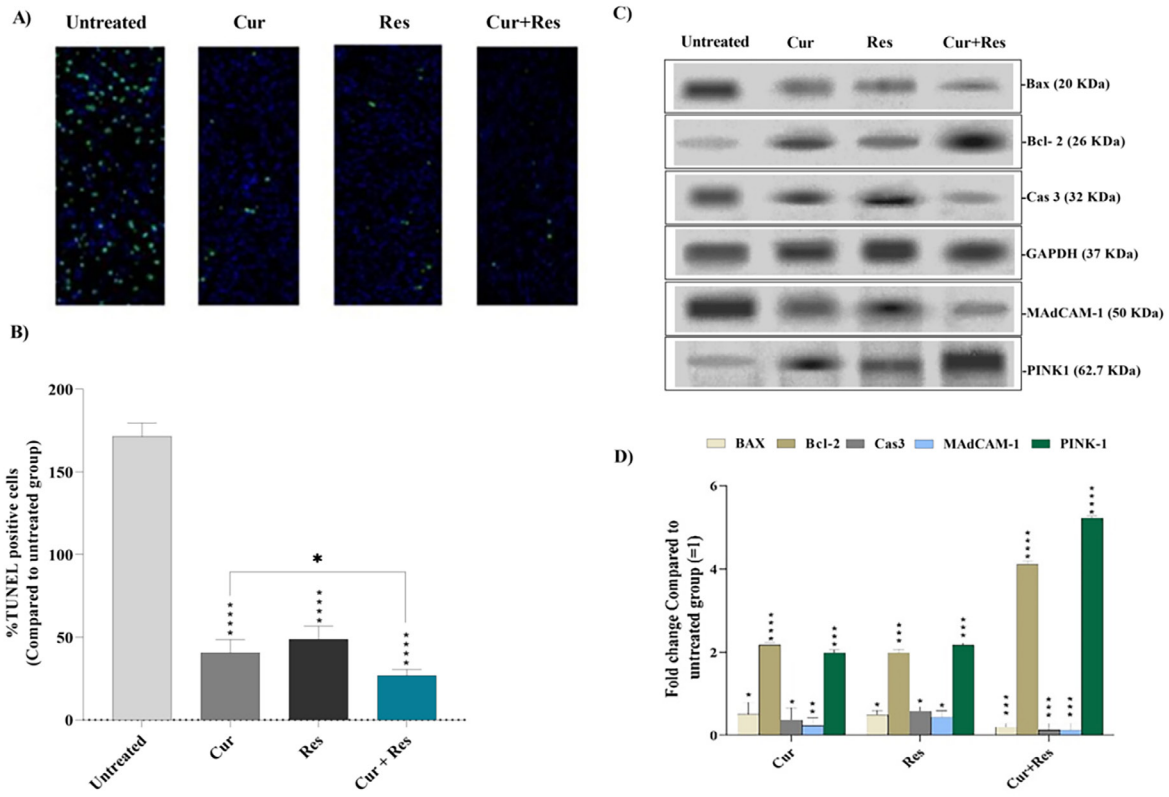


**Figure 4:** Curcumin and resveratrol synergistically reduce hepatocyte apoptosis and modulate the expression of key apoptosis-related proteins. (A) Representative images of liver sections stained with TUNEL to detect apoptotic cells (green nuclei) and DAPI for total nuclear counterstaining (blue). (B) Quantification of the percentage of TUNEL-positive apoptotic cells relative to the total number of cells. (C) Representative Western blot analysis showing the expression of pro-apoptotic proteins (Bax, Caspase-3), anti-apoptotic protein (Bcl-2), the adhesion molecule MAdCAM-1, and the mitochondrial protective protein PINK1. GAPDH was used as a loading control. (D) Densitometric quantification of the Western blot bands, normalized to GAPDH and expressed as a fold change relative to the untreated group. Data are presented as the Mean±Standard Deviation (SD). For TUNEL analysis (A, B), $n$=20 mice per group. For Western blot analysis (C, D), $n$=6 mice per group. Statistical significance was determined by a one-way analysis of variance (ANOVA) with Tukey's *post hoc* test. In panel B, ****$p$<0.0001 compared to the untreated group; *$p$<0.05 for the combination treatment (Cur+Res) compared to either monotherapy group. In panel D, ***$p$<0.001 compared to the untreated group.

in apoptotic markers (Bax, Caspase-3) alongside the increase in PINK1 may thus reflect a treatment-induced shift from cell death towards cellular repair and survival, a mechanism potentially mediated through mTOR modulation. The initiation of controlled cellular demise in hepatic cells is a vital element in liver ailments and plays a prominent role in most liver-related issues. The proficient regulation of programmed cell mortality is vital for the appropriate development of tissues, the preservation of a balanced internal setting, and the elimination of unhealthy or irregular cells. The essential factor in liver diseases and a key component in the majority of liver dysfunctions is the apoptosis of liver cells.[44,45] Numerous research projects have been carried out on animals and people, and the results have consistently shown that hindering the occurrence of cell death in liver cells can efficiently reverse or postpone the progression of liver disease. These convincing findings suggest that utilizing caspase inhibitors could be a promising approach for effectively treating liver conditions. Other research has shown a significant reduction in the levels of serum Alanine Aminotransferase (ALT) among people with Non-Alcoholic Steatohepatitis (NASH) who were prescribed the caspase inhibitor GS-9450.[46] The inclusion of IDN-6556 in both the refrigeration storage and washing liquids led to a noteworthy decrease in the effects of cell death caused by the combination of low temperature preservation and subsequent reoxygenation during liver transplant operations.[47] It was observed in the study that apoptosis occurs in cells during ACLF. Experiments conducted in a controlled setting revealed that an increase in PINK1 levels resulted in enhanced cell survival due to the inhibition of apoptosis. In contrast, a PINK1 deficiency caused cell death through apoptosis. In ACLF mice, elevating PINK1 levels significantly improved liver function and reduced liver damage and apoptosis. Hence, PINK1 appears to have a protective function for ACLF by preventing apoptosis and mitigating liver damage.[48]

We acknowledge that this study has several limitations. Our investigation focused on key downstream protein markers of inflammation and apoptosis but did not include a direct assessment of upstream signaling pathway activation, such as the phosphorylation state of NF-κB or mTOR components. Therefore, our proposed mechanisms remain associative. Future

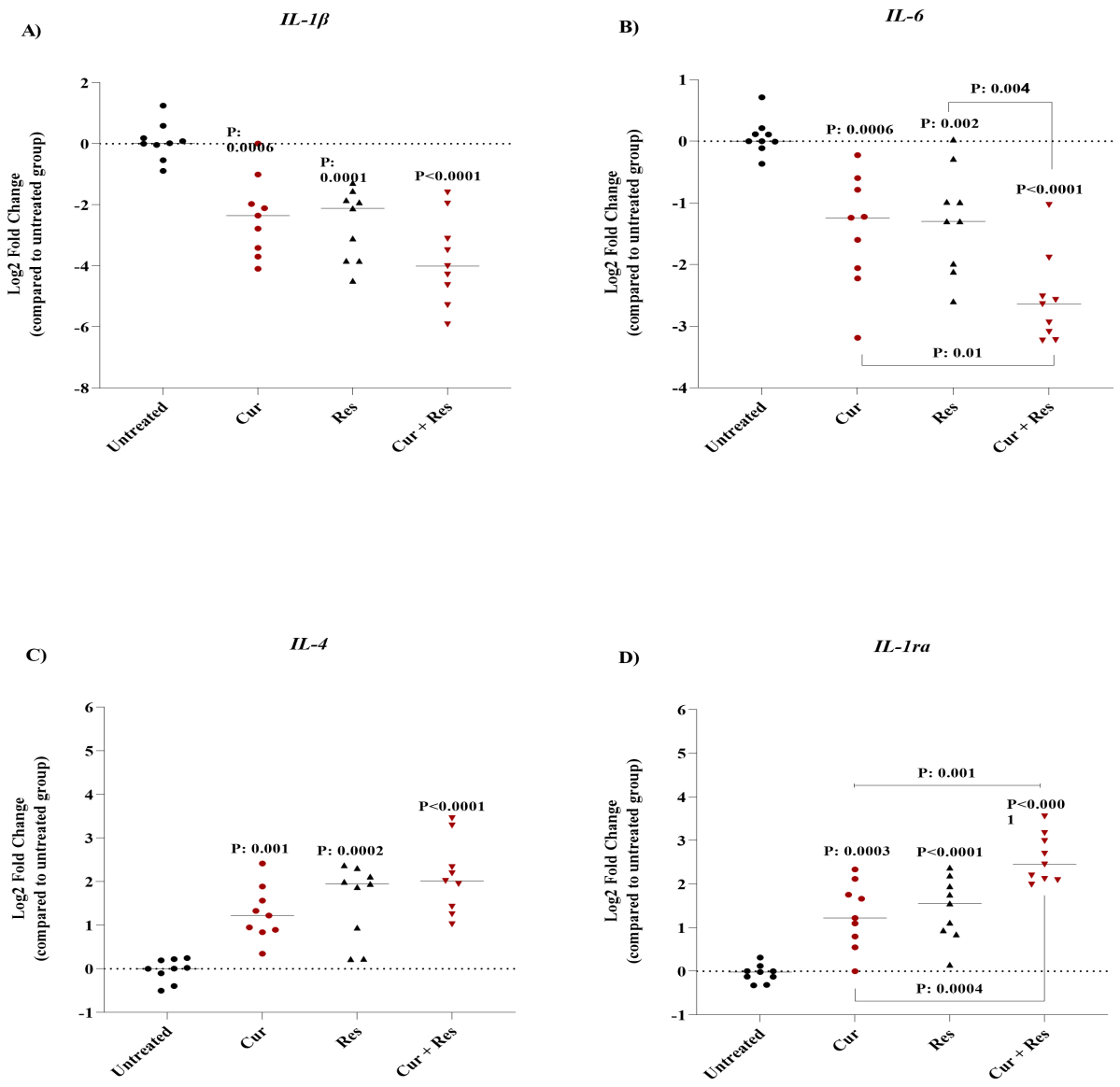


**Figure 5:** Combination treatment with curcumin and resveratrol shifts the hepatic cytokine mRNA expression profile towards an anti-inflammatory state. Hepatic gene expression of the pro-inflammatory cytokines (A) interleukin-1 beta (IL-1β) and (B) interleukin-6 (IL-6), and the anti-inflammatory cytokines (C) interleukin-4 (IL-4) and (D) interleukin-1 receptor antagonist (IL-1ra) was measured by quantitative reverse-transcriptase PCR (RT-PCR). Results are plotted as the Log2 fold change relative to the mean of the untreated control group. Each data point represents an individual mouse ($n$=10 per group), and the horizontal line indicates the group mean. Statistical significance was determined using a one-way Analysis of Variance (ANOVA) followed by Tukey's *post hoc* test. Relevant $p$-values for significant comparisons are indicated directly on the graph.

studies are warranted to dissect the precise signaling events that drive the synergistic effects observed here.

## CONCLUSION

This study demonstrates that resveratrol, curcumin, and their combination exert significant hepatoprotective effects in a murine model of Acute-on-Chronic Liver Failure (ACLF). The treatments effectively reduced serum levels of Alanine Transaminase (ALT) and pro-inflammatory cytokines (TNF-α, IFN-γ, IL-18), while increasing the anti-inflammatory cytokine IL-10. Histological analyses revealed preserved liver architecture, and TUNEL assays confirmed a reduction in hepatocyte apoptosis, corroborated by decreased expression of pro-apoptotic proteins (caspase 3 and Bax) as well as MAdCAM1 along with increased expression of anti-apoptotic proteins (PINK1, Bcl-2). Additionally, RT-PCR results indicated a shift toward an anti-inflammatory profile, with reduced IL-6 and IL-1β and elevated IL-4 and IL-1ra levels. The reduction in chemokine CCL25 and MAdCAM1 expression further suggests modulation of immune cell trafficking and inflammation. In summary, the combination of resveratrol and curcumin offers a promising therapeutic approach for ACLF, warranting further investigation in clinical models.

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## ABBREVIATIONS

**ACLF:** Acute-on-Chronic Liver Failure; **AD:** Acute Decompensation; **ALT:** Alanine Transaminase; **APAP:** Acetaminophen; **Bax:** Bcl-2-associated X protein; **Bcl-2:** B-cell lymphoma 2; **$CCl_4$:** Carbon Tetrachloride; **CCL25:** Chemokine (C-C motif) Ligand 25; **CCR9:** C-C Chemokine Receptor 9; **EASL:** European Association for the Study of the Liver; **ELISA:** Enzyme-Linked Immunosorbent Assay; **HE:** Hepatic Encephalopathy; **H&E:** Hematoxylin and Eosin; **HRP:** Horseradish Peroxidase; **IBD:** Inflammatory Bowel Disease; **IFN-γ:** Interferon-gamma; **IL-1β:** Interleukin-1 beta; **IL-1ra:** Interleukin-1 receptor antagonist; **IL-4:** Interleukin-4; **IL-6:** Interleukin-6; **IL-10:** Interleukin-10; **IL-18:** Interleukin-18; **LPS:** Lipopolysaccharide; **LT:** Liver Transplantation; **MAdCAM1:** Mucosal Addressin Cell Adhesion Molecule-1; **NASH:** Non-Alcoholic Steatohepatitis; **NF-κB:** Nuclear Factor kappa B; **PINK1:** PTEN-induced kinase 1; **PRRs:** Pattern Recognition Receptors; **PVT:** Portal Vein Thrombosis; **RT-PCR:** Reverse Transcriptase-Polymerase Chain Reaction; **SDC:** Stable Decompensated Cirrhosis; **TNF-α:** Tumor Necrosis Factor-alpha; **TUNEL:** Terminal deoxynucleotidyl transferase dUTP nick end labeling; **UDC:** Unstable Decompensated Cirrhosis; **VCAM-1:** Vascular Cell Adhesion Molecule-1; **ICAM-1:** Intercellular Adhesion Molecule-1; **α4β7:** Alpha-4 beta-7 integrin.

## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

## SUMMARY

In a murine model of Acute-on-Chronic Liver Failure (ACLF) induced by carbon tetrachloride and acetaminophen/lipopolysaccharide in C57BL6 mice, resveratrol (40 mg/kg), curcumin (25 mg/kg), or their combination (15 mg/kg + 26 mg/kg) significantly alleviated liver damage. Treatments reduced serum ALT, pro-inflammatory cytokines (TNF-α, IFN-γ, IL-18), and CCL25, while increasing anti-inflammatory IL-10. Histology showed preserved liver architecture, and TUNEL assays indicated reduced hepatocyte apoptosis, supported by lower pro-apoptotic (caspase 3, Bax, MAdCAM1) and higher anti-apoptotic (PINK1, Bcl-2) protein levels. RT-PCR revealed decreased IL-6/IL-1β and increased IL-4/IL-1ra, with reduced MAdCAM1 expression, suggesting resveratrol and curcumin's therapeutic potential in ACLF by modulating inflammation and apoptosis.

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