

RESEARCH ARTICLE

PHYTOCHEMICAL COMBINATION THERAPY FOR LIVER FIBROSIS: INVESTIGATING APOCYNIN AND URSOLIC ACID IN A CCl₄ INDUCED RAT MODEL

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ABSTRACT:

Background: Hepatic fibrosis is characterized by excessive accumulation of extracellular matrix, leading to progressive disruption of liver function. Activation of hepatic stellate cells (HSCs), often driven by oxidative stress, plays a central role in fibrogenesis. Apocynin and Ursolic acid are plant-derived compounds with reported antioxidant and antifibrotic properties. This study aimed to evaluate their individual and combined therapeutic effects in a carbon tetrachloride (CCl₄)-induced hepatic fibrosis rat model.

Objective: To investigate the potential synergistic antifibrotic effects of Apocynin and Ursolic acid in CCl₄-induced hepatic fibrosis.

Method: Thirty Wistar rats were divided into five groups: normal control, disease control (CCl₄), and three treatment groups receiving Apocynin (3 mg/kg), Ursolic acid (20 mg/kg), or their combination. Hepatic fibrosis was induced with CCl₄ (1 ml/kg, intraperitoneally, twice weekly for 3 weeks), followed by a 21-day oral treatment period. Biochemical parameters

including SGPT, SGOT, ALP, GGT, hydroxyproline, total protein, and albumin were evaluated. Oxidative stress markers such as reduced glutathione, catalase activity, TBARS, and tissue hydroxyproline were measured. Histopathological analysis was performed using H&E and Masson's trichrome staining.

Results: Both Apocynin and Ursolic acid significantly reduced serum and tissue hydroxyproline levels and improved antioxidant enzyme activity. Histological analysis indicated attenuation of fibrosis in treated groups. However, no significant synergistic enhancement was observed with the combination therapy.

Conclusion: Apocynin and Ursolic acid exhibit promising antifibrotic and antioxidant activity in a CCl_4 -induced hepatic fibrosis model. Further studies with prolonged treatment durations are warranted to explore potential synergistic effects.

Keywords: Apocynin, Ursolic acid, Hepatic fibrosis, Oxidative stress, Antioxidant therapy, Carbon tetrachloride (CCl_4)

INTRODUCTION:

Hepatitis B or C virus infection, alcohol, non-alcoholic fatty liver disease (NAFLD), autoimmune diseases, and genetic conditions like hemochromatosis (iron overload) and Wilson's disease (copper accumulation) are among the chronic liver tissue injuries that cause liver fibrosis, which is defined as a reversible wound-healing process [1]. The liver contains a dense network of phagocytes that uphold tolerance in non-inflammatory conditions and rapidly detect signals of hepatocyte stress and injury, resulting in the activation of pro-inflammatory pathways [35]. The prevalence of NAFLD rose by 58%, from 37.41 per 1,000 person-years (95% CI: 24.36–57.06) during 1994–2006, to 52.74 (95% CI: 36.15–76.34) in 2007–2008, and reaching 59.11 per 1,000 (95% CI: 39.64–87.26) in the 2010–2014 survey

period. The Prevalence of MASLD is notable, with symptoms ranging from uncomplicated steatosis to metabolic dysfunction-associated steatohepatitis (MASH), which may progress to advanced fibrosis and cirrhosis [2]. The build-up of extracellular matrix (ECM) proteins, primarily crosslinked collagens type I and type III, that replace injured normal tissue causes hepatic fibrosis, a fibrous scar [3]. Hepatic stellate cells (HSCs) reside the perisinusoidal space between hepatocytes and sinusoidal endothelial cell, serving as the principal source of activated myofibroblasts and portal fibroblast that facilitate the fibro genic process [4]. Multiple intrahepatic and extrahepatic factors can stimulate HSC activation, leading to the development of liver fibrosis. Molecular signalling pathways play a crucial role in regulating HSC activation [36].

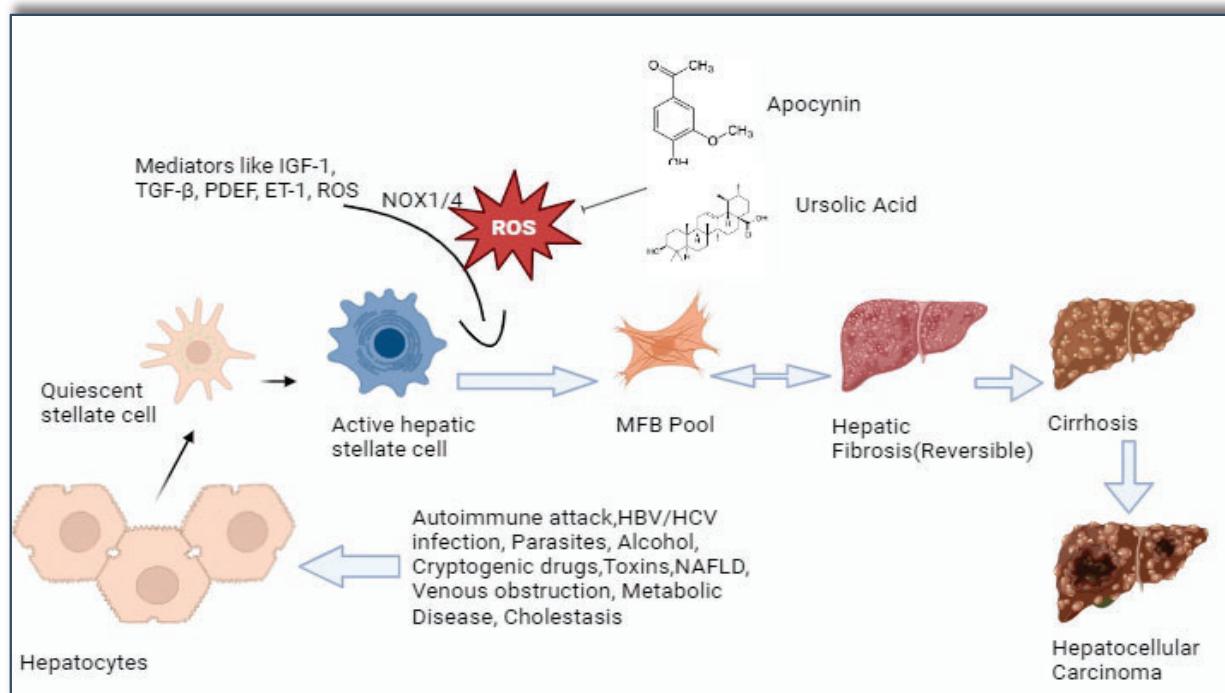


Figure 1: Pathophysiology of Hepatic Fibrosis with hypothesis (Apocynin and Ursolic acid may reverse liver fibrosis by inhibiting the NOX1/4 and ROS pathway) (NOX: NADPH oxidase, IGF-1: Insulin like growth factor-1, TGF-β: Tissue growth factor), PDEF: Prostate derived Ets factor, ET-1: Endothelin-1, ROS: Reactive oxygen species, MFB: Myofibroblast)

In response to liver injury, inflammatory mediators facilitate HSC activation and subsequent differentiation into myofibroblasts as shown in figure 1. Activated HSCs (aHSCs) are a major source of collagen in the liver and can abundantly secrete ECM proteins, tissue inhibitors of metalloproteinases, and matrix metalloproteinases (MMPs) that elicit liver architecture remodelling [5,6]. The proliferation and collagen synthesis of hepatic stellate cells is initiated by lipid peroxidation caused by oxidative stress [7–9].

Among various models, carbon tetrachloride (CCl_4) is the most widely

used hepatotoxin due to its high reproducibility, simplicity, and rapid induction of liver fibrosis within 4–12 weeks through intraperitoneal injection or inhalation. [40] Liver microsomal P450 must bio transform CCl_4 , a well-known model compound for the generation of chemically induced liver injury, in order to produce hepatotoxic metabolites, specifically trichloromethyl free radicals (CCl_3 and/or CCl_3OO).^[34] Protein thiols and sulfhydryl groups, including glutathione (GSH), can react with trichloromethyl (CCl_3) free radicals. The covalent binding of trichloromethyl free radicals to cell proteins is thought to be the

first step in a series of events that ultimately result in membrane lipid peroxidation and, ultimately, cell necrosis [10].

Another commonly used model is thioacetamide (TAA), which induces persistent hepatocyte damage and bridging fibrosis through oxidative stress and toxic metabolites. However, TAA is highly toxic and has a longer induction period. Dimethyl nitrosamine (DMN) causes severe liver fibrosis characterized by iron deposition, haemorrhagic necrosis, and advanced ECM cross-linking. Diet-induced models such as high-fat diet (HFD) and methionine- and choline-deficient (MCD) or choline-deficient, L-amino acid-defined (CDAA) diets mimic metabolic features of NAFLD/NASH and induce fibrosis over extended periods. Bile duct ligation (BDL) and DDC diet are used to model cholestatic liver injury and biliary fibrosis [41] while genetically modified models like *Mdr2*^{-/-} and *TAK1*^{-/-} mice replicate primary sclerosing cholangitis and other genetic cholangiopathies [42]. Alcohol-induced models alone show minimal fibrosis but become effective when combined with a second insult such as CCl_4 or high-fat diets [43].

The gold standard for evaluating fibrosis and necro-inflammatory activity is liver biopsy; however, this invasive method is expensive, linked to morbidity and mortality, and susceptible to sampling error because of the variability in fibrosis distribution and interpretation [38].

Current treatment options for hepatic fibrosis include several drug categories. Anti-hepatitis viral infection drugs, such as faldaprevir, ribavirin, and peginterferon alfa-2a, are commonly used, with additional agents like simeprevir, daclatasvir, and sofosbuvir currently under clinical evaluation [11–13]. For primary biliary cholangitis, the FDA has approved obeticholic acid and ursodeoxycholic acid, both of which may help mitigate cholangitis-induced liver fibrosis [14]. Furthermore, fibroblast growth factor (FGF) regulators and analogues, including pegbelfermin, have shown promise in treatment strategies [15]. FXR agonists, notably obeticholic acid, are also utilized [16], while farglitazar (GI262570), a peroxisome proliferator-activated receptor- γ (PPAR γ) agonist, has demonstrated the ability to inhibit hepatic stellate cell activation [17]. Picoside-I and kutkoside are free radical scavengers (superoxide anion $\text{O}_2^\bullet$) and inhibit lipid peroxidation in liver tissue [37]. These treatments represent a range of therapeutic approaches aimed at addressing the underlying mechanisms of hepatic fibrosis. Rats testing has revealed that silymarin supports liver fibrosis treatment by re-establishing the concentration of smooth muscle α -actin (α -SMA) in CCl_4 [18]. Curcumin significantly reduced the progression of liver fibrosis in CCl_4 injected rats [19]. Naringenin, a flavonoid found in citrus fruits, bergamot, and tomatoes, was administered orally at a dose of 50 mg/day in the form of nanoparticles for 2 months

to male Wistar albino rats (200–250 g), previously induced with liver fibrosis using CCl₄ (2 ml/kg body weight, intraperitoneally, twice a week for 8 weeks). The treatment reduced fibrosis and upregulated MMP-2 expression, as confirmed by gelatin zymography [39].

The phytochemicals such as apocynin, Ursolic acid act on the mediators and inhibit the HSC activation stage. Apocynin and Ursolic acid are NOX inhibitors and act as antioxidants and this study helps to

determine the effect of phytochemicals on hepatic fibrosis[20,21]. As mentioned earlier these phytochemicals affects different mediators individually so the purpose of this study aimed to find out if combinations of these phytochemicals can produce synergistic effect in reversing of liver fibrosis by biochemical analysis as well as liver fibrosis quantification by tissue parameters [22]. Study was carried out to determine the effect of rational phytochemicals' combination in chemical induced hepatic fibrosis.

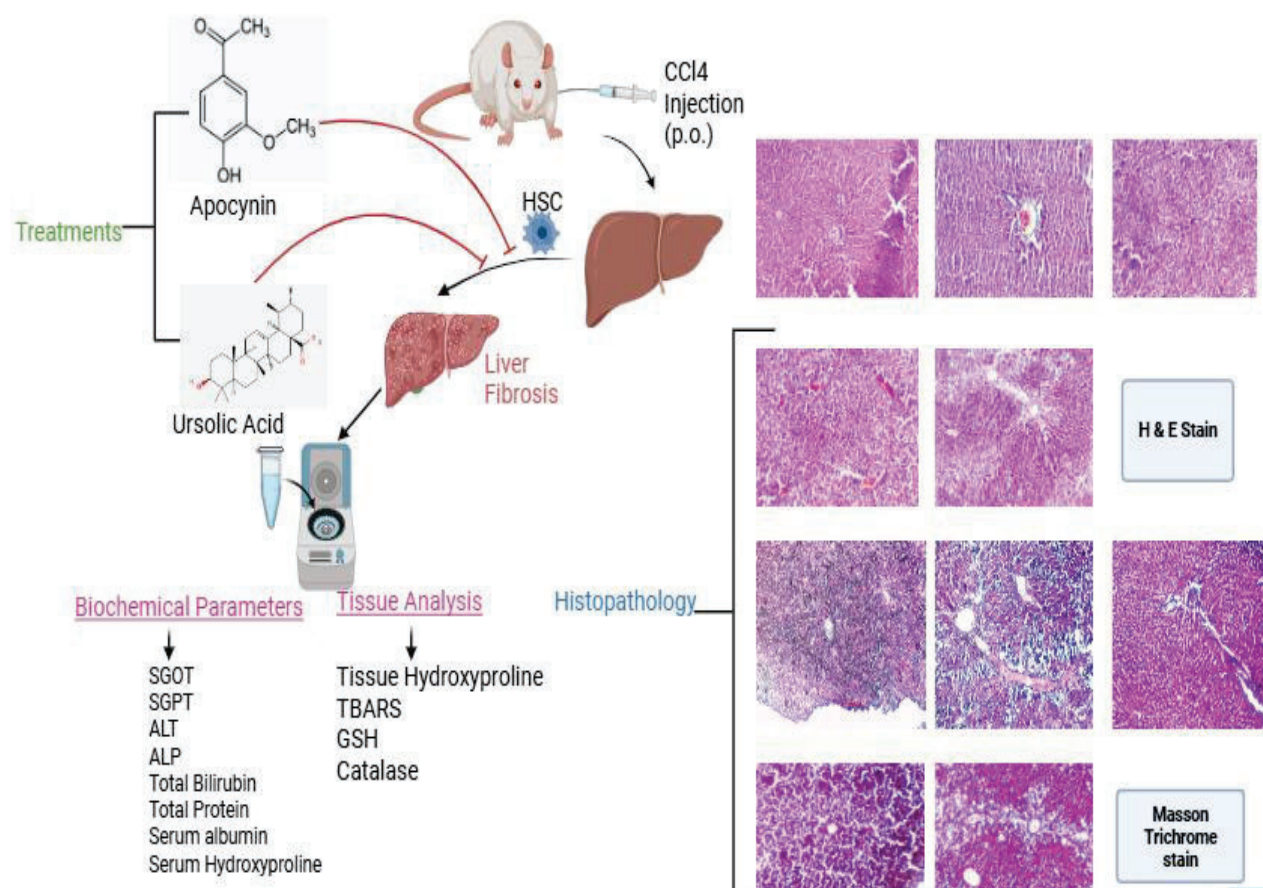


Figure 2: Evaluation of Apocynin and Ursolic Acid in CCl₄-Induced Liver Fibrosis: Experimental Design and Histopathology

Materials and Methods:

Ethics Approval:

Prior permission of experiments on animals were taken from the Institutional Animal Ethics Committee. (Protocol no-LMCP/IAEC/22/0007). All the experiments on animals were conducted as per the guidelines of committee for the purpose of control and supervision of experiments on animals (CPCSEA), Ministry of Environment, forests & climate change, Animal Welfare division, Government of India.

Animals:

Adult Wistar albino rats, both male and female, with an average weight of approximately 300 g were taken. After an acclimatization period, the animals were randomized in five experimental groups, ensuring an equal distribution of males and females within each group to assess the gender-specific differences in fibrosis progression.

Induction of Liver fibrosis:

Fibrosis was induced by using CCl₄ (1ml/kg, in olive oil, 1:1 ratio, p.o.)[23]. CCl₄ was administered twice a week via oral route for 6 weeks[24]

Treatment drugs:

Phytochemicals like apocynin and Ursolic acid was scientifically proved to reverse the liver fibrosis. Apocynin 3mg/kg p.o.

and Ursolic acid 20 mg/kg p.o., both were dissolved in DMSO.

Design of the study:

Figure 2 shows that rats were randomized in 5 different groups (each group had 3 male and 3 female). Rats have received CCl₄ dose as per body weight for twice a week for 6 weeks. Group 1: normal control (0.9% saline). Group 2: disease control (CCl₄ {1ml/kg} in olive oil, P.O.) Group 3: CCl₄ (1ml/kg, p.o.) + Apocynin (3mg/kg, p.o) Group 4: CCl₄ (1ml/kg, p.o.) + Ursolic acid (20mg/kg, P.O.) Group 5: Combination: CCl₄ (1ml/kg, P.O.) + Apocynin (3mg/kg, P.O.) + Ursolic acid (20mg/kg, P.O.)[25] To induce hepatic fibrosis, carbon tetrachloride (CCl₄) was administered twice a week for 21 consecutive days. After this induction period, treatment groups received treatments along with CCl₄ for the assessment of the protective effects of the phytochemicals, both individually and in combination, against CCl₄-induced liver fibrosis. The food-water intake and changes in body weight has been recorded weekly. The SGPT and SGOT parameters were checked every week and serum hydroxyproline was checked every week after 21 days. The remaining parameters alkaline phosphate, GGT, Total serum protein, Serum albumin, tissue hydroxyproline, catalase, TBARS, reduced glutathione, total bilirubin was checked at the end of the treatment[26].

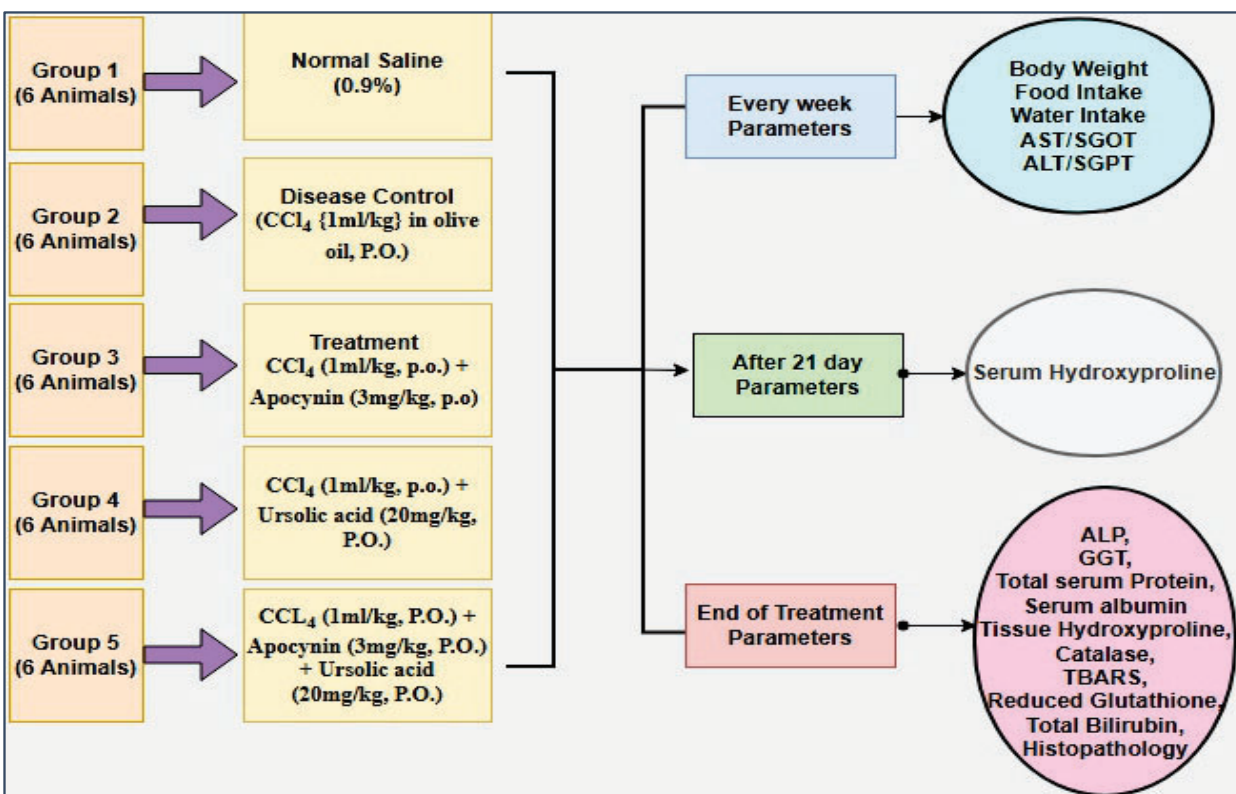


Figure 3: Detailed study design with timeline and description (CCl₄: carbon tetrachloride, P.O.: per Oral, AST: Aspartate Transaminase, SGOT: Serum glutamic-oxaloacetic transaminase, ALT: Alanine aminotransferase, SGPT: Serum glutamate pyruvate transaminase, ALP: Alkaline phosphate, GGT: Gamma Glutamyl transferase, TBARS: Thiobarbituric acid reactive substances)

Preparation of blood samples

The blood samples were collected by puncturing the retro-orbital plexus under the light ether anaesthesia. The blood was be allowed to lot and serum was separate by centrifugation at 3000rpm for 15 min and used for assessment of liver function tests by using available kit and data will be record[27].

Preparation of liver homogenate:

Liver homogenate, derived from 1 g of hepatic tissue, was collected from all rats at the conclusion of the experiment,

subsequently washed and homogenized in an ice-cold which has 1.15% potassium chloride solution within a 50 mmol potassium phosphate buffer (pH 7.4) to achieve a 10% (W/V) liver homogenate (weight of liver tissue in grams per volume of buffer in millilitres) [26]. The liver tissue was homogenized utilizing a sonicator. The homogenate was subsequently centrifuged at 4000 rpm for 5 minutes at 4 °C. The obtained supernatant was utilized to ascertain the content of reduced glutathione (GSH), TBARS, and to evaluate the activities of hydroxyproline and catalase (CAT).[26]

Assay methods:

Liver function tests: The activity of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphate, GGT, Total serum protein, Serum albumin, total bilirubin, Serum Hydroxyproline was performed [33].

Oxidative markers in liver homogenate: The level of lipid peroxidation (TBARS), GSH, catalase, tissue hydroxyproline was performed.

Histopathology:

At the end of the experiment all the animals sacrificed by cervical dislocation and quickly liver was excised and immediately stored at -20°C. A portion of liver stored separately in formalin (10% formaldehyde solution) for histopathological studies. 4 mm thick section of liver were cut and processed, and stained with either haematoxylin and eosin (H&E) for studying histopathological changes and Masson's trichrome stain for determination of the area-based percentage of reactive collagen.[22]

Statistical Analysis:

The results are presented as mean $\pm$ standard error of the mean (SEM) for each experimental group. One-way analysis of variance (ANOVA) was conducted for statistical analysis, followed by Tukey's post hoc test for multiple comparisons, using GraphPad Prism software. A p-value less than 0.05 was deemed statistically significant.

Results:

The disease control (CCl₄) group exhibited a notable decrease in body weight, food intake, and water intake due to hepatic fibrosis. In contrast, the treatment groups maintained better food and water intake levels, with only a moderate reduction in body weight compared to the normal control group. Additionally, water intake in the treatment groups showed only a 10–20% variation, demonstrating a protective effect against severe metabolic disturbances seen in the disease control group.

Biochemical Parameter analysis:

1. Liver Function Tests

Treatment with Apocynin, Ursolic acid, and their combination resulted in significant improvements in liver function parameters, suggesting their hepatoprotective potential against CCl₄-induced liver damage. Serum SGOT (AST) and SGPT (ALT) levels were markedly decreased in all treatment groups compared to the disease control (*p<0.05), with Apocynin and Ursolic acid alone showing a more pronounced effect than their combination (i & ii). Similarly, alkaline phosphatase (ALP) levels exhibited a significant decline in the treatment groups (*p<0.05), though the reduction was slightly less in the combination group compared to individual treatments(iii). Gamma-glutamyl transferase (GGT) levels increased in all

treated groups, indicating a response to liver injury repair mechanisms(iv).

2. Protein Metabolism

All treatment groups exhibited reduced total protein and albumin levels in comparison to the disease control (* $p < 0.05$). The combination treatment did not show a superior effect over Apocynin or Ursolic acid alone, suggesting that individual treatments might be more effective in maintaining protein synthesis (v & vi).

3. Collagen Turnover and Fibrosis Markers

Serum hydroxyproline levels were significantly reduced in the Apocynin and Ursolic acid treatment groups, reflecting a reduction in collagen synthesis and liver fibrosis (* $p < 0.05$) (vii). However, the combination treatment displayed higher variability, indicating no additional benefit over individual treatments. Similarly, tissue hydroxyproline levels were markedly decreased in all treated groups (* $p < 0.05$), further supporting their anti-fibrotic potential(viii).

4. Oxidative Stress Markers

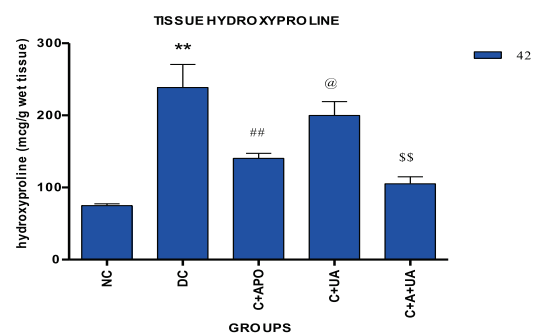
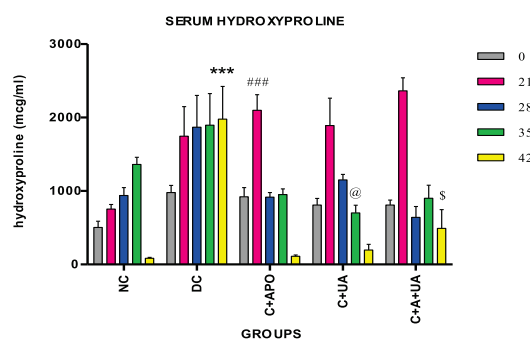
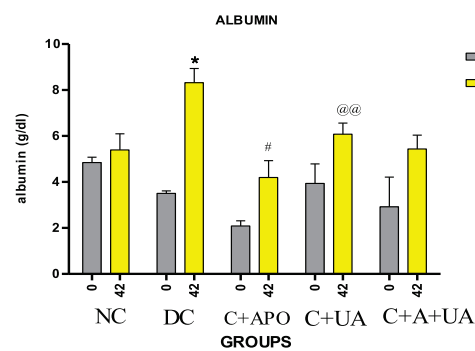
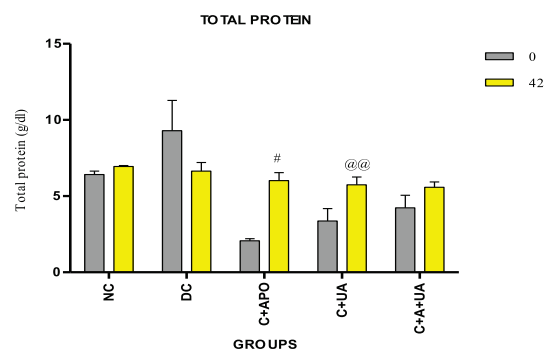
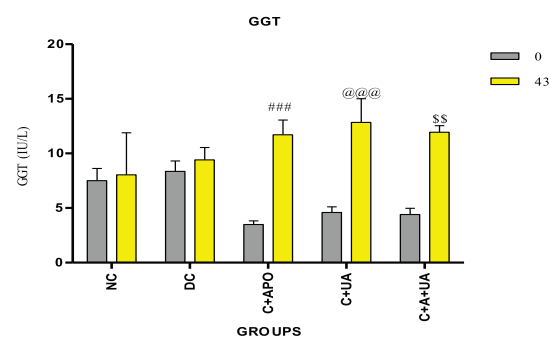
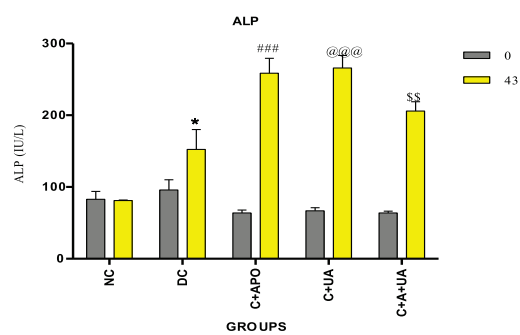
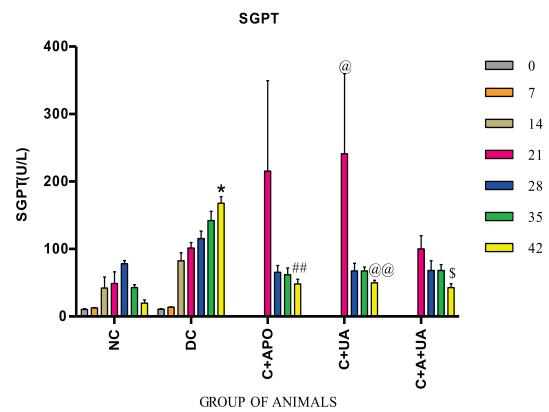
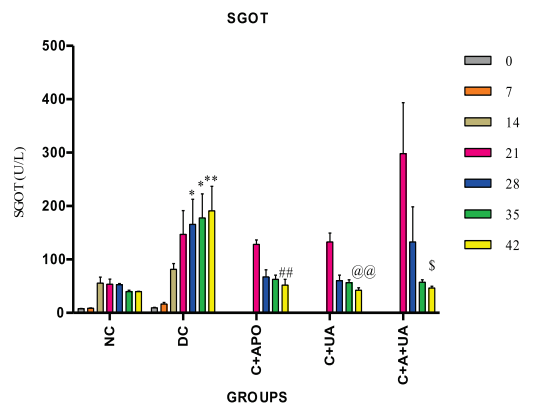
The levels of reduced glutathione (GSH) significantly increased across all treatment groups (* $p < 0.05$), highlighting their

antioxidant effects in mitigating oxidative stress(x). However, Thiobarbituric acid reactive substances (TBARS), an indicator of lipid peroxidation, exhibited no significant changes, suggesting minimal impact on lipid peroxidation pathways(xi)(28).

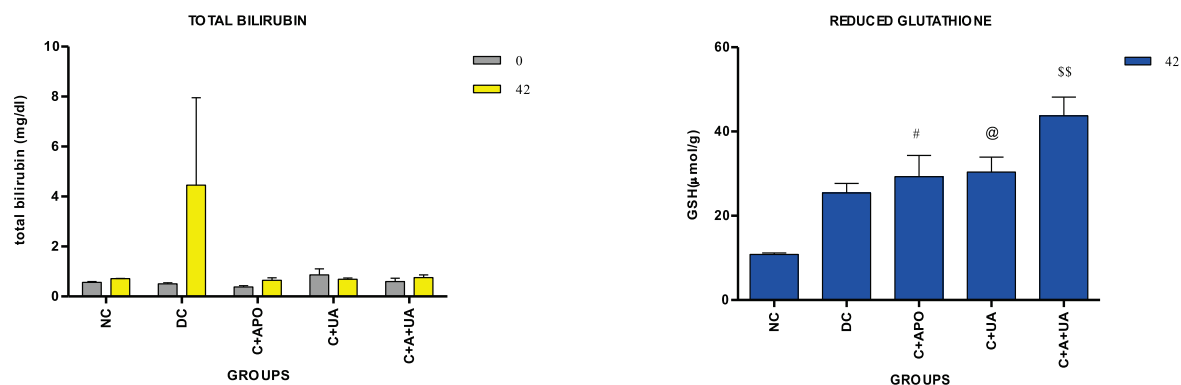
5. Biliary Function and Enzymatic Antioxidant Activity

Serum bilirubin levels remained stable across all groups, indicating no significant effect on bile metabolism(ix). Catalase (CAT) activity was comparable across all treatment groups and the disease control, suggesting that these treatments did not significantly influence enzymatic antioxidant defence mechanisms(xii).

Overall, the individual treatments of Apocynin and Ursolic acid showed more significant improvements in liver function and fibrosis markers compared to the combination, suggesting that while both compounds have therapeutic potential, the combination treatment did not provide the expected synergistic benefit. Both compounds exhibited notable effects on oxidative stress, liver damage markers, and collagen accumulation, suggesting a beneficial role in the reversal of liver fibrosis.

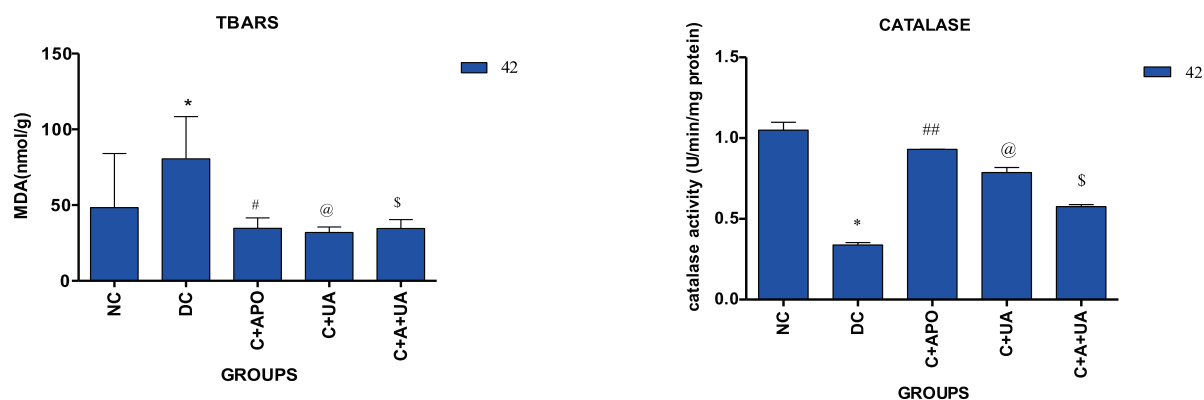


vii



X

ix



xii

xi

Figure 4: Result of analytical parameters

Figure Ligand: Effect of Apocynin, Ursolic acid, and their combination on biochemical parameters in CCl_4 -induced liver damage. Treatment with Apocynin and Ursolic acid alone significantly reduced SGOT, SGPT, and ALP levels compared to disease control, with a more pronounced effect than the combination treatment. Serum and tissue hydroxyproline levels were reduced in both individual treatments, indicating decreased collagen synthesis and liver fibrosis, while the combination treatment showed variability without superior effects.

Glutathione levels were significantly increased in all treatment groups, highlighting antioxidant effects, though no significant changes in TBARS, catalase activity, or bilirubin levels were observed. These findings suggest that Apocynin and Ursolic acid exhibit therapeutic potential in reversing liver fibrosis, with the combination treatment not providing a synergistic benefit. (* $p < 0.05$, # $p < 0.05$, @ $p < 0.05$, \$ $p < 0.05$). NC: Normal control, DC: Disease control, C+APO: CCl_4 (carbon

tetrachloride) + Apocynin, C+UA: CCl₄(carbon tetrachloride) + Ursolic Acid, C+A+UA: CCl₄(carbon tetrachloride) + Apocynin+ Ursolic acid.

Histopathology:

(A) Haematoxylin & Eosin (H&E stain):

Figure A, histopathological examination of liver tissue from male and female rats, stained with Haematoxylin and Eosin (H&E), reveals notable differences in the structure of the central vein, sinusoidal space, and the arrangement of hepatocytes across various experimental groups, including normal control, disease control, and treatment groups. Figures a and b: In the normal control group, the liver architecture is well-preserved, exhibiting typical hepatocyte arrangement, a regular central vein, and evenly spaced sinusoidal structures. Figures c and d: The disease control group demonstrates significant histopathological alterations, including disorganized hepatocytes, disruption of the central vein, and irregularities in the sinusoidal spaces, indicating liver damage.

Figures e and f: The Apocynin-treated group shows improved hepatic architecture, with better-preserved hepatocytes, less damage to the central vein, and more uniform sinusoidal spaces compared to the disease control group, suggesting a protective effect. Figures g and h: The Ursolic acid-treated group also exhibits reduced hepatocyte damage, improved arrangement, and less central vein disruption, along with a more regular sinusoidal structure, similar to the Apocynin-treated group, indicating comparable therapeutic efficacy. Figures i and j: The combination treatment group demonstrates a histopathological profile similar to that of the individual treatment groups (Apocynin and Ursolic acid), with less hepatocyte damage, better preservation of central vein integrity, and improved sinusoidal structure relative to the disease control group. This histological analysis suggests that both Apocynin and Ursolic acid, individually and in combination, provide significant hepatoprotection against the structural damage induced in the disease control group.

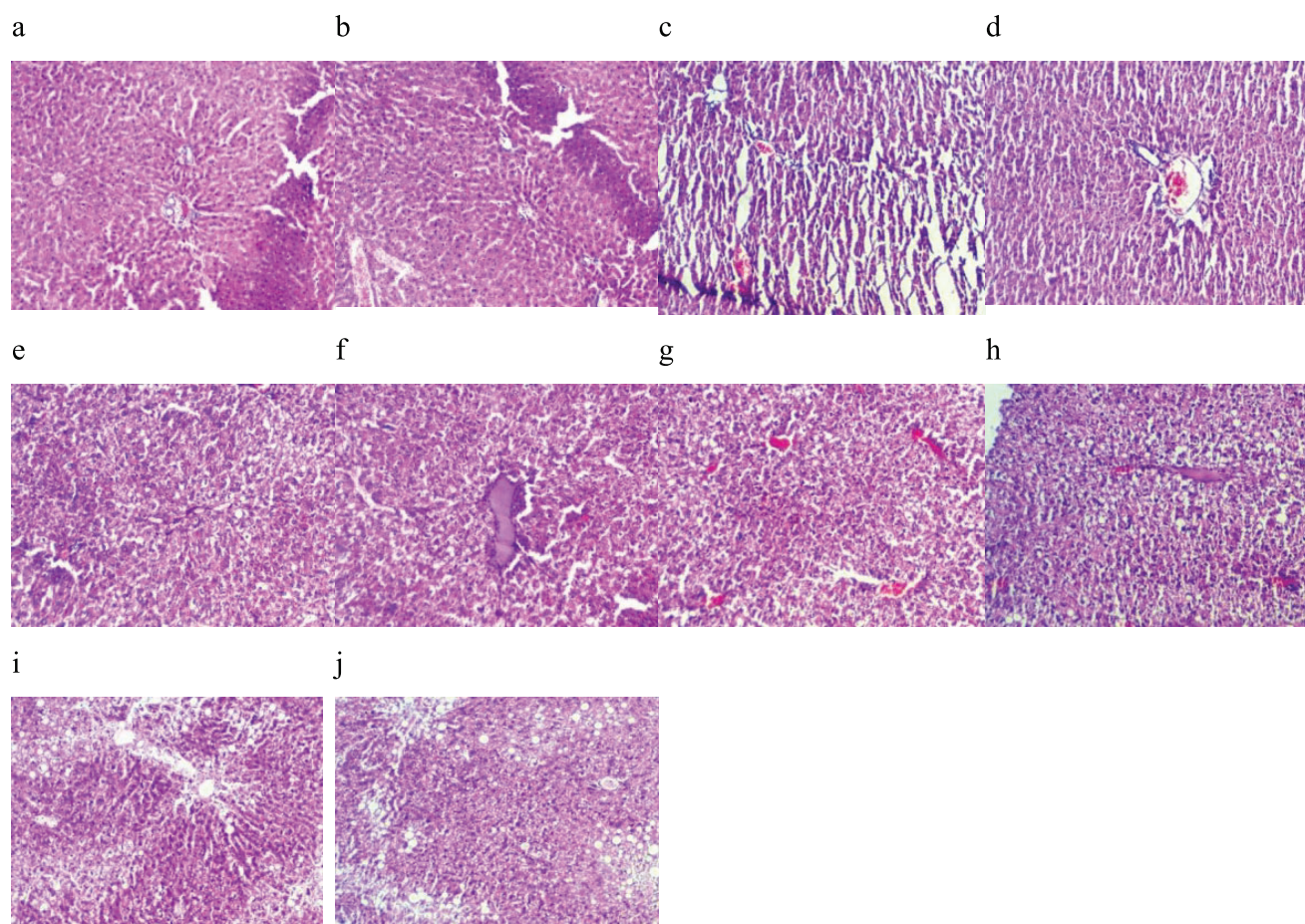


Figure 5: Histopathology of liver with hematoxylin and eosin(H&E) stain:

Histopathological analysis of liver tissue stained with H&E. **(a, b)** Normal control: Intact liver structure with regular hepatocyte arrangement, central vein, and sinusoidal spaces. **(c, d)** Disease control: Disrupted hepatocytes, central vein, and sinusoidal irregularities, indicating liver damage. **(e, f)** Apocynin-treated: Improved architecture with preserved hepatocytes and less central vein disruption. **(g, h)** Ursolic acid-treated: Similar protective effects as Apocynin, with better hepatocyte arrangement and sinusoidal integrity. **(i, j)** Combination treatment: Comparable protection to individual treatments. Both Apocynin and Ursolic acid provide hepatoprotection against liver damage.

(B) Masson Trichrome:

In Figure B, histopathological analysis of liver tissue from male and female rats, stained with Masson's Trichrome (MT), reveals significant differences in collagen

accumulation, hepatocyte arrangement, and the structural integrity of the central vein and sinusoidal spaces across the experimental groups, including normal control, disease control, and treatment groups. Figures a and b: In the normal

control group, the liver architecture is intact, with a regular arrangement of hepatocytes, a well-formed central vein, and evenly spaced sinusoidal structures, indicative of healthy liver tissue. Figures c and d: In the disease control group, there is marked collagen accumulation, disorganization of hepatocytes, severe damage to the central vein, and significant alterations in the sinusoidal spaces, reflecting substantial hepatic injury. Figures e and f: The Apocynin-treated group demonstrates a significant reduction in collagen deposition, with a more preserved hepatocyte arrangement, less damage to the central vein, and improved sinusoidal architecture compared to the disease control group, indicating a protective effect on liver structure. Figures g and h: The Ursolic acid-treated group exhibits a similar protective profile to the Apocynin-treated group, with minimal

collagen accumulation, better-preserved hepatocyte arrangement, and reduced damage to the central vein and sinusoidal spaces, suggesting comparable efficacy in maintaining liver architecture. Figures i and j: The combination treatment group shows comparable effects to the individual treatments in terms of collagen accumulation, hepatocyte arrangement, central vein structure, and sinusoidal space preservation. The combined treatment appears to offer synergistic benefits in reducing liver damage and improving tissue integrity. These findings highlight the potential of both Apocynin and Ursolic acid, either individually or in combination, in significantly attenuating liver damage, promoting the preservation of normal liver architecture, and reducing pathological collagen deposition in response to hepatic injury.

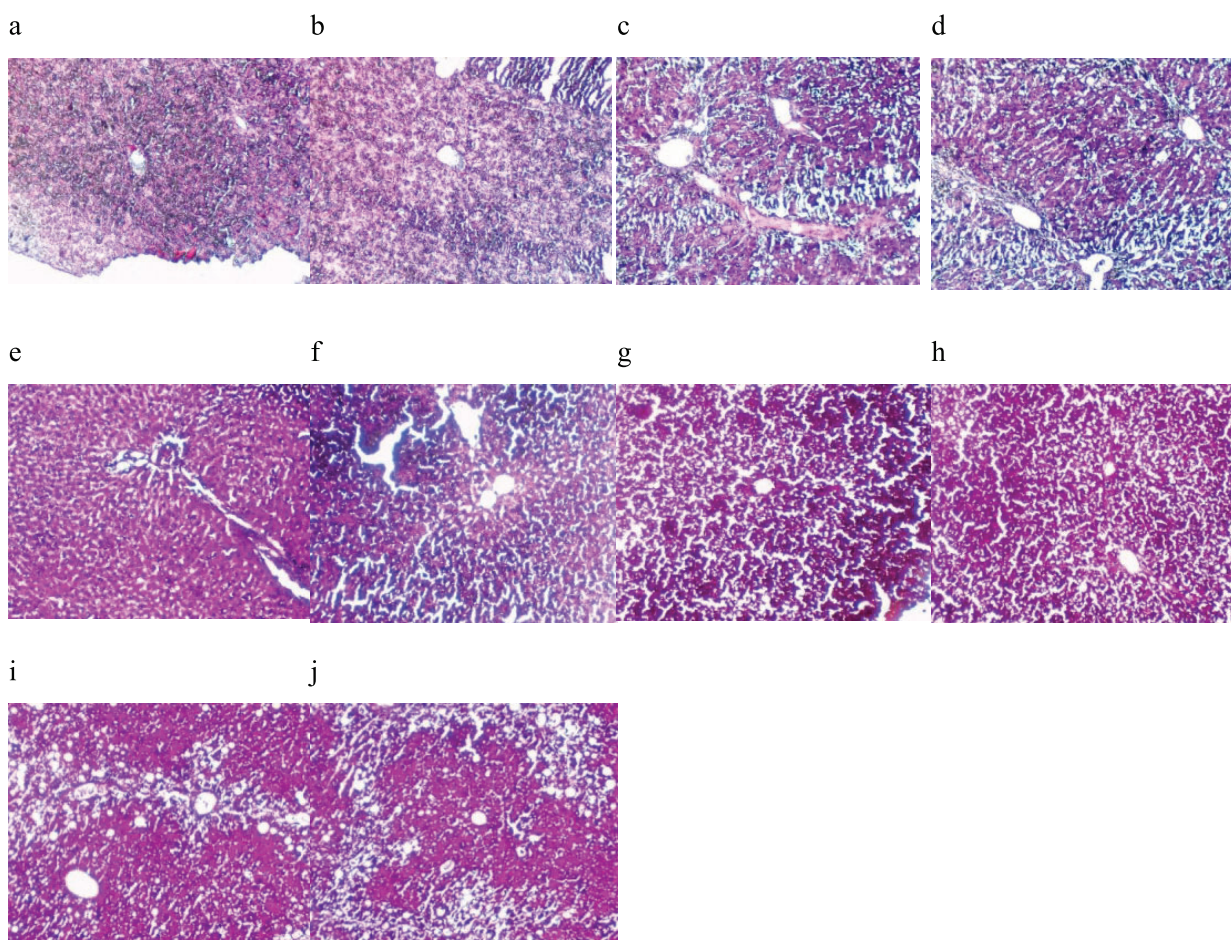


Figure 6: Histopathology of liver with Masson trichrome stain: Histopathological analysis of liver tissue stained with Masson's Trichrome (MT) showing collagen accumulation and liver architecture. **(a, b)** Normal control: Intact liver structure with regular hepatocyte arrangement, central vein, and sinusoidal spaces. **(c, d)** Disease control: Marked collagen deposition, disorganized hepatocytes, and severe damage to central vein and sinusoidal spaces, indicating significant liver injury. **(e, f)** Apocynin-treated: Reduced collagen accumulation, better-preserved hepatocytes, and improved central vein and sinusoidal structure. **(g, h)** Ursolic acid-treated: Similar protective effects to Apocynin, with minimal collagen deposition and improved liver architecture. **(i, j)** Combination treatment: Comparable effects to individual treatments, showing enhanced protection against liver damage. Both Apocynin and Ursolic acid attenuate collagen deposition and preserve liver tissue integrity.

Discussion:

The purpose of this study was to investigate if combination of Apocynin and

Ursolic acid work better than individual in CCl_4 induced hepatic fibrosis in rat model [33]. Liver fibrosis is a progressive pathological condition that arises as a

consequence of chronic liver injury due to various etiologies, such as alcohol consumption, viral infections, chemical-induced toxicity, and genetic disorders. HSCs have been identified as the principal cells responsible for matrix production during liver fibrosis. Despite the significant clinical burden of liver fibrosis, current therapeutic strategies to reverse fibrosis are still limited. Previous studies have demonstrated the potential of phytochemicals, such as Apocynin and Ursolic Acid, in ameliorating liver fibrosis induced by carbon tetrachloride (CCl_4) in rodent models[29,30]. However, the commonly used CCl_4 dosing regimen, administered once a week, induces hepatotoxicity but does not fully result in fibrosis. Therefore, modifying the CCl_4 dosing regimen to twice a week for six weeks was deemed necessary to reliably induce liver fibrosis and better assess the effects of Apocynin and Ursolic Acid as antifibrotic agents.

In this study, male and female rats were divided into five experimental groups: (1) normal control, (2) CCl_4 (disease control), (3) CCl_4 + Apocynin, (4) CCl_4 + Ursolic Acid, and (5) CCl_4 + Apocynin + Ursolic Acid. The dose of CCl_4 used was 1 mL/kg, administered twice a week for six weeks, dissolved in olive oil. Apocynin was administered at 3 mg/kg, and Ursolic Acid at 20 mg/kg, both by oral gavage. Treatment with the phytochemicals began on day 21 after CCl_4 administration and continued for the remaining 21 days of the study. The primary aim was to evaluate the

antifibrotic effects of Apocynin, Ursolic Acid, and their combination in the context of oxidative stress-mediated liver fibrosis.

Oxidative stress plays crucial role in the pathogenesis of liver fibrosis, contributing to hepatocyte injury, activation of hepatic stellate cells (HSCs), and excessive extracellular matrix (ECM) accumulation[28]. Both Apocynin and Ursolic Acid have demonstrated antioxidant properties, targeting reactive oxygen species (ROS) and modulating the activity of NADPH oxidases (NOX1/4), which are involved in fibrogenesis. In this study, we evaluated oxidative stress markers, including reduced glutathione (GSH), lipid peroxidation (TBARS), and catalase activity, to assess the impact of the phytochemicals on oxidative damage and their potential to reverse fibrosis.

The present investigation demonstrated a significant elevation in the activities of ALT, AST, GGT, ALP, and bilirubin, as well as serum hydroxyproline, indicating hepatic tissue damage in the CCl_4 -treated group following three weeks of treatment. Tissue hydroxyproline also increase after CCl_4 treatment. This may come from inflammation and hepatocellular injury, which increases permeability of cell membrane and releases transaminases into the bloodstream [26]. Elevated GGT activity is related with several primary and secondary hepatobiliary disorders; increased blood levels of GGT correlate with cholestasis resulting from intrahepatic or extrahepatic biliary obstruction.

Significant reductions in serum ALT, AST, and GGT levels, bilirubin, serum hydroxyproline levels in rats co-treated with CCl₄+Apocynin+Ursolic acid were recorded. In this study, rats administered CCl₄ exhibited diminished GSH levels and CAT activity after three weeks of treatment, alongside an increase in LPO, as indicated by elevated MDA levels ^[26]. This effect may have arisen from damage to cellular constituents and peroxidation of membrane lipids[31]. The principal mechanism of CCl₄-induced hepatic injury is oxidative stress, in which hepatic cytochrome P450 metabolizes CCl₄ to generate the trichloromethyl radical, leading to hepatocyte necrosis, compromised liver function, and disruption of hepatic architecture due to free radicals produced by CCl₄ exposure. The reactive oxygen species (ROS) deplete endogenous antioxidants and interact with cellular lipids which leading to lipid-peroxidation. [26][32]

Biochemical parameters such as SGOT, SGPT, serum and tissue hydroxyproline, and liver function markers were significantly improved with the phytochemical treatments. SGOT and SGPT levels, indicators of liver damage, were markedly reduced in the treatment groups compared to the disease control group. Furthermore, serum and tissue hydroxyproline levels, which reflect collagen deposition and fibrosis, were significantly decreased, further supporting the antifibrotic effects of Apocynin and Ursolic Acid. The disease control group

showed elevated levels of these biomarkers, consistent with severe liver damage and fibrosis.

Histopathological evaluation using Haematoxylin and Eosin (H&E) staining revealed extensive hepatocyte damage, increased sinusoidal space, and disrupted liver architecture in the CCl₄-treated group. In contrast, the treatment groups (both individual and combination treatments) exhibited improved liver morphology, with less hepatocyte damage, more organized hepatocyte arrangement, and reduced sinusoidal space, suggesting a protective effect on liver tissue.

Masson's Trichrome staining, which highlights collagen deposition, confirmed the biochemical findings. The disease control group exhibited significant collagen accumulation, a hallmark of liver fibrosis. Both Apocynin and Ursolic Acid treatments reduced collagen deposition compared to the disease control group, while the combination treatment showed similar effects but with slightly higher collagen accumulation compared to the individual treatments. This suggests that while the combination of Apocynin and Ursolic Acid provides some antifibrotic effects, the individual phytochemicals were more effective in reducing ECM accumulation and reversing liver fibrosis.

In conclusion, this study demonstrates that both Apocynin and Ursolic Acid, either alone or in combination, exert beneficial effects in reducing oxidative stress and

reversing liver fibrosis induced by CCl₄ in rats. The individual treatments showed superior efficacy compared to the combination treatment, likely due to their shared target (NOX1/4) and the potential lack of an additive effect when used together. These findings highlight the potential of Apocynin and Ursolic Acid as promising therapeutic agents for liver fibrosis, with further studies needed to explore optimal dosing regimens and combination therapies for enhanced antifibrotic effects.

Conclusion:

Biochemical analyses (serum and tissue) and histopathological evaluations (H&E and Masson's trichrome staining) revealed significant liver damage in the disease control group, characterized by severe distortion of hepatocyte arrangement around the central vein, increased sinusoidal spaces, and extensive collagen deposition. In contrast, treatment groups exhibited less damage to hepatocyte architecture, reduced sinusoidal space alterations, and decreased collagen accumulation when compared to the disease control group. However, the combination therapy did not demonstrate enhanced efficacy compared to the individual treatments with Apocynin and Ursolic acid. Rather, the combination produced results similar to those observed with each phytochemical administered alone.

One possible explanation for the lack of synergistic effects could be the shared mechanism of action of both Apocynin and Ursolic acid, particularly their inhibition of NADPH oxidases (NOX1/4). It is plausible that the interaction between these two compounds may have led to an attenuated effect when used together. Additionally, the duration of treatment (21 days) may not have been sufficient to fully capture the potential synergistic benefits of the combination. Extending the treatment period for an additional week may provide further insight into whether the combined therapy could outperform the individual treatments, as a longer exposure may be necessary to observe the full therapeutic potential of the combined phytochemicals.

Abbreviations:

CCl₄ – Carbon tetrachloride

MMPs- Matrix metalloproteinases

HSCs – Hepatic stellate cells

ECM – Extracellular matrix

SGPT – Serum glutamate pyruvate transaminase

SGOT – Serum glutamate oxaloacetate transaminase

ALP – Alkaline phosphatase

GGT – Gamma-glutamyl transferase

α-SMA- Smooth muscle alpha-actin

TBARS – Thiobarbituric acid reactive

substances

GSH – Reduced glutathione

NOX – NADPH oxidase

H&E – Haematoxylin and eosin

MASLD -Metabolic Dysfunction-Associated Steatotic Liver Disease

NAFLD- Non-alcoholic fatty liver disease

FDA- Food and Drug Administration

FGF- Fibroblast growth factor

FXR- Farnesoid X Receptor

PPAR γ - Peroxisome proliferator-activated receptor-gamma

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