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Exploring the remarkable effect of ursodeoxycholic acid and *Allium sativum* combination on MMP-9 and TIMP-1 levels in cholestatic rat's model

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ABSTRACT

Background: Cholestasis is a disturbance in the production or flow of bile that causes excessive accumulation of bile fluid and damage in the liver. Chronic liver damage will lead to liver fibrosis. Matrix metalloproteinase-9 (MMP-9) and tissue inhibitors of metalloprotease-1 (TIMP-1) play a critical role in liver fibrosis formation. Although ursodeoxycholic acid (UDCA) and Allium sativum extracts have long been renowned for improving liver function, their combination in alleviating liver fibrosis through the MMP-9 and TIMP-1 inhibitory pathways has yet to be studied.
Objective: This study was conducted to assess the efficacy of UDCA and *Allium sativum* extract combination in altering MMP-9 and TIMP-1 levels, which are the main factors in the progression of liver fibrosis.
Methods: We performed an experimental study with a post-test-only control group on 36 Sprague-Dawley rats, which were randomly grouped into healthy, negative, positive, and three treatment groups with UDCA (13.5 mg) and graded doses of Allium sativum extract (3.6 mg; 7.2 mg; and 14.4 mg). Cholestasis induction was done by a choledochal duct ligation, and treatment was given for 14 days. Levels of MMP-9 and TIMP-1 were analyzed after treatment.
Results: Combining UDCA and *Allium sativum* improved the MMP-9 levels significantly in cholestatic rats, compared to administrating UDCA only ($p < 0.05$). Combining UDCA and *Allium sativum* increased the TIMP-1 level ($p < 0.05$). Although the MMP-9 level results were under our existing hypothesis, TIMP-1 results showed surprising results. Matrix metalloproteinase-9 levels were strongly negatively correlated with TIMP-1 levels, with a r-value= 0.981.
Conclusion: Combining *Allium sativum* and UDCA alleviates liver fibrosis progression through lowering levels of MMP-9 and increasing levels of TIMP-1.

INTRODUCTION

Cholestasis refers to a disruption in the production or flow of bile. It can be caused by obstructions at any stage of the bile secretion pathway, from the liver parenchymal cells on the basolateral (sinusoidal) membrane of the hepatocytes to the ampulla of Vater in the duodenum.[1] Increased bile acid levels in the serum or liver result from cholestasis. An overabundance of bile fluid buildup in the liver can harm hepatocytes and produce excessive free radicals.[2] High amounts of free radicals and cell damage are some of the risk factors for the formation of liver fibrosis, cholestatic liver injury, and liver function failure.[1,2]

Fibrosis occurs due to the accumulation of extracellular matrix following the activation and proliferation of hepatic stellate cells (HSCs).[3] Extracellular matrix is carefully controlled by a constant turnover in healthy liver tissue homeostasis, which is coordinated by a class of enzymes called matrix metalloproteinases (MMPs) and their particular inhibitors, tissue inhibitors of

metalloproteases (TIMPs), which are generated by activated HSCs. Hepatic stellate cells become extremely active and differentiate into their fibroblast phenotype following chronic injury. This equilibrium is upset during fibrogenesis, when TIMPs expression is higher than MMPs expression, impeding matrix breakdown.[4,5] In liver fibrosis, fibrolysis, liver carcinogenesis, and liver regeneration, nearly all known MMPs are important players. One of the MMPs that is most associated with the regulation of liver fibrogenesis is MMP-9. It is also associated with tissue remodeling when liver fibrosis develops in biliary atresia.[4]

Matrix metalloproteinase-9 encourages the apoptosis of HSCs, which aids in fibrogenesis. It is well known that TIMP-1 levels rise during hepatic fibrogenesis, preventing MMPs and extracellular matrix breakdown and ultimately resulting in fibrosis in sick livers.[5] Tissue inhibitors of metalloprotease-1 expression levels have been linked to both profibrogenic and carcinogenic activities, and there is evidence linking TIMP-1 to the development of hepatocellular carcinoma and liver fibrosis.[6]

Approximately 3% of all bile acids in the body are hydrophilic, such as UDCA or 3α, 7β-dihydroxy-5β-cholanic acid, which is a bile acid produced by the body. UDCA protects hepatocytes and cholangiocytes from bile acid-induced harm, including inflammation and mitochondrial malfunction. In patients with primary biliary cholangitis, a daily dose of 13–15 mg/kg body weight of UDCA raises the amount of UDCA in the bile acids by about 40–50%. When UDCA is administered, the amount of hydrophilic bile acids in stasis bile will vary. Due to its higher hydrophilicity and lower cytotoxicity, bile rich in UDCA causes less harm to bile duct cells, portal inflammation, and ductal proliferation. It has been demonstrated that UDCA triggers anti-apoptotic pathways, stopping Kupffer cells and liver macrophages from producing reactive oxygen species, thereby lowering stress and liver damage caused by oxidation. While UDCA can shield the liver from bile acid-induced damage, a previous study reveals that 35–40% of primary biliary cholangitis patients do not react well to UDCA treatment and have a dismal prognosis.[7]

In addition to its medicinal ingredients, *Allium sativum* (garlic) has long been used as a dietary component to prevent illness. It has long been used in traditional medicine as well as a preventative strategy against ailments like diabetes, cancer, aging, and cardiovascular disease.[8] Many research in the last ten years have documented the benefits of *Allium sativum* and its active components in a variety of diseases, including liver disorders. These benefits are mainly attributed to its ability to suppress inflammation and oxidative stress. The impact of garlic extract on cholestasis and liver fibrosis has been previously examined.[9]

Garlic extract reduces oxidative stress levels by producing glutathione, myeloperoxidase activity, and pro-inflammatory cytokines like Tumor Necrosis Factor Alpha (TNF-α) and Transforming Growth Factor Beta 1 (TGF-β1) to control protein levels, including matrix metalloproteinase, and oxidative stress levels. These results validate garlic extract as an adjuvant therapy for cholestasis and prevent cholestasis-related liver fibrosis.[10] However, there is no previous research about how *Allium sativum* extract, either by itself or in conjunction with UDCA, affects MMP-9 and TIMP-1, the primary mediators of the liver fibrosis process. This study aimed to evaluate the effectiveness of the combination of UDCA and *Allium sativum* extract in modifying MMP-9 and TIMP-1 levels, which are key elements in advancing liver fibrosis. Our results will significantly contribute to using one of the Indonesian herbals in managing cholestasis.

METHODS

We performed an experimental study with a post-test-only control group design in November 2023 at Universitas Gajah Mada Experimental Animal Laboratory.

Allium sativum extract

Allium sativum extract was used from PT. Sido Muncul Tbk. as a fine powder in capsule form. About 400 mg of *Allium sativum* extract, or 3.5% of the active ingredient *alliin*, and 3,500 fresh garlic are contained in each capsule. Adults should take 2 to 6 g (one to two cloves) of raw *Allium sativum* daily or one 400 mg pill (standardized to 1.3 percent alliin or 0.6 percent allicin) two to three times a day, as previously described.[11]

Animal models and bile duct ligation procedure

The minimum sample size was determined using the Institutional Animal Care and Use Committee Guidebook and the World Health Organization, which has five animals per group, adhering to the 3R principle (Replacement, Reduction, and Refinement). There were six groups with five rats per treatment group (the minimum sample size used was 30 rats). One rat was added to each treatment group to anticipate the mice exclusion due to drop-out criteria, making the total number of research samples 36 rats.

Acclimatization was administered to the 36 male Sprague-Dawley rats, weighing between 150 and 200 grams at the beginning of the study, before the experiment began. The bile duct ligation (BDL) technique, which requires laparotomy surgery, was used to produce cholestasis. The rats received an intramuscular injection of ketamine hydrochloride (10 mg/200 grBW)[12] as an anesthetic and 80 mg/200 grBW[13] of prophylactic antibiotic prior to surgery. A laparotomy, including an abdominal incision in the midline, was carried out under sterile conditions. A 4-0 silk suture was used to ligate the common bile duct. Three days of oral ibuprofen 3 mg/200 grBW was administered every eight hours to reduce surgical discomfort.[14]

The same surgical process was performed on sham-operated rats, except BDL. Following surgery, 30 BDL-operated rats were randomized into the other five groups, while six sham-operated rats were placed in the C1 group: Six rats were in each of the following groups: (C2) BDL, (C3) BDL + UDCA, (T1) BDL + UDCA + 3.6 mg *Allium sativum* extract, (T2) BDL + UDCA + 7.2 mg *Allium sativum* extract, and (T3) BDL + UDCA + 14.4 mg *Allium sativum* extract. Food and drink were provided according to the same schedule. UDCA was administered orally at a dose of 13.5 mg derived from the human therapeutic dose conversion.[15] *Allium sativum* extracts were administered orally by the laboratory attendant and researchers. For fourteen days, every treatment was administered nonstop.

Analysis of MMP-9 and TIMP-1 level

Blood from the rats' orbital vein was taken on the 15th day to measure MMP-9 and TIMP-1 serum levels with the ELISA Kit from Antibody-Sunlong Biotech Co. Ltd (Cat No: EL0019Hu and EL0028Ra).

Statistical analysis

The data from MMP-9 and TIMP-1 was initially processed utilizing coding, editing, data entry, and cleaning procedures. SPSS software was used for statistical analysis. The homogeneity test was conducted using the Levene test, while the normality test was conducted using the Shapiro-Wilk test. Given the anomalous distribution of the MMP-9 and TIMP-1 values, we performed the Kruskal-Wallis test, a nonparametric test, followed by the Mann-Whitney post hoc test. A 95% confidence range was selected, and a p-value of less than 0.05 was deemed significant. The Spearman's test was used to determine whether the MMP-9 and TIMP-1 levels were correlated, and the relationship levels were measured using the correlation coefficient.[16]

Ethics

The Health Research Ethics Committee of the Faculty of Medicine at Diponegoro University (No. 149/EC-H/KEPK/FK-UNDIP/XII/2023) has approved this study.

RESULTS

The descriptive and normality analysis of the MMP-9 level was addressed in Table 1. The MMP-9 level data were not normally distributed from the Shapiro-Wilk test. Therefore, we then continued with Kruskal Wallis as a nonparametric test. The lowest MMP-9 level was in the C1 group (0.34 pg/ml), while the highest was in the C2 group (1.25 pg/ml), indicating that the BDL-only group significantly increased the MMP-9 level and experienced hepatic injury. MMP-9 level decreased in the T1-T3 groups and was identified as the lowest in the T3 group (0.49 pg/ml), almost hitting the same level as the C1 group. These results showed that the combination of UDCA

and *Allium sativum* administration significantly improved the MMP-9 levels in cholestatic rats, compared to administrating UDCA only.

Table 1. Descriptive and normality analysis of matrix metalloproteinase-9 level

Groups	Median (min – max)	p£	pβ
Sham-operated group (C1)	0.34 (0.33 – 0.36)		
Bile duct ligated group (C2)	1.25 (1.21 – 1.29)		
Bile duct ligation + 13.5 mg UDCA group (C3)	1.04 (0.99 – 1.08)		
Bile duct ligation + 13.5 mg UDCA + 3.6 mg *Allium sativum* extract group (T1)	0.78 (0.76 – 0.8)	0.043*	<0.001**
Bile duct ligation + 13.5 mg UDCA + 7.2 mg *Allium sativum* extract group (T2)	0.61 (0.57 – 0.64)		
Bile duct ligation + 13.5 mg UDCA + 14.4 mg *Allium sativum* extract group (T3)	0.49 (0.46 – 0.5)		

£ Shapiro-Wilk; * Normal (p>0.05); β Kruskall wallis ** significant (p<0.05); UDCA: ursodeoxycholic acid

We found a significant difference in MMP-9 levels between the groups with a p-value of 0.001 (p<0.05), then continued with the Mann-Whitney as the comparison test (Table 2). Comparison of MMP-9 levels among all groups showed a significant result with a p-value of 0.004.

Table 2. Matrix metalloproteinase-9 level on Mann-Whitney p-value analysis between groups

Groups	C1	C2	C3	T1	T2	T3
Sham-operated group (C1)						
Bile duct ligated group (C2)	0.004*					
Bile duct ligation + 13.5 mg UDCA group (C3)	0.004*	0.004*				
Bile duct ligation + 13.5 mg UDCA + 3.6 mg *Allium sativum* extract group (T1)	0.004*	0.004*	0.004*			
Bile duct ligation + 13.5 mg UDCA + 7.2 mg *Allium sativum* extract group (T2)	0.004*	0.004*	0.004*	0.004*		
Bile duct ligation + 13.5 mg UDCA + 14.4 mg *Allium sativum* extract group (T3)	0.004*	0.004*	0.004*	0.004*	0.004*	

Notes: * significant (p<0.05); UDCA: ursodeoxycholic acid

The descriptive and normality analysis of the TIMP-1 level was addressed in Table 3. The TIMP-1 level data were not normally distributed according to the Shapiro-Wilk test. The BDL-only group had significantly decreased TIMP-1 levels (the lowest score: 131.1 pg/ml). The TIMP-1 levels increased in the T1-T3 groups and was identified as the highest in the T3 group (338.35 pg/ml. These results showed that DCA and *Allium sativum* combination significantly increased the TIMP-1 level in cholestatic rats, compared to administrating UDCA only.

Table 3. The descriptive and normality analysis of tissue inhibitors of metalloprotease–1 level

Groups	Median (min – max)	p£	pβ
Sham-operated group (C1)	367.22 (360.4 – 374.56)	0.003*	<0.001**
Bile duct ligated group (C2)	131.10 (125.11 – 137.64)		
Bile duct ligation + 13.5 mg UDCA group (C3)	226.97 (224.24 – 229.14)		
Bile duct ligation + 13.5 mg UDCA + 3.6 mg *Allium sativum* extract group (T1)	281.70 (277.07 – 283.61)		
Bile duct ligation + 13.5 mg UDCA + 7.2 mg *Allium sativum* extract group (T2)	311.93 (303.21 – 314.65)		
Bile duct ligation + 13.5 mg UDCA + 14.4 mg *Allium sativum* extract group (T3)	338.35 (335.89 – 346.24)		

£ Shapiro-Wilk; * Normal (p>0.05); β Kruskall wallis; ** significant (p<0.05, UDCA: ursodeoxycholic acid

Since the data was not normally distributed, therefore, we then continued with Kruskal

Wallis as a nonparametric test. We found a significant difference in TIMP-1 level between the groups with a p-value of 0.001 (p<0.05) (Tabale 4), then continued with the Mann-Whitney as the comparison test. Comparison of TIMP-1 level among all groups showed a significant result with a p-value of 0.004 (p<0.05).

Table 4. Mann-Whitney p-value of tissue inhibitors of metalloprotease–1 level between groups

Groups	C1	C2	C3	T1	T2	T3
Sham-operated group (C1)						
Bile duct ligated group (C2)	0.004*					
Bile duct ligation + 13.5 mg UDCA group (C3)	0.004*	0.004*				
Bile duct ligation + 13.5 mg UDCA + 3.6 mg *Allium sativum* extract group (T1)	0.004*	0.004*	0.004*			
Bile duct ligation + 13.5 mg UDCA + 7.2 mg *Allium sativum* extract group (T2)	0.004*	0.004*	0.004*	0.004*		
Bile duct ligation + 13.5 mg UDCA + 14.4 mg *Allium sativum* extract group (T3)	0.004*	0.004*	0.004*	0.004*	0.004*	

Notes: * significant (p<0.05); UDCA: ursodeoxycholic acid

Correlation between MMP-9 and TIMP-1 level

The association test between MMP-9 and TIMP-1 levels was performed using the Spearman Test as a nonparametric test to assess the correlation and strength between those variables. The results showed a p-value < 0.001 and an r-value = - 0.981 (CI 95%), so it can be concluded that MMP-9 levels are correlated with TIMP- levels, with the direction and strength of the correlation negatively strong (Figure 3).

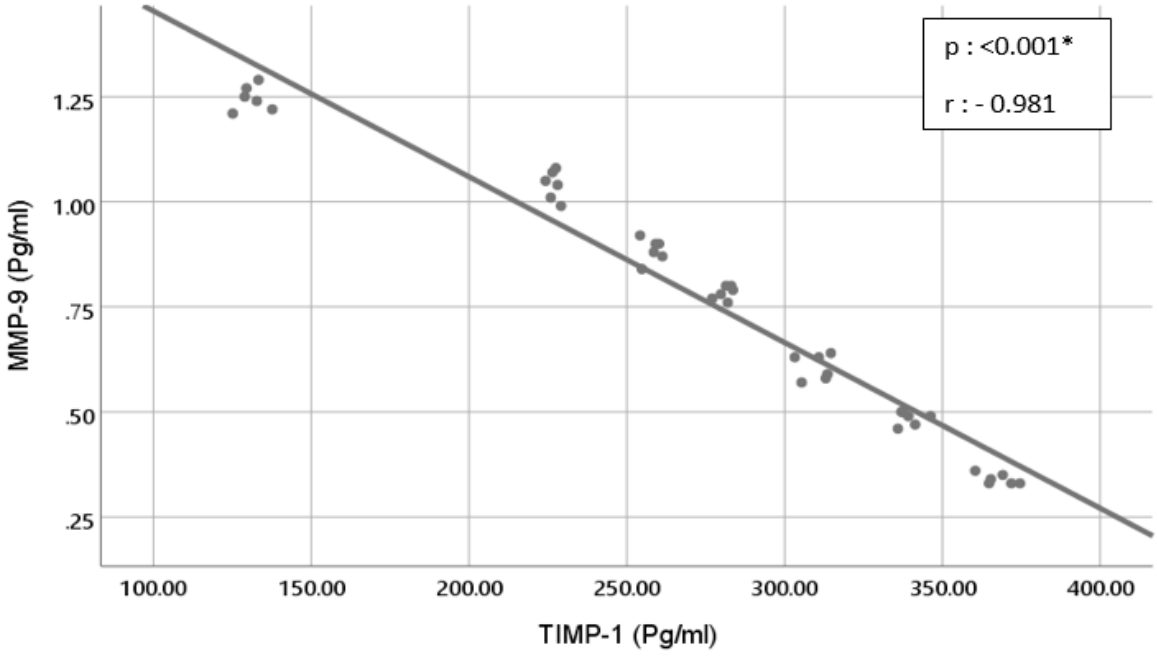

Figure 3. Scatterplot graph of the correlation between MMP-9 and TIMP-1 level. £Spearman; * significant (p < 0.05); MMP-9: matrix metalloproteinase-9; TIMP-1: tissue inhibitors of metalloproteinase-1

DISCUSSION

This study showed a significant difference in MMP-9 and TIMP-1 levels between groups, and between the averages of the control and treatment groups. We found that the combination of UDCA and *Allium sativum* reduced MMP-9 levels and increased TIMP-1 compared to UDCA administration in Sprague-Dawley rats with ligated common bile ducts. The basic process of liver fibrosis is the persistent inflammatory process within the liver parenchyma. Earlier preclinical studies highlighted the importance of maintaining a balance between MMP and TIMP levels to

mitigate the fibrogenesis process. Following liver cell injury, an inflammatory response is initiated, marked by the release of IL, TNF, and TGF-β cytokines. These cytokines collaborate to activate HSC in the liver. Once activated, HSC undergoes differentiation into myofibroblasts, leading to the production of collagen-rich extracellular matrix. The regulation of collagen production is regulated by MMP and its inhibitory counterpart, the TIMP.[16]

Bile duct occlusion results from common bile duct ligation. This technique is frequently applied to lab animals to ascertain the pathophysiology of cholestasis-induced liver fibrosis. Aspartate transaminase and aminotransferase levels rise due to acute biliary infarction and hepatocellular injury accompanying bile duct ligation-induced cholestasis.[16] The TGF-β is released as the primary profibrogenic agent when the injured hepatocytes experience necrosis and apoptosis following bile duct ligation. Hepatic stellate cells that are positive for α-smooth muscle actin (α-SMA) and resemble myofibroblasts become more numerous as the injury to the liver worsens.[17] Large levels of collagen in the HSCs can lead to extracellular matrix synthesis, and ultimately to liver fibrosis and cirrhosis. The activation and multiplication of HSCs led to the accumulation of extracellular matrix, which in turn caused fibrosis. Hepatic fibrosis is primarily caused by activated HSCs depositing large amounts of collagen after liver damage. Matrix metalloproteinases and TIMPs play a crucial role in fibrogenesis and fibrolysis by promoting the destruction of collagen. Among the MMPs, MMP-9 relates to tissue remodeling in the pathogenesis of hepatic fibrosis in biliary tract atresia. It is linked to the regulation of hepatic fibrogenesis by stimulating HSC death, aiding in fibrogenesis .[17]

In contrast, animals in the C3 group that received 13.5 mg of UDCA after bile duct ligation surgery exhibited lower MMP-9 levels than those in the C2 group. Previous studies have demonstrated that the UDCA is an effective liver-protective agent. It works to treat cholestasis by protecting bile duct cells from hydrophobic bile acids that are cytotoxic, encouraging choleretic hepatobiliary secretion, and protecting hepatocytes from apoptosis caused by bile acids (anti-apoptosis).[15]

The T1-T3 treatment group's MMP-9 levels decreased as the dosage of *Allium sativum* extract increased. These results displayed an inverse connection between the levels of MMP-9 in the serum of rats with ligated choledochal ducts and greater doses of *Allium sativum* extract. Notable differences between the treatment and control groups substantiate the study's original premise, which states that combining UDCA and Allium sativum extract effectively reduces MMP-9 levels. As an enzyme, MMP breaks down collagen and other proteins found in the extracellular matrix. The MMP-9 can be harmful at high concentrations because it can damage normal cells and have an excessively inflammatory effect.[5,17] It has been observed that administering garlic extract has antifibrotic and antioxidant properties. Bile duct ligation was used to directly examine the effects of garlic extract on samples. Aqueous garlic extract effectively reduced oxidative stress and liver fibrosis in mice with biliary obstruction.[10,18]

Additionally, treatment with aqueous garlic extract reduced the inflammatory process triggered by BDL. Renal and liver functions were also enhanced. The primary outcome was the inhibition of TGF-β1 and MMP-13 transcription, which postpones or stops the development of cholestasis into fibrosis and cirrhosis. It was previously discovered in a model of bile duct-ligated rats that the MMP-13 gene's expression was decreased by both aqueous garlic extract and enalapril, providing compelling evidence that garlic extract slows down the process of liver fibrogenesis.[19]

The assessment of serum TIMP-1 levels in this investigation yielded unexpected findings. The lowest TIMP-1 level was found in the C2 (bile duct ligated) group, while the highest was in the C1 (Sham-operated) group. These results indicate that the BDL-only group had the lowest TIMP-1 levels among all groups. Analyzing the control group against the treatment group revealed significant differences. The analytical tests in this study indicated a positive correlation between the dosage of Allium sativum extract and the levels of TIMP-1 in bile duct ligated rats. Interestingly, this outcome contradicts most previously gathered literature on the subject. Several previous investigations have documented that increased TIMP-1 levels are involved in hepatic fibrogenesis, which in turn causes fibrosis in injured livers by degrading the extracellular

matrix and inhibiting MMP. Prior studies have demonstrated a relationship between raised TIMP levels and more severe liver fibrosis, as seen by patients' greater TIMP-1 gene expression. When TIMP is overexpressed, MMP should break down and accumulate in the extracellular matrix, which eventually causes fibrosis. Like MMP, TIMP acts as a pro-fibrotic cytokine by increasing TGF-B activity and actively contributing to the fibrogenesis process.[5,19,20]

Although TIMP-1's profibrogenic action has been discussed extensively in earlier research, our findings were the contrary. Compared to the negative control (K1) and positive control (K2) groups, levels of TIMP-1 in experimental animals were more remarkable in all treatment groups. These results were consistent with studies by Wang et al., using a mouse model that has a TIMP-1 deficit.[21] Their research unexpectedly showed that TIMP-1-deficient animals were more vulnerable to CCl4-induced liver cell damage, pointing to TIMP-1's potential hepatoprotective function in liver injury. One identified mechanism for TIMP-1's hepatoprotective action is the suppression of hepatocyte apoptosis, albeit the rationale for this effect is yet unknown. Further study is necessary to understand the processes behind TIMP-1's anti-apoptotic effect on hepatocytes. Wang et al. propose a dual role for TIMP-1, despite prior knowledge labeling it as pro-fibrogenic in liver fibrosis, as indicated by TIMP-1 transgenic animals resistant to fibrosis resolution and studies indicating fibrosis cessation with TIMP-1 neutralizing antibodies. According to their theories, TIMP-1 both promotes and prevents fibrosis by preserving the existence of HSCs and averting liver damage. The TIMP-1's overall effect on liver fibrosis depends on how well its stimulatory and inhibitory actions are balanced. Additionally, this study shows that TIMP-1 rises with increasing Allium sativum extract doses, indicating that the extract may reduce liver fibrosis by raising TIMP-1 levels.[21]

Our study is the first to evaluate the impact of UDCA and *Allium sativum* extract on MMP-9 and TIMP-1 levels, which differ slightly from previous studies on TIMP-1 levels. We used the ELISA technology, which has broad clinical application and great sensitivity. Our study limitation is that the serum samples may not accurately reflect the actual tissue conditions. Previous research on MMP and TIMP in liver fibrosis frequently used tissue samples and techniques such as immunohistochemistry, Western blot, and RT-qPCR.

CONCLUSION

In conclusion, the combination of UDCA and *Allum sativum* alleviates liver fibrosis progression by lowering levels of MMP-9 and increasing levels of TIMP-1. The efficacy of UDCA and *Allium sativum* increased when the dose of *Allium sativum* was increased. These findings suggest potential implications for further clinical research into new combination therapies for treating cholestasis with novel drug combinations. Future research utilizing liver tissue samples is essential for more specific insights, especially on the effect of *Allium sativum* extract on the MMP-9 and TIMP-1 activities in the liver tissue.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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AUTHORS CONTRIBUTIONS

ADS contributes to conceptualization, formal analysis, investigation, project administration, software, and writing original draft; SAP contributes to methodology, project administration, resources, validation, visualization, and review-editing; MM contributes to investigation, supervision, validation, and review-editing; EP contributes to investigation, supervision, validation, and review-editing; EM contributes to investigation, supervision, validation, and review-editing.

LIST OF ABBREVIATIONS

UDCA: Ursodeoxycholic Acid; MMP-9: Matrix Metalloproteinase-9; TIMP-1: Tissue Inhibitors of Metalloproteases-1; HSC: Hepatic Stellate Cells; BDL: Bile Duct Ligation; TNF-α: Tumor Necrosis Factor-α; TGF-β1: Transforming Growth Factor-β1; α-SMA: α-smooth muscle actin

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