



HEPATOPROTECTIVE AND ANTIOXIDANT EFFECTS OF CAFFEINE AND SILYMARIN COMBINATION BY DECREASING LYSOPHOSPHATIDIC ACID RECEPTOR 3 TISSUE AND GENE EXPRESSION IN RATS

Salma M. Eraky^{1*}, Mohamed El-Mesery¹, Amro El-Karef², Laila A. Eissa¹ and Amal M. El-Gayar¹

¹Department of Biochemistry, Faculty of Pharmacy, Mansoura University, Mansoura 35516, Egypt

²Department of Pathology, Faculty of Medicine, Mansoura University, Mansoura 35516, Egypt

Liver disease causes about 2 million deaths yearly in the world. Caffeine (Caff) is the most common psychoactive substance utilized around the world. Caff has been demonstrated to possess anti-fibrotic actions in the liver. Silymarin (Sily) is a natural product largely used for its hepatoprotective actions through antioxidant, anti-inflammatory, and anti-fibrotic effects. **Aim of the study:** The current study aims to investigate the hepatoprotective and antioxidant effects of Caff and Sily alone or in combination in a rat model of liver fibrosis focusing on their effects on lysophosphatidic acid receptor 3 (LPAR3) expression. **Materials and methods:** 200 mg/kg of thioacetamide (TAA) was injected intraperitoneally twice weekly for 8 weeks to induce liver fibrosis. Caff, Sily, and their combination were orally administered to rats at a dose of 50 mg/kg/day for 8 weeks together with TAA injection. **Results:** Caff and Sily alone or in combination significantly improved liver function tests compared with fibrotic group. Moreover, they significantly decreased hepatic malondialdehyde (MDA) content and significantly increased the hepatic glutathione (GSH) concentration as compared with the fibrotic group. They significantly decreased fibrosis and necro-inflammatory scores with a superior significant effect in the necro-inflammatory score for the combination group each drug alone. Caff and Sily alone or in combination significantly decreased the hepatic LPAR3 gene and tissue expression, which was significantly correlated to their hepatoprotective effects. **Conclusion:** Caff and Sily alone or in combination protected against hepatic fibrosis through down-regulation of LPAR3 as well as antioxidant effects.

Keywords: Caffeine; Glutathione; Liver fibrosis; Lysophosphatidic acid receptor; Malondialdehyde; Oxidative stress; Silymarin.

INTRODUCTION

Liver fibrosis is a leading cause of morbidity and mortality worldwide. All chronic liver disease, if left untreated, can lead to fibrosis. It is an excessively abundant, wound healing process in which extreme connective tissue accumulates in the liver. Advanced liver fibrosis leads to liver failure, cirrhosis, and portal hypertension and usually needs liver transplantation^{1&2}.

Lysophosphatidic acid (LPA) is a small glycerophospholipid molecule that regulates various cell functions including survival, proliferation, and migration^{3&4}. LPA performs its functions by binding to G-protein-coupled receptors, known as lysophosphatidic acid receptors (LPARs) of which 7 have been characterized to date (LPARs1–7)^{3&5-7}. LPARs 1-3 are known as endothelial differentiation gene (EDG) family⁸. LPAR3 is a 40-kD protein that shares sequence homology with LPAR1 (~54%)⁹.

Increased expression of LPARs was shown in clinical and experimental studies of liver fibrosis¹⁰⁻¹⁴. Moreover, LPA is thought to be involved in liver fibrosis through activation of hepatic myofibroblasts and increasing the proliferation and survival of hepatic stellate cells (HSCs)¹⁵⁻¹⁷. Correlations have been established between plasma LPA levels and different histological grades and complications of liver fibrosis in subjects with chronic Hepatitis C virus¹⁸ and in rodents with chemically induced fibrosis^{19&20}.

Oxidative stress represents the imbalance between the formation and elimination of free radicals together with reduced production of antioxidants²¹. Reactive oxygen species (ROS) can initiate lipid peroxidation, leading to DNA strand breaks and aimlessly oxidizing all molecules in biological membranes and tissues, finally leading to injury^{22&23}. Malondialdehyde (MDA) is a lipid peroxidation product and an important marker of oxidative stress^{24&25}. Glutathione (GSH) is one of the most studied antioxidants. It is known to decrease the lipid peroxidation of cellular membranes and other such targets²⁶.

The liver is a primary organ attacked by ROS²⁷. Oxidative stress can stimulate Kupffer cells to produce cytokines such as tumor necrosis factor- α , which may increase apoptosis and inflammation. Furthermore, the proliferation and collagen synthesis of hepatic stellate cells are stimulated by lipid peroxidation that results from oxidative stress²⁸⁻³⁰.

Caffeine (Caff) (3,7-dihydro-1,3,7-trimethyl-1H-purine-2,6-dione) is contained in many types of food, including chocolate, cocoa, coffee, and tea³¹. Although there is a popular thought that Caff has negative effects on health, a large prospective study found that coffee intake was inversely associated with mortality³². Moreover, Caff gained attention in the last two decades for its hepatoprotective effects³³⁻³⁵, which were mainly attributed to its antioxidant properties³⁶.

Silymarin (Sily) is the extract of milk thistle (*Silybum marianum* (L.) Gaertn.). It is well-known for its antioxidant and chemoprotective actions on the liver. Therefore, it has been the gold standard drug to ameliorate liver disorders of different etiologies³⁷. Experimental studies demonstrated

that Sily protects against liver damage through its antioxidant and anti-fibrotic effects^{38&39}.

The purpose of this study is to investigate the potential antifibrotic and antioxidant effects of combining Caff to Sily against experimentally induced liver fibrosis and to further understand the molecular mechanism through the LPAR3 gene and tissue expression.

MATERIALS AND METHODS

Chemicals and drugs

TAA and Caff were purchased from Sigma Aldrich, USA. Sily was obtained as legalon capsules produced by Chemical Industries Development (CID), under license of MADAUS AG, MADAUS GmbH., Germany (Batch number.01190380).

Animals and diet

Sixty male Sprague–Dawley rats (180–200 g) were purchased from Holding Company for Biological products & Vaccines, Egypt. Standard conditions were set as 12h-light/12h-dark cycles. Rats were allowed ad libitum to food and tap water. The study follows the “Research Ethics Committee” of the Faculty of Pharmacy, Mansoura University, Egypt under the guidelines of “Principles of Laboratory Animal Care” (NIH publication No. 85-23, revised 1985) (Code number: 2020-90).

Experimental design

Rats were divided into five groups (n=12) as shown in **Fig. 1**: control group; rats were injected intraperitoneally with sterile saline twice weekly for 8 weeks, a fibrotic group in which liver fibrosis was induced by injecting TAA (200 mg/kg, two times a week for 8 weeks intraperitoneally)⁴⁰; Caff group, received Caff (50 mg/kg) orally⁴¹; Sily group, received Sily (50 mg/kg) orally⁴²; and Sily+ Caff group, received both Sily (50 mg/kg) orally, and Caff (50 mg/kg) orally. All treatments started at the beginning of the experiment along with TAA injection and continued to the end of the study.

At the end of the experiment rats were fasted for 12 hrs. Blood samples were collected by retro-orbital puncture and centrifuged for serum preparation. Liver tissues were separated. A small part of the liver was flash frozen in liquid nitrogen then stored at -80°C for Reverse transcription polymerase chain

reaction (RT-PCR) analysis. Another small part of liver was cut and fixed in 10% phosphate-buffered formalin for histopathological examination and immunohistochemical analysis. Further part of liver tissue was homogenized in cold phosphate buffered saline then centrifuged for determination of hepatic MDA and GSH contents.

Liver function tests

Serum was used for determination of serum albumin and total protein concentrations, ALT and AST activities using commercial kits

(Human Diagnostics, Germany) and total bilirubin concentration using commercial kit (Diamond Diagnostics, Egypt).

Oxidative stress

Determination of hepatic MDA content

MDA content was measured spectrophotometrically in rat liver homogenate according to the previous method⁴³, where MDA in acidic medium reacts with thiobarbituric acid at a temperature of 95°C for 30 min releasing a thiobarbituric acid reactive product that is measured at 534 nm.

8 Weeks

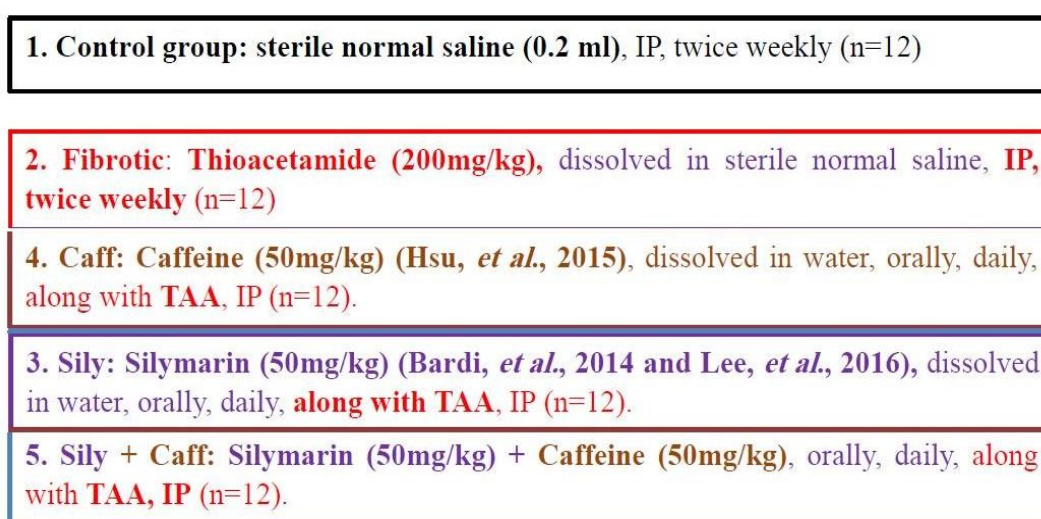


Fig.1: Experimental design of the study.

Table 1: Primer sets used in RT-PCR (Biosearch technologies, CA, USA).

Name	Sequence	Reference sequence	Product length
HPRT1-F	5` - CCTAAAAGACAGCGGCAAGT-3`	<u>NM_012583.2</u>	173
HPRT1-R	5` - AATCAAAAAGGGACGCAGCAA-3`		
LPAR3-F	5` - GCAAGGGTGGAGGTGTAGAA-3`	<u>NM_023969.1</u>	157
LPAR3-R	5` - GGTCTAAACTCGCCATCACG-3`		

- HPRT1 : Hypoxanthine phosphoribosyl transferase 1 (the housekeeping gene).
- F : Forward.
- R : Reverse.
- LPAR : Lysophosphatidic acid receptor.
- ACTA2 : Alpha 2 Actin (alpha-smooth muscle actin gene).
- RT-PCR : Reverse transcription-polymerase chain reaction.

Determination of hepatic GSH content:

GSH content in the liver was measured according to the method of⁴⁴ since GSH reduces Ellman's Reagent (5,5'-dithio-bis-(2-nitrobenzoic acid) producing a yellow compound. The absorbance of the produced chromogen at 405 nm is directly proportional to GSH content.

Histopathology

Formalin-fixed liver tissues were embedded in paraffin, cut into 5 µm sections, and stained using hematoxylin and eosin (H&E) stain for further evaluation of necro-inflammation using Ishak's histological activity index⁴⁶. Other sections were stained using Masson's trichrome (MT) stain for the quantification of fibrotic areas. Under a light microscope, sections were investigated, and digital pictures were captured using a digital camera. Histopathology was read by a single independent pathologist, blinded to experimental design. The collagenous areas stained with MT were extracted and analyzed by NIH Image software. The fibrosis percentage was described as the ratio of the stained area to the total tissue area using 30 random fields in the same slide⁴⁷.

Immunohistochemistry

Sections were deparaffinized, rehydrated then LPAR3 rabbit polyclonal antibody (Mybiosource, USA) was applied to them. Diaminobenzidine kit was used for the analysis of antibody binding, along with hematoxylin as the counterstain. Subsequently, the slides were visualized using a light microscope to detect LPAR3 expression in the liver, using an internal control (Brain). The number of positive cells were counted in 10 high-power fields in the cytoplasm of fibrotic areas.

RT-PCR

RNA was extracted from flash-frozen liver sections using Direct-zol RNA MiniPrep kit (ZYMORESEARCH CO, USA) following the protocols given by the suppliers. Afterward, c-DNA was synthesized from RNA by SensiFAST cDNA Synthesis Kit (Bioline, USA) following the protocols given by the suppliers. Subsequently, the qPCR reactions

were carried out according to the protocols mentioned in the instruction manual of SensiFAST SYBR Hi-ROX Kit (Bioline, USA) using a thermocycler Piko Real-PCR (Thermo Fisher Scientific Inc., USA). Ultimately, relative mRNA expression was calculated using the $2^{-\Delta\Delta C_t}$ method where hypoxanthine phosphoribosyltransferase 1 (HPRT1) was used as a housekeeping gene.

Statistical analysis

The statistical analysis was performed using statistical software SPSS version 17 (USA), while graphs were plotted by GraphPad Prism V 5.02 (GraphPad Software Inc., San Diego, CA USA). All results were expressed as mean \pm standard error (SE). If P values were < 0.05 , it was considered significant. One-way analysis of variance (ANOVA) followed by Bonferroni test (for parametric data) or Mann-Whitney U test (for non-parametric data of necro-inflammatory and immunohistochemical scoring) was used for analyzing statistically significant differences between groups. Pearson correlation was used to assess the correlation between different parameters.

RESULTS AND DISCUSSION

Results

Effects of Caff and Sily on liver function

Serum albumin and total protein concentrations were significantly decreased in the fibrotic group compared with the control group (**P** < **0.005**, **P** < **0.05** respectively). Moreover, there was a significant elevation in serum total bilirubin concentrations, serum ALT, and AST activities (**P** < **0.005**) compared with the control group. Administration of Caff and/or Sily significantly elevated serum albumin and total protein concentrations (**P** < **0.005**), compared with the fibrotic group. On the other hand, administration of Caff and/or Sily significantly decreased serum total bilirubin concentrations, as well as serum ALT and AST activities (**P** < **0.005**), compared with the fibrotic group. Interestingly, Sily and the combination treatment significantly decreased serum ALT and AST activities (**P** < **0.005**) compared with Caff (**Table 2**).

Table 2: Effects of Sily (50 mg/kg/day) and/or Caff (50 mg/kg/day) on serum albumin, total protein, and total bilirubin concentrations, ALT and AST activities in rats.

Group	Serum albumin concentration (g/dl)	Serum total protein concentration (g/dl)	Serum total bilirubin concentration (mg/dl)	ALT Activity (U/L)	AST Activity (U/L)
Control	2.807 ± 0.063	6.38 ± 0.108	0.147 ± 0.034	114.667 ± 5.857	216.833 ± 21.067
Fibrotic	2.037 ± 0.055##	5.453 ± 0.088 #	0.964 ± 0.074 ##	4571 ± 211.487 ##	4298.4 ± 400.731 ##
Caff	2.967 ± 0.025**	7.6872 ± 0.199 ** ##	0.496 ± 0.068**	348.667 ± 11.467 **	1067.778 ± 81.509 ** ##
Sily	3.029 ± 0.08 **	7.663 ± 0.195 ** ##	0.35 ± 0.063**	324.909 ± 12.999 **	486.546 ± 42.049 ** @@
Sily+ Caff	3.18 ± 0.112** #	8.1289 ± 0.209 ** ##	0.439 ± 0.122**	279.3 ± 26.447 **	334.2 ± 17.931 ** @@

The number of animals in each group is 12.

Values are presented as mean ± SE.

: Significant against the control group (P<0.05).

: Significant against the control group (P<0.005).

** : Significant against the fibrotic group (P<0.005).

@@ : Significant against the Caff group (P<0.005).

Caff : Caffeine

Sily : Silymarin

Effects of Caff and Sily on oxidative stress

The fibrotic group showed a significant elevation in hepatic MDA content, as well as a significant decrease in hepatic GSH content compared with the control group (P<0.005). Caff and/or Sily treatment caused a significant decrease in hepatic MDA content compared with the fibrotic group (P<0.005, P<0.05,

P<0.005, respectively). Also, Sily caused a significant decrease in hepatic MDA content compared with the Caff group (P<0.005) (Table 3). On the other hand, Caff and/or Sily treatment significantly increased hepatic GSH content (P<0.05, P<0.05, P<0.005, respectively), compared with the fibrotic group (Table 3).

Table 3: Effects of Sily (50 mg/kg/day) and/or Caff (50 mg/kg/day) on hepatic malondialdehyde (MDA) and hepatic glutathione (GSH) contents in rats.

Group	Hepatic MDA content (nmol/g tissue)	Hepatic GSH content (µmol/g tissue)
Control	3.615 ± 1.958	3.285 ± 0.493
Fibrotic	25.63 ± 0.739 ##	0.578 ± 0.074#
Caff	19.401 ± 0.725 *	2.866 ± 0.403 *
Sily	8.647 ± 0.764 ** @@	3.016 ± 0.343 *
Sily+ Caff	16.253 ± 1.781 ** \$	4.526 ± 0.592 **

The number of animals in each group is 12.

Values are presented as mean ± SE.

: Significant against the control group (P<0.05).

: Significant against the control group (P<0.005).

* : Significant against the fibrotic group (P<0.005).

** : Significant against the fibrotic group (P<0.05).

@@ : Significant against the Caff group (P<0.05).

\$: Significant against the Sily group (P<0.05).

Caff : Caffeine

Sily : Silymarin

Effects of Caff and Sily on liver necro-inflammation and fibrosis score

Isolated livers from the fibrotic group showed rough surfaces with small nodules. On the other hand, liver surfaces of the control and all treated groups were smooth and nearly intact (**Fig. 2**).

In liver sections stained with Hematoxylin-Eosin; the fibrotic group showed marked necrosis, moderate to marked portal inflammation. The fibrotic group showed moderate to severe necro-inflammatory scores with a mean score of (13.7 ± 0.3) . Pre-treatment with Caff and/or Sily caused a significant reduction of the necro-inflammation

compared with the fibrotic group ($P < 0.005$). Interestingly, the combination group significantly reduced the necro-inflammatory score compared with Caff and Sily groups ($P < 0.005$) (**Fig. 3, Table 4**).

The Masson's trichrome stained liver sections showed that the amount of collagen deposition was significantly elevated in the fibrotic group. Caff and/or Sily administration significantly reduced the fibrosis score (%) and collagen content compared with the fibrotic group ($P < 0.005$) (**Fig. 4, Table 4**).

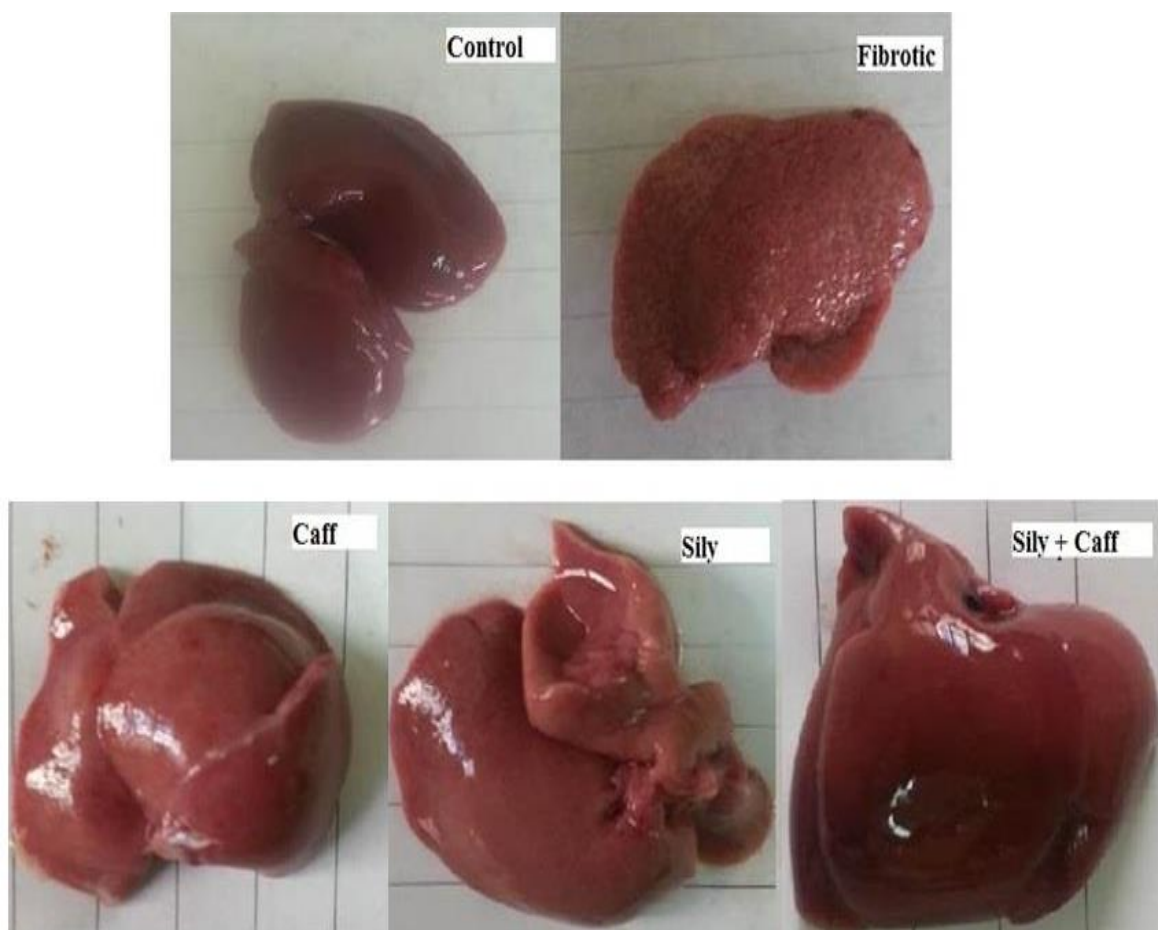


Fig. 2: Livers isolated from control group, fibrotic group (treated with IP injection of 200 mg/kg thioacetamide two times/week) and groups treated orally with caffeine (Caff) (50 mg/kg/day), silymarin (Sily) (50 mg/kg/day) alone or in combination (Sily+ Caff) along with thioacetamide injection for 8 weeks.

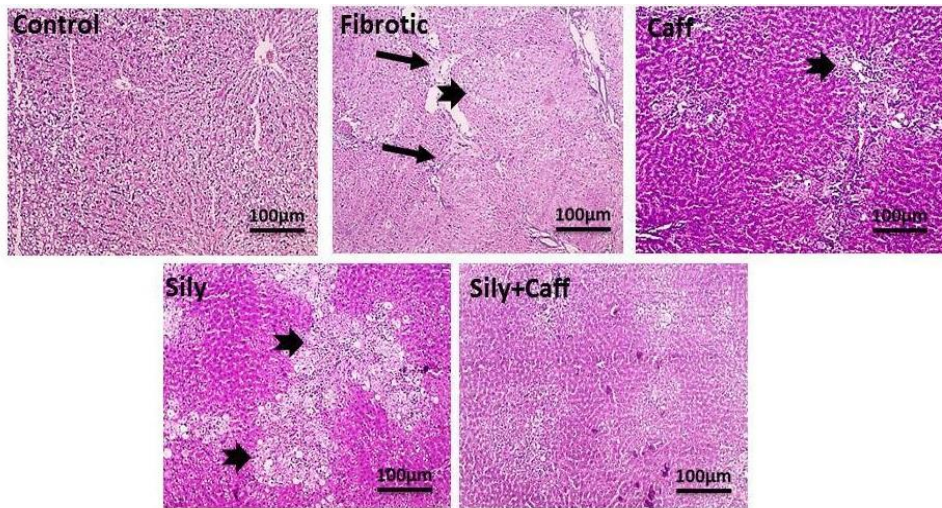


Fig. 3: Caffeine (Caff) (50 mg/kg/day), silymarin (sily) (50 mg/kg/day) alone or in combination (Sily+Caff) improved hepatic necro-inflammation in rats (hematoxylin-eosin staining).

Microscopic pictures of hematoxylin-eosin-stained hepatic sections show regular arrangement of hepatic cords around central veins in the control group. Hepatic sections from the fibrotic group show communicating fibrosis between portal areas and central veins (thin black arrows) and large patchy areas of hydropic and ballooning degeneration (thick black arrows). Individual treatment with Caff or Sily showed a reduction of necro-inflammatory changes. Combined treatment showed a marked reduction of necro-inflammation to levels comparable to the control group. X: 100 bar 100.

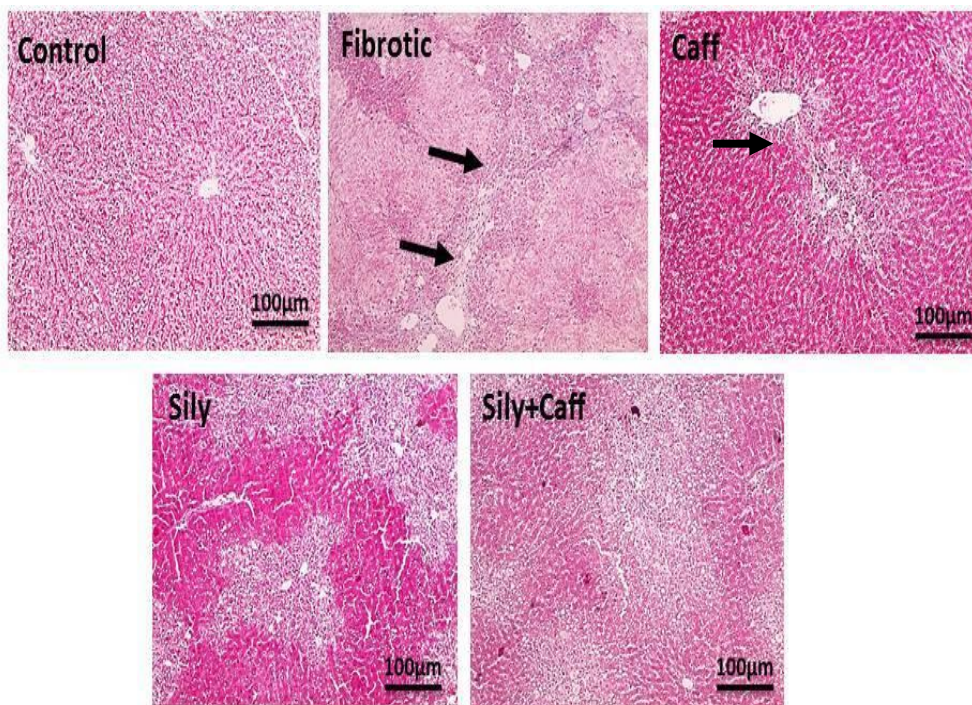


Fig. 4: Caffeine (Caff) (50 mg/kg/day), silymarin (Sily) (50 mg/kg/day) alone or in combination (Sily+Caff) protected the liver from fibrosis and collagen deposition in rats (Masson's trichrome staining).

Microscopic pictures of Masson trichrome-stained hepatic sections show no fibrosis in the control group. Hepatic sections from the fibrotic group show marked bluish communicating fibrous tissue deposition between portal areas and central veins. Individual treatment with Caff or Sily showed a reduction of collagen deposition. Combined treatment showed a marked improvement of fibrosis comparable to the control group. Arrows indicate areas of fibrosis.

Table 4: Effects of Sily (50 mg/kg/day) and/or Caff (50 mg/kg/day) on hepatic necro-inflammatory score and fibrosis score (%).

Group	Hepatic necro-inflammatory score	Hepatic fibrosis score (%)
Control	-	1.01 ± 0.023
Fibrotic	13.7 ± 0.3	2.88 ± 0.241 ^{##}
Caff	9.2 ± 0.291 ^{**}	1.535 ± 0.09 ^{** #}
Sily	9.6 ± 0.371 ^{**}	1.693 ± 0.079 ^{** ##}
Sily+ Caff	7.5 ± 0.167 ^{**\$\$ @@}	1.415 ± 0.078 ^{**}

The number of animals in each group is 12.

Values are presented as mean±SE.

^{##} : Significant against the control group (P<0.005).

^{**} : Significant against the fibrotic group (P<0.05).

^{\$\$} : Significant against the Sily group (P<0.005).

^{@@} : Significant against the Caff group (P<0.005).

Caff : Caffeine

Sily : Silymarin

Effects of Caff and Sily on hepatic LPAR3 tissue expression

Immunohistochemical analysis of LPAR3 tissue expression indicated that the fibrotic group showed a significant increase in tissue expression of LPAR3 compared with the control group (P< 0.005). Caff and/or Sily

down-regulated LPAR3 tissue expression compared with the fibrotic group (P<0.005). Moreover, the combination therapy significantly down-regulated LPAR3 tissue expression (P<0.05) compared with the Sily group (Table 5, Fig. 5).

Table 5: Effects of Sily (50 mg/kg/day) and/or Caff (50 mg/kg/day) on hepatic lysophosphatidic acid receptor 3 (LPAR3) mRNA gene and tissue expression.

Group	Hepatic LPAR3 mRNA gene expression	Hepatic LPAR3 tissue expression
Control	0.904159 ± 0.12	1.5 ± 0.224
Fibrotic	1.716861 ± 0.163 ^{##}	4.8 ± 0.291 ^{##}
Caff	0.579 ± 0.067 ^{**}	2.5 ± 0.224 ^{**#}
Sily	0.59 ± 0.037 ^{**}	3.3 ± 0.335 ^{*##}
Sily+ Caff	0.388 ± 0.02 ^{**}	2.0 ± 0.258 ^{** \$}

The number of animals in each group is 12.

Values are presented as mean±SE.

[#] : Significant against the control group (P<0.05).

^{##} : Significant against the control group (P<0.005).

^{*} : Significant against the fibrotic group (P<0.05).

^{**} : Significant against the fibrotic group (P<0.005).

^{\$} : Significant against the Sily group (P<0.05).

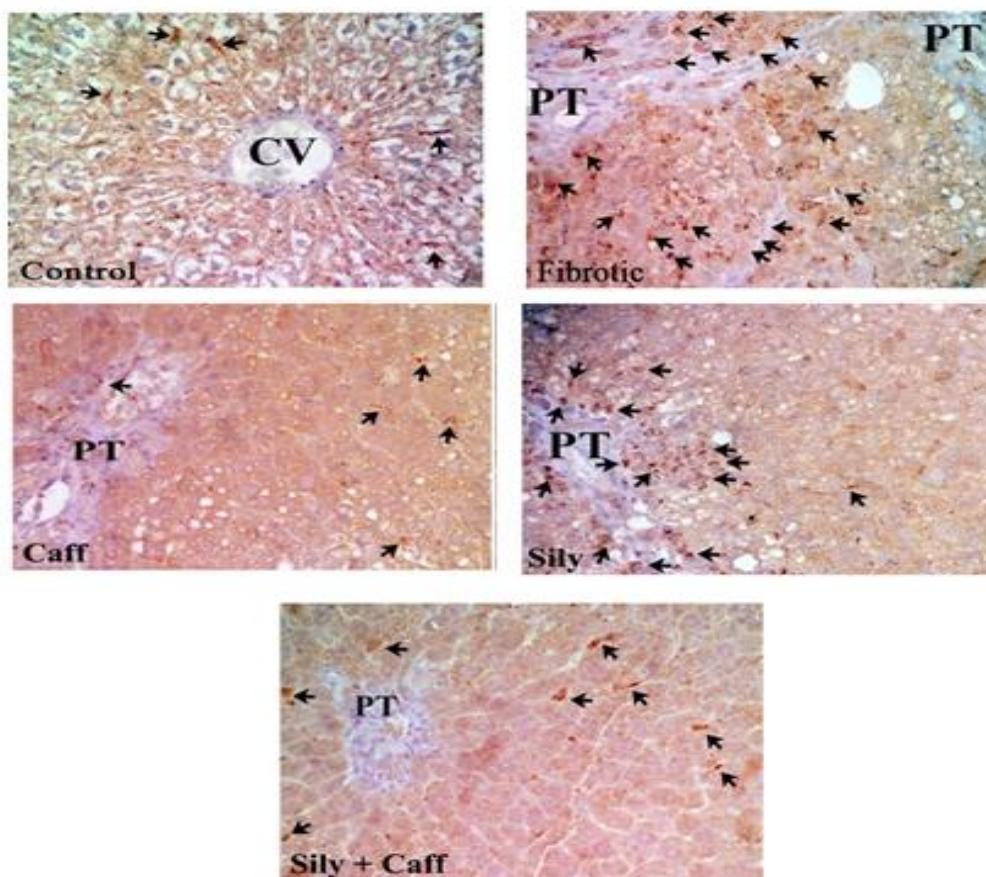


Fig. 5: Caffeine (Caff) (50 mg/kg/day), silymarin (Sily) (50 mg/kg/day) alone or in combination (Sily+Caff) decreased hepatic lysophosphatidic acid receptor 3 (LPAR3) tissue expression in rats.

Immunohistochemical staining of LPAR3 showed higher cytoplasmic tissue expression in fibrotic rats (arrows) with mild expression in Caff or Sily individual treatment and a marked reduction of expression with persistent positivity in non-parenchymal cells in the combined treatment.

Effects of Caff and Sily on hepatic LPAR3 gene expression

LPAR3 mRNA gene expression was significantly elevated in the fibrotic group compared with the control group ($P < 0.005$). Administration of Caff and/or Sily significantly reduced the LPAR3 mRNA gene expression compared with the fibrotic group (**Table 5**).

Correlation studies

There was a significant negative correlation between LPAR3 expression and serum albumin and total protein concentrations (**Table 6**). Alternatively, there was a significant

positive correlation between LPAR3 expression and total bilirubin concentration, ALT, and AST activities (**Table 6**). Moreover, there was a significant positive correlation between LPAR3 expression and both fibrosis and necro-inflammatory scores (**Table 6**). There was a significant positive correlation between LPAR3 tissue expression and hepatic MDA content, while mRNA and tissue expression of LPAR3 showed a significant negative correlation with hepatic GSH content (**Table 6**).

Table 6: Correlation between LPAR3 mRNA gene and tissue expression, liver function tests, fibrosis score (%), necro-inflammatory score, hepatic malondialdehyde (MDA), glutathione (GSH) contents.

	LPAR3 mRNA gene expression	LPAR3 tissue expression
Albumin concentration (r)	-0.864**	-0.546**
Total protein concentration (r)	-0.807**	-0.364*
Total bilirubin concentration (r)	0.588**	0.634**
ALT activity (r)	0.869**	0.530**
AST activity (r)	0.838**	0.539**
Fibrosis score (%) (r)	0.789**	0.697**
Necro-inflammatory score (r)	0.864**	
Hepatic MDA content (r)	0.212	
Hepatic GSH content (r)	-0.639*	
LPAR3 mRNA gene expression (r)	-	

- ALT : Alanine aminotransferase
 AST : Aspartate aminotransferase
 LPAR : Lysophosphatidic acid receptor
 MDA : Malondialdehyde.
 GSH : Glutathione.
 * = P<0.05.
 ** = P<0.005.

Discussion

Liver fibrosis is a complicated inflammatory and fibrogenic process which will progress to liver cirrhosis, and finally to hepatocellular carcinoma if left untreated. Fibrosis involves the excessive accumulation of extracellular matrix, which is formed of a condensed mesh of polysaccharides, macromolecules, and proteins, involving various kinds of collagen, forming insoluble fibers and microfibrils⁴⁸.

The use of medicines of herbal sources is a very old routine in scientific research. Caff consumption has been related to decreased prevalence of chronic liver disorders⁴⁹. Moreover, Sily is well-known for its antioxidant and chemoprotective actions on the liver. Therefore, it has been the gold standard drug to ameliorate liver disorders of different etiologies³⁷. Thus, we were motivated in this study to investigate the hepatoprotective effects of combining Caff and Sily in a rat model of liver fibrosis, and to study the effects of Caff and Sily alone or in combination on LPAR3 expression, as well as the oxidative stress marker MDA and the antioxidant GSH.

In the current study, Caff and/or Sily recovered the normal synthetic and excretory

capacity of the liver as well as the integrity of hepatocytes. This was confirmed by the normalization of liver functions as well as the decrease in necro-inflammatory and fibrosis scores. For our interest, the current study found that the combination treatment showed a significant decrease in necro-inflammation when compared with individual treatment with Caff and Sily. Moreover, the combination therapy reduced fibrosis score to values that were comparable to the control group.

LPA is a mediator that acts on G-protein coupled receptors (LPAR1- LPAR6) subtypes. Some studies proved the contribution of LPA in the etiology of fibrosis and the correlation between fibrosis and both increased LPA production and expression of some LPAR subtypes, especially LPAR3 in some organs. The involvement of LPAR in pulmonary, renal, and vascular fibrosis was proven. Also, this was strongly suspected in liver fibrosis⁵⁰.

LPAR3 is coupled to G α i/o and G α q of the heterotrimeric G protein, mediating phospholipase C activation, Ca²⁺ mobilization, inhibition and activation of adenylate cyclase, and protein kinase activation that mediates mitogen activation⁵¹, stimulating profibrotic behavior in hepatic stellate cells⁵².

Regarding liver damage, studies have demonstrated that LPA may increase GSH levels, leading to blockage of c-Jun N-terminal kinase (JNK) phosphorylation⁵³. Since sustained P-JNK activation was identified in liver diseases, LPA may have an important role against liver diseases. Moreover, the LPA signaling stimulates Phosphoinositide 3-kinase and stabilizes hypoxia inducible factor-1-alpha which is a key transcription factor that promotes many features of liver fibrosis⁵⁴. Interestingly, this process may be related to LPAR1 and LPAR3 receptors⁵⁵.

In the current study, LPAR3 was shown to be involved in the pathogenesis of liver fibrosis, since the LPAR3 gene and tissue expression were significantly elevated in the fibrotic group compared with the control group. Moreover, LPAR3 gene and tissue expression showed positive correlation with necro-inflammatory score, fibrosis score, ALT, AST activities, and a significant negative correlation with total protein and albumin concentrations. In line with our results, previous studies demonstrated the significant correlation between increased LPAR3 expression and hepatocellular carcinoma^{56&57}.

Results of the current study indicated that the hepatoprotective effect of Caff and Sily alone or in combination is maintained through the downregulation of LPAR3 expression. Interestingly, the combination group significantly down-regulated hepatic tissue expression of LPAR3 compared with Sily individual treatment. This can explain the reason for the significant down-regulation of hepatic necro-inflammatory score caused by the combination treatment when compared with Sily individual treatment. Moreover, the combination of Caff and Sily normalized tissue expression of LPAR3, since it showed no significant change compared with the control group. This can explain the reason for the normalization of hepatic fibrosis score (%) in the combination group.

Oxidative stress is the imbalance between reactive oxygen species and reactive nitrogen species generation and elimination. It could be stimulated in the liver by different agents like toxins, viruses, and alcohol. Oxidative stress can lead to fundamental cellular and tissue damage. Lipids, proteins, and DNA, and their signaling molecules are targets of ROS. Free

radicals interact with lipids to generate endoperoxides and hydroperoxides that undergo fragmentation to produce reactive intermediates, like MDA that irreversibly bind DNA, phospholipids, and proteins, leading to cell death. ROS can activate oxidation-induced hepatocyte apoptosis, causing injury to hepatocytes⁵⁸.

The involvement of oxidative stress in liver diseases has been widely studied²⁵. Oxidative stress can mediate tissue and cellular actions participated in the pathogenesis of hepatic fibrosis. Increased ROS participates in fibrogenesis through direct interaction with HSCs, production of collagen from activated HSCs as well as secretion of pro-fibrogenic cytokines^{27,59-62}. Lipid peroxidation products such as MDA were shown to stimulate the immune response, participating in the progression of fatty liver to fibrosis⁶³.

In the current study, content of hepatic MDA, a lipid peroxidation product and marker of oxidative stress, was significantly elevated in the fibrotic group. This can be elucidated by the hepatotoxic mechanisms of TAA which depend mainly on the production of ROS and lipid peroxidation through reactive metabolites of TAA as TAA-S-dioxide^{64&65}. Moreover, this study showed a significant decrease in hepatic GSH content in the fibrotic group. This agrees with previous studies in different animal models of liver fibrosis^{35, 66&67}. GSH is the most abundant intracellular free thiol and an important water-soluble antioxidant in different types of cells. A decreased GSH content was observed in clinical and experimental fibrosis models. Thus, GSH and N-acetylcysteine, a precursor of GSH, have been extensively used for the treatment of fibrotic diseases⁶⁸.

The antioxidant activity of Caff may be due to the presence of large amounts of phytochemicals like triterpenes, flavonoids, or polyphenols. Moreover, phenolic diterpenes of Caff like cafestol and kahweol were found to inhibit lipid peroxidation^{35,69}. Moreover, the antioxidant mechanisms of Caff may be achieved through restoration of catalase activity and activation of the antioxidant nuclear factor erythroid 2-related factor 2 (Nrf-2) as it was recently demonstrated in renal fibrosis⁷⁰. Sily keeps the intact integrity of hepatocyte membrane due to its antioxidant characteristics, which could be correlated to its

phenolic nature due to the presence of flavonoids in the composition of Sily. Sily not only prevents the entry of xenobiotics but it is also able to stabilize free radicals and ROS and increase intracellular glutathione content, inhibiting membrane lipid peroxidation^{71&72}.

The involvement of LPAR3 in the lipid peroxidation and oxidative stress can be illustrated by the significant positive correlation between hepatic LPAR3 expression and MDA content as well as a significant negative correlation with GSH content. This agrees with previous study that showed that blocking of LPAR3 caused a significant decrease in MDA content in a dose-dependent manner⁷³. Moreover, some studies showed that exogenous LPA treatment stimulated lipid peroxidation and caused a dose-dependent increase in MDA content^{74&75}. As well, another study showed that LPA decreased intracellular GSH levels⁷⁶. To best of our knowledge, the current study was the first to demonstrate the effects of Caff and Sily alone or in combination on the expression of LPAR3 and linking this with oxidative stress in TAA-induced liver fibrosis.

Conclusion

Caff and Sily alone or in combination showed hepatoprotective, antioxidant effects in a rat model of liver fibrosis through down-regulation of LPAR3. To our interest, combination of Caff and Sily significantly decreased both necro-inflammation and LPAR3 tissue expression than single treatment with Sily. Moreover, combination of Caff and Sily was able to normalize the fibrosis score.

Abbreviations

GSH; glutathione, LPA; lysophosphatidic acid, LPAR; lysophosphatidic acid receptor, MDA; malondialdehyde, TAA; thioacetamide.

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نشرة العلوم الصيدلانية جامعة أسيوط



التأثيرات الوقائية للكبد والمضادة للأكسدة لمزيج الكافيين والسيليمارين عن طريق تقليل التعبير النسيجي و الجيني لمستقبلات حمض الليزوفوسفاتيدك ٣ في الجرذان

سلمى عراقي^{١*} - محمد المسيري^١ - عمرو الكارف^٢ - ليلى عيسى^١ - آمال الجيار^١

^١ قسم الكيمياء الحيوية ، كلية الصيدلة ، جامعة المنصورة ، المنصورة ٣٥٥١٦ ، مصر

^٢ قسم علم الأمراض ، كلية الطب ، جامعة المنصورة ، المنصورة ٣٥٥١٦ ، مصر

تسبب أمراض الكبد حوالي ٢ مليون حالة وفاة سنويًا في العالم. يعد السيليمارين منتجًا طبيعيًا يستخدم بصورة كبيرة لتأثيراته الوقائية للكبد من خلال التأثيرات المضادة للأكسدة والالتهابات والتليف. يعتبر الكافيين هو أكثر المواد ذات التأثير النفساني شيوعًا في جميع أنحاء العالم. ثبت أن الكافيين يمتلك تأثيرات مضادة للالتهاب في الكبد.

هدف الدراسة: تهدف الدراسة الحالية إلى التحقق من التأثيرات الوقائية للكبد ومضادات الأكسدة لكل من السيليمارين و الكافيين بمفردهما أو معًا في نموذج الجرذان لتليف الكبد مع التركيز على تأثيرهما على تعبير مستقبل حمض الليزوفوسفاتيدك^٣ (LPAR3).

المواد والطرق: تم حقن ٢٠٠ مجم / كجم من ثيوأسيتاميد (TAA) في البطن مرتين أسبوعيًا لمدة ٨ أسابيع للحث على تليف الكبد. تم إعطاء السيليمارين و الكافيين بمفردهما أو معًا عن طريق الفم للفئران بجرعة ٥٠ مجم / كجم / يوم لكل منهما لمدة ٨ أسابيع مع حقن TAA.

نتائج: أدى استخدام السيليمارين و الكافيين بمفردهما أو معًا إلى تحسن ملحوظ في اختبارات وظائف الكبد مقارنة بمجموعة التليف. علاوة على ذلك ، فقد قللوا بشكل ملحوظ محتوى المالوندايالدهيد الكبدي وزادوا بشكل ملحوظ تركيز الجلوتاثيون الكبدي مقارنة بمجموعة التليف. لقد قللوا بشكل كبير من درجات التليف والالتهابات النخرية مع تأثيرًا دلالة احصائية كبيرة في درجة الالتهاب النخري للمجموعة المركبة مقارنة كل دواء على حدة. قلل السيليمارين و الكافيين بمفردهما أو معًا بشكل كبير من التعبير الجيني و النسيجي ل LPAR3 في الكبد، والذي كان مرتبطًا بشكل كبير بتأثيرات الكبد.

الخلاصة: السيليمارين و الكافيين لوحدهما أو معًا يحميان من التليف الكبدي من خلال التنظيم الخافض لـ LPAR3 وكذلك التأثيرات المضادة للأكسدة.