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Anti-Fibrotic Effect of Michelia Champaca L. Against Carbon Tetrachloride Induced Liver Injury in Rats

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ABSTRACT

The purpose of the study is to evaluate the antifibrotic effect of *Michelia champaca* in CCl4 induced rats. The animals were divided into 5 groups consisting of six animals in each group. Group I rats received saline (0.5 ml/kg b.wt) orally. Group II rats administered with CCl_4 (0.5ml/kg b.wt) dissolved in olive oil (1:1 ratio) injected intraperitoneally on alternate days. Group III administered with CCl_4 treated with methanolic extract of *M.champaca* (300 mg/kg b.w) orally. Group VI, the CCl_4 induced rats were treated with Silymarin (25 mg/kg b.w) orally. Group V rats were treated with methanolic extract of *M.champaca* alone (300 mg/kg/b.w) orally. Matrix metal protease-8 (MMP-8) mRNA expression was assayed by RT-PCR and the protein expression of tissue inhibitor of metallo protenase-1(TIMP-1) was analyzed by immunohistochemistry. CCl_4 treatment resulted in elevate the expression of MMP-8 gene and TIMP1 protein. *Michelia champaca* flower extract suppressed MMP -8 and TIMP1 expression following CCl_4 treatment. These findings revealed that the anti fibrotic effects of *Michelia champaca* on liver fibrogenesis might be due to the presence of phytochemicals.
Keywords: anit-fibrotic, Michelia Champaca L, liver.

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INTRODUCTION

Liver is the key organ of metabolism and excretion. It is often exposed to a variety xenobiotics and therapeutic agents. The hepatotoxin carbon tetrachloride (CCl_4) is frequently used to induce liver fibrosis in animal models [1]. Treatment with CCl_4generates free radicals that trigger a cascade of events that result in hepatic fibrosis, mimicking the oxidative stress that has a fibrogenic effect on HSC [2–4]. . Liver fibrosis is a wound-healing process involving the activation of hepatic stellate cells (HSCs) and an increased deposition of extracellular matrix (ECM) components, including type I/III collagen, metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) [5-6]. HSCs play pivotal roles in fibrogenesis in the liver [7-8]. Following injury, HSCs are activated and undergo complex transdifferentiation, leading to increased proliferation, migration and contraction and a shift towards synthesis and deposition of ECM materials, contributing to scar formation and eventually cirrhosis [9-11]. A characteristic feature of activated HSCs is the activation of several cytokine mediators, including transforming growth factor-β (TGF-β), platelet derived growth factor (PDGF) and angiotensin [12-14]. Therefore, investigation of gene expression and identification of the potential therapeutic agent is extremely important.

Michelia champaca L. (Magnoliaceae) commonly known as Svarna champa, a tall handsome tree with yellow fragrant blossoms, is commonly used by many traditional herbal preparations. The plant is also reported to have significant wound healing [15], antimicrobial [16] , antidiabetic [17], antitumor [18], anti-inflammatory [19], antioxidant [20] and antiinfective [21] properties. The present study investigated the antifibrotic effect of *Michelia champaca* on inflammation via studying the MMP-8 and TIMP1genes expression in CCl_4 induced rats.

MATERIALS AND METHODS

Collection of plant material

The *Michelia champaca* flowers were procured from the local areas of Udumalaipettai, Coimbatore District,Tamilnadu. The collected plant material was botanically identified and confirmed by Dr.S.John Britto, The Director, Rapinat Herbarium, St. Joseph's College, Tiruchirappalli, Tamilnadu.

Preparation of Extract

Flowers were shade dried and were finely powdered. The 150g of powdered material was dissolved with 250ml of 70% methanol and extract was prepared using soxhlet apparatus for 30-40 hours. The extract was filtered and concentrated on a water bath at temperature below 50º C to syrub consistency (yield: 12%). Then it was stored in refrigerated condition for further use.

Experimental Animals

Healthy Wistar albino rats of male, weighing about 150-200g were obtained from Tamil Nadu Veterinary and Animal Science University, Chennai, India. Animals were maintained under standard conditions (12 h light / dark cycle; 25 ± 2º C with 65 ± 5% humidity) and were fed with standard rat feed (Sai Durga feeds and Foods, Bangalore, India) and water *ad libitum*. All the animals were acclimatized to laboratory conditions for a week before commencement of the experiment. All the experimental protocols were reviewed and approved by the Institutional Animal Ethical Committee (IAEC) prior to the initiation of the experiment and the care of the laboratory animals was taken as per the CPCSEA regulations (Registration Number: 790/03/ac/CPCSEA).

Experimental design

The animals were divided into 5 groups consisting of 6 animals in each group. Group I rats received saline (0.5 ml/kgb.wt) orally for 21 days. Group II rats administered with CCl_4 (0.5ml/kg b.wt) dissolved in olive oil (1:1 ratio) injected intraperitoneally for 21 days alternatively. Group III administered with CCl_4 treated with methanolic extract of *M.champaca* (300 mg/kg b.w) orally for 21 days. Group VI, the CCl_4 induced rats were treated with silymarin (25 mg/kg b.w) orally for 21 days. Group V rats were treated with methanolic extract of

M.champaca alone (300 mg/kg/b.w) orally for 21 days. The animals were sacrificed at the end of the experimental period by cervical decapitation under mild anesthesia. Blood sample was collected in centrifuging tubes and allowed to clot for 45 min at room temperature. Serum was separated by centrifugation at 2500 rpm for 15 minutes

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Gene Expression

RNA Extraction and RT-PCR

Total RNA was extracted from liver tissue samples (approximately 100 mg per sample) of experimental rats. Liver samples were flash frozen in liquid nitrogen and subsequently stored at −70°C in 1 ml Qiazol (QIAGEN, Valencia, CA, USA). Frozen samples were homogenized using a Polytron 300 D homogenizer. Then, 0.3 ml chloroform was added to the homogenate. The mixtures were shaken for 30 seconds followed by centrifugation at 4°C and 16,400 × *g* for 15 min. The supernatant was transferred to a new set of tubes, and an equal volume of isopropanol was added to the samples, shaken for 15 seconds and centrifuged at 4°C and 16,400 × *g* for 15 min. The RNA pellets were washed with 70% ethanol, briefly dries up, and then dissolved in diethylpyrocarbonate (DEPC) water. RNA concentration and purity were determined spectrophotometrically at 260 nm. Reverse transcriptase-PCR was carried out with the extracted total RNA as a template using kit (Qiagen One Step RT-PCR, USA). The following primers were used for MMP 8: 5'TGGCCATTCTTTGGGGCTCGC3'(forward) and 5'TGGGGTCACAGGGTTTGGGTGT3' (reverse) and β-actin as control gene;5`-GGAGAAGATGACCCAGATCA-3`(forward),5`-GATCTTCATGAGGTAGTCAG-3'(reverse). Reactions were performed as follows: an initial step at 94 ºC for 1 min 30s, followed by 35 cycles (1 min at 94 ºC, 1 min at 64 ºC and 1 min at 72 ºC) and finally a 3 min extension step at 72 ºC. After PCR, products were electrophoresed on agarose gels, stained with ethidium bromide, and images were recorded using gel documentation system (Bio-Rad Inc. USA).

Immunohistochemistry

Using poly-L-lysine-coated slides, liver sections were prepared and heated in an oven for 25 minutes at 60°C. After heating, tissue sections were deparaffinized and treated with 3% H_2O_2 for 10 min to inactivate endogenous peroxidases, heated in 10 mM citrate buffer at 121°C for 30 min for antigen retrieval, blocked in 5% normal serum for 20 min, and incubated with a primary polyclonal rabbit anti-rat antibody specific for MMP-8 (1:100 in PBS) overnight at 4°C. After three extensive washes with PBS, sections were incubated with a biotin-conjugated secondary antibody (1:2,000 in PBS) for 20 min at 32°C. After further incubation with horseradish peroxidase (HRP)-labeled streptavidin, antibody binding was visualized with diaminobenzidine (DAB) and sections were counterstained with hematoxylin for 10 seconds at room temperature based on manufacture instruction. For negative control, primary antibody was replaced with PBS alone. Tissue slides were examined using a microscope and examine the brown –stained positive antigens.

Statistical Analysis

Statistical analysis of the results was performed using one-way ANOVA followed by Dunken's Multiple Range Test (DMRT) using SPSS (Version 13, SPSS Inc., Chicago, IL, USA). A value of $P < 0.05$ was considered statistically significant between the measurements of the two compared groups. All values were reported as mean ± SD.

RESULTS AND DISCUSSION

The effect of CCl_4 and *Michelia champaca* on the oxidative stress, MMP 8 gene expression level was measured in liver tissues using real time (RT-PCR) analysis. In the CCl_4 group, MMP 8 gene expression was significantly ($p<0.05$) increased in mRNA level by 3.37 fold higher than that in the control rats *M.champaca* flower extract supplementation in combination with CCl_4 resulted in complete reversal of CCl_4 aberrant effect on the MMP 8 gene expression levels to their normal values as in the control group. These reversal changes were observed as significant ($p<0.05$) decrease in gene expression by 1.37 fold compared to CCl_4 group. Administration of flower extract alone resulted in non significant increase in expression level compared to the control group (Fig 1&2).

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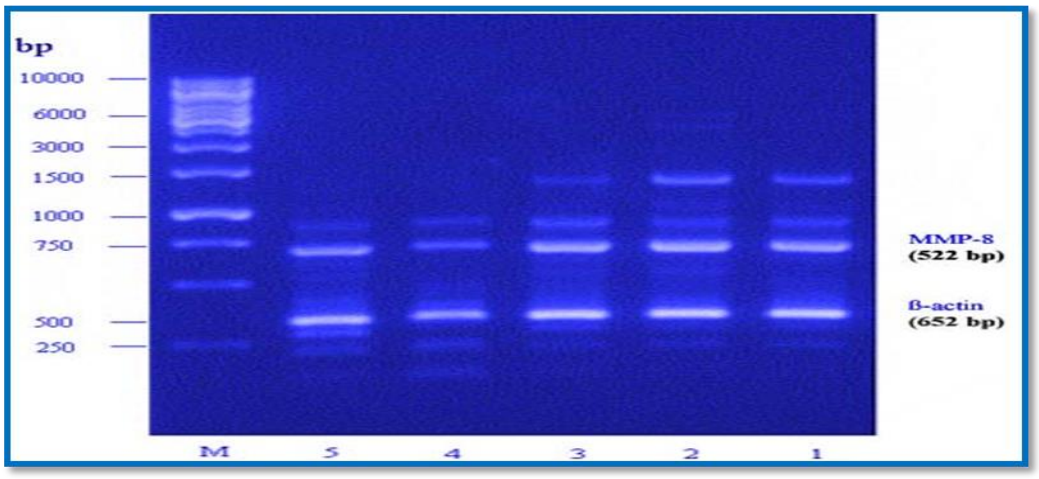

Figure 1: Agarose gel electrophoresis of gene expression of MMP-8 by RT-PCR

Lane I	**:**	**Control**
Lane II	**:**	**CCl_4 (0.5 ml/kg)**
Lane III	**:**	***M.champaca* flower extract (300 mg/kg) + CCl_4**
Lane IV	**:**	**Silymarin (25 mg/kg) + CCl_4**
Lane V	**:**	***M.champaca* flower extract alone (300 mg/kg).**

β-Actin was used as control

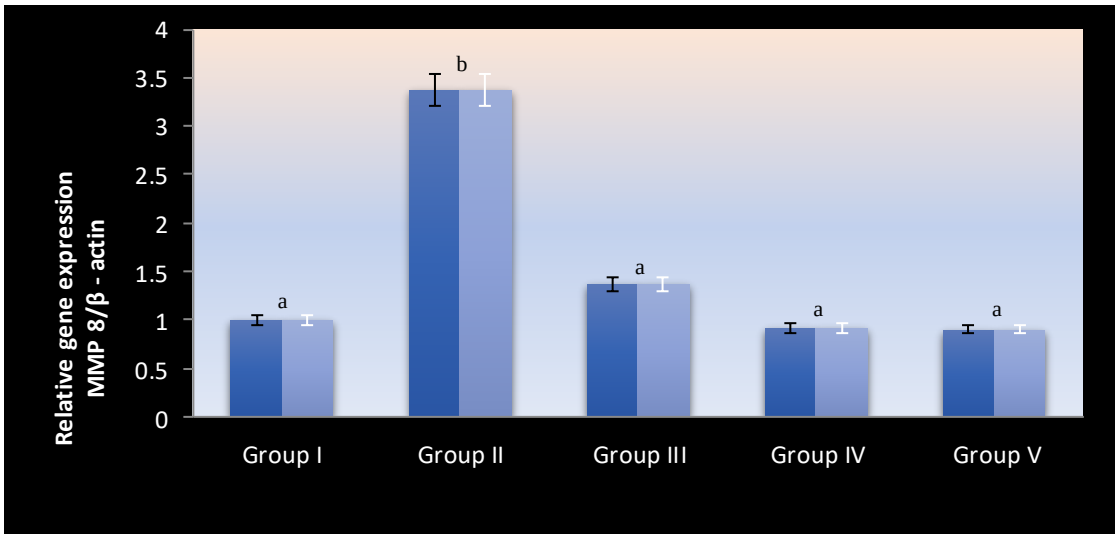

Figure 2: Semi-quantitative RT-PCR analysis of MMP-8 gene in rats

Values are given as mean ± S.D (n=6).Values not sharing a common superscript letter significantly at ($p<0.05$) (DMRT)

Gene expression represents a key role in the liver tissue response to pro-inflammatory and pro-fibrotic stimuli, which expressed by an intense transcriptional activation of a wide variety of genes, in different way involved in tissue repair. Oxidative stress and lipid peroxidation derived aldehydes, appears able to activate AP-1, a transcription factor which has been demonstrated to be essential for optimal transcription of many genes, whose some of primary interest in liver fibrosis, like TGF–β1, collagen type I and MMP2 [22-23].

Immunohistochemistry

Immunostaining of TIMP1 to detect liver fibrosis. TIMP1 staining of hepatocytes from the livers of *Michelia champaca* treated groups is shown in Fig 3(A-E). Hepatocytes of liver tissues from CCl_4 treated rats

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marked increase in ECM content and displayed bundles of collagen surrounding the lobules, which resulted in fibrous septa. These septa surrounded by TIMP1 cells which are characteristic of fibrosis. Treatment with *M.champaca* prevented the activation of HSC and only traces of TIMP1 positive cells were detected. However, flower extract alone group the mild expression was noted in portal areas. These findings support the *M.champaca* extract induced hepatoprotective activities against progressive liver damage by inhibiting fibrosis of hepatocytes and ameliorating their proliferation.

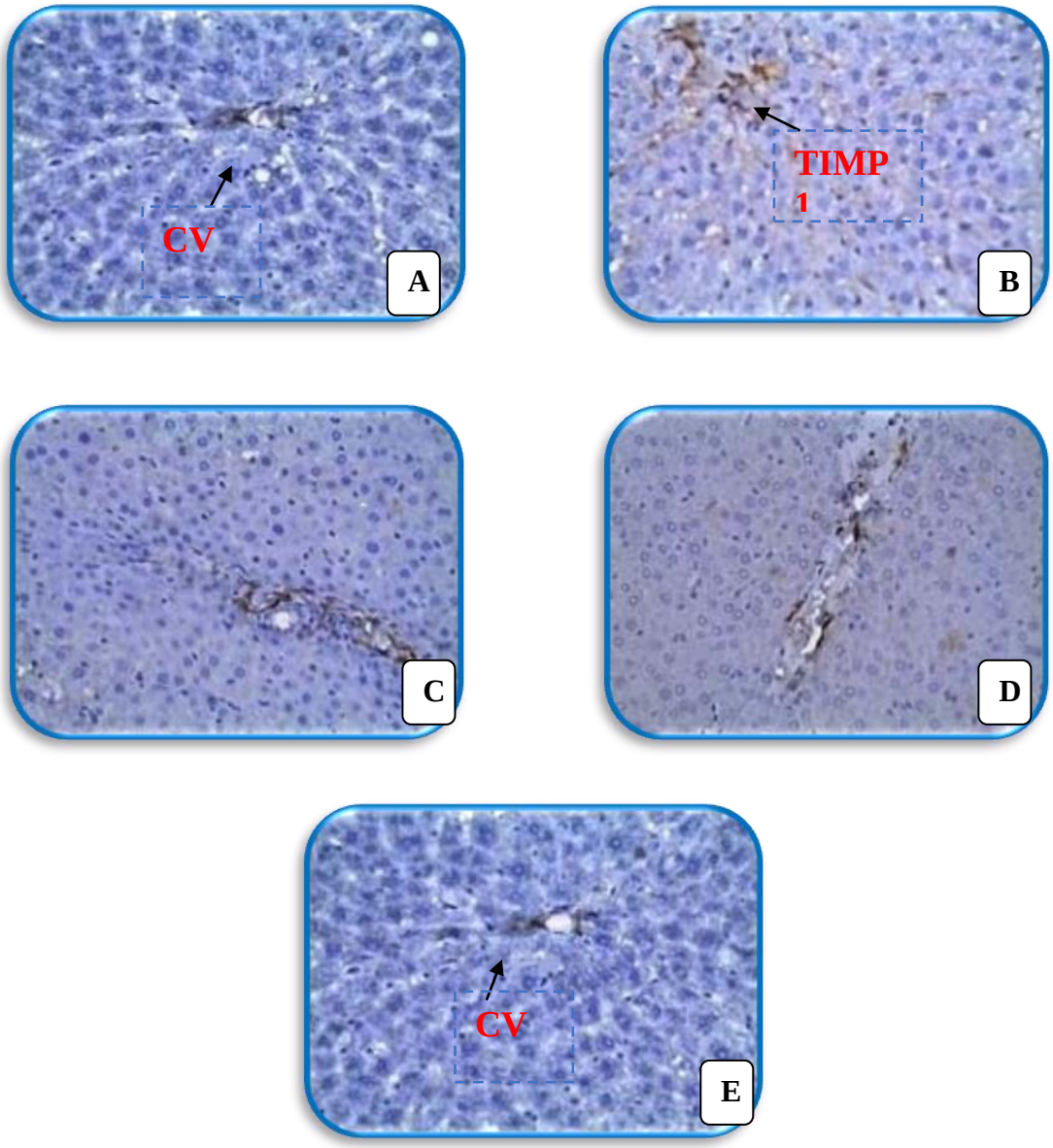

Figure 3: Photomicrographs of liver sections were immunostained with TIMP1

A: Group I (0.5ml normal saline) shows few cells were stained with TIMP1 within the lobule. **B:** Group II (0.5 mL of CCl_4 /corn oil (1:1, v/v)/kg, i.p) shows numerous TIMP1 positive cells around portal triad and central vein. **C:** Group III (CCl_4 +*M.champaca* (300mg/kg b.wt) shows small number of TIMP1 positive cells around portal triad and central vein indicated less fibrosis and improvement by treatment. **D:** Group IV (CCl_4 + silymarin (25mg/kg b.wt) shows few TIMP1 positive cells around portal triad and central vein are present. **E:** Group V (*Michelia champaca* flower extract alone) shows few cells were stained with TIMP1 similar to control.

Extracellular degradation of matrix proteins is regulated by a family of enzymes known as MMPs [24-26] types I, II, and III collagenases degrade interstitial collagens. Type IV collagenases/gelatinases degrade basement membrane collagen and gelatins and stromelysins which degrade a broad range of substrates including proteoglycans, laminin, gelatins, and fibronectin. However, the activated forms of MMPs in turn are

inhibited specifically by TIMPs, such as TIMP-1 and -2 [27-31]. In CCl_4 treated groups, the strong expression was noted in portal areas. After the rats treated with *M.champaca*, TIMP1 protein expression decreased significantly in liver cells, indicating that the benefits may be related to reducing hepatic collagen deposits, thereby improving liver metabolic functions

CONCLUSION

The results indicated that *Michelia champaca* flower extract was effective in reversing hepatic fibrosis after CCl_4 administration and the flower extract has the ability to down-regulate MMP and TIMP1 gene expression. Therefore, *M.champaca* may be considered an effective therapeutic antifibrotic agent for the treatment of hepatic fibrosis by suppressing proinflammatory mediators.

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