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Boswellia serrata and *Salvia miltiorrhiza* extracts reduce DMN-induced hepatic fibrosis in mice by TGF-β1 downregulation

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Abstract. – BACKGROUND: Hepatic fibrosis is characterised by a progressive accumulation of fibrillar extracellular matrix (ECM) proteins, including collagen that occurs in chronic liver diseases. Transforming growth factor-beta1 (TGF-beta)/Smad3 signalling plays a major role in tissue fibrogenesis acting as a potent stimulus of ECM accumulation.

AIM: To evaluate the effects of a combined therapy with anti-inflammatory *Boswellia* and anti-fibrotic *Salvia* extracts on the course of chronic hepatitis-associated fibrosis induced by dimethylnitrosamine (DMN) in mice, as well as on the hepatic expression of TGF-beta1 and Smad proteins.

METHODS: Chronic hepatitis-associated fibrosis was induced in mice by intraperitoneal DMN administration. Mice were assigned to 5 groups: controls; DMN without any treatment; DMN treated orally with *Boswellia* extracts (50 mg/kg/day); DMN treated orally with *Salvia* extracts (150 mg/kg/day); DMN treated orally with both *Boswellia* (50 mg/kg/day) and *Salvia* extracts (150 mg/kg/day). The liver was excised for macroscopic examination and histological, morphometric and immunohistochemical (IHC) analyses. For IHC, alpha-smooth muscle actin (alpha-SMA), collagen types I-III, TGF-beta1, connective tissue growth factor (CTGF), Smad3, Smad7, CD3, PCNA and TUNEL antibodies were used.

RESULTS: The combined oral administration of *Boswellia* and *Salvia* extracts improved the course and the macroscopic findings of DMN-induced chronic hepatitis-associated fibrosis. The histological severity of the hepatic fibrosis showed a marked improvement following treatment and was associated with a reduction in the hepatic expression of alpha-SMA, collagen I-III, CTGF, TGF-beta1, Smad3, and Smad7.

CONCLUSIONS: These data demonstrate that the treatment of *Boswellia* plus *Salvia* extracts is effective in preventing hepatic fibrosis in DMN-induced chronic hepatitis. The anti-fibrotic properties are mainly related to *Salvia* extracts and appear to be mediated by the inhibition of the TGF-beta1/Smad3 pathway.

Key Words:
DMN-induced hepatitis, Chronic hepatitis-associated fibrosis, Liver fibrosis, TGF-β, Smad proteins, ECM, Boswellia, Salvia, Anti-fibrotic.

Introduction

Hepatic fibrosis is characterised by a progressive accumulation of fibrillar extracellular matrix (ECM) proteins, mainly collagen, that occurs in response to a variety of insults, including viral hepatitis, alcohol abuse, drug use, metabolic disease, autoimmune disease[1,2]. Fibrosis is a chronic and progressive process regulated by a complex system of cell/matrix/cytokine and growth factors, but it may be a reversible event[3-5]. The physiological fibrogenesis process triggered by the onset of inflammation may lead to tissue repair or to fibrosis depending on the balance between extracellular matrix (ECM) synthesis or degradation. ECM degradation is mediated by the metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs)[3,4]. The pathological fibrosis leads to a progressive deterioration of the organ-specific function[2-4].

During liver injury, hepatic stellate cells (HSCs), a major fibrogenic liver cell type, are activated and transdifferentiated to become myofibroblasts (MFBs)[6]. MFBs proliferate and acquire fibrogenetic characteristics, such as expression of α-smooth muscle actin (α-SMA) as well as synthesis and secretion of ECM proteins, in particular type I and type III collagen[2,3,6]. During hepatic fibrogenesis, HSCs are activated by several inflammatory cytokines and growth factors. Of these, transforming growth factor-β1 (TGF-β1) seems to be the most important fibrogenic factor for MFBs[2,3,7]. TGF-β, via intracellu-

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lar TGF-β/Smad signalling pathway, is recognized as the strongest factor inducing ECM deposition by simultaneously stimulating the synthesis of new matrix components, increasing the synthesis of enzymes that inhibit ECM degradation, and decreasing the synthesis of matrix-degrading proteases[2,3,7].

Several anti-fibrotic drugs (chemical and biological) have been tested in experimental models of tissue fibrosis and shown to inhibit, mitigate or even reverse the fibrogenesis/fibrosis process[1]. Their anti-fibrotic activity would appear to be related to various mechanisms of action, by reducing activated ECM-producing cells and their profibrogenic effects (proliferation, motility, contraction, ECM deposition), by promoting ECM-producing cells apoptosis, or by promoting ECM degradation. Not only the efficacy, but also the safety of the anti-fibrotic drugs is important as these have to be administered for a prolonged period of time. Numerous pharmaceutical agents have been experimented with varying degrees of success, but with unacceptable side-effects in long-term treatment[1]. Therefore, the research of new therapeutic strategies, based on the as[illegible]tion of drugs with anti-inflammatory and [illegible]brotic properties, is needed in fibrotic dise[illegible].

Among the numerous drugs investigated [illegible]r for their anti-fibrotic potential, botanicals re[illegible]sent a remarkable component[1,8][illegible] develo[illegible]ment of anti-fibrotics from n[illegible]cts ma[illegible] reduce the risk of toxicity [illegible]lst mai[illegible]ning the therapeutic effectiveness [illegible] the dr[illegible] used in the clinical setting. B[illegible]anic[illegible] [illegible]fibrotic properties [illegible]e been [illegible]ted both in experimental and [illegible]ical studies [illegible]g efficacy and safety i[illegible]ans[1,8-17].

Of the bota[illegible]icals, [illegible]tention has been focused on [illegible]swellia Serr[illegible]nd *Salvia Miltiorrhiza*.

Th[illegible]um resin extracts from *Boswellia Serrata* ha[illegible] re[illegible]ed to have an anti-inflammatory and i[illegible]odulat[illegible] effect[11,18]. Boswellic [illegible]ids, ur[illegible]pe of [illegible]tacyclic triterpenes, have [illegible]identi[illegible]e main biologically active co[illegible]nents of [illegible]wellia extracts[11].

[illegible]ia Miltiorrhiza* has been reported to exert i[illegible]ects, both *in vitro* and *in vivo,* [illegible]ainst hepatic fibrosis[8,13,14,19-23]. Furthemore, [illegible]ia Miltiorrhiza* has been shown to decrease th[illegible]oduction of the ECM components fibronectin, laminin and collagen I and III protein[19,20]. Several compounds have been extracted and analysed from *Salvia Miltiorrhiza* roots, the most potent of which would appear to be salvianolic acid and Tanshinone II-A[12-16,19,20].

Aim of the present study was to evaluate: (1) the prophylactic effect of anti-inflam[illegible] Boswellia extracts, alone or in combi[illegible] anti-fibrotic Salvia extracts, on the [illegible]velopment of chronic hepatitis-associated f[illegible]sis induced by dimethylnitrosamine (DMN) i[illegible] and (2) their potential regulatory e[illegible]cts o[illegible]ro-fibrotic TGF-β/Smads path[illegible].

DMN is a potent he[illegible]toxin and is an [illegible]lished model of liver[illegible]sis w[illegible]a pathophy[illegible]ology closely resembli[illegible]rrhosis [illegible] man. This model has [illegible]n show[illegible]roduce[illegible]ny decompensating [illegible]ures of hu[illegible] fibrosis, such as po[illegible]ertension, a[illegible], hypoproteinaemi[illegible]nd b[illegible]mical abnormalities[24-26]. Therefore, it may r[illegible]nt a useful model for scre[illegible]nti-fibrotic a[illegible][27].

Mate[illegible]s and Methods

An[illegible]

For[illegible]k Swiss 10 week-old mice were [illegible]ed (Charles River Laboratories, Calco, Lecco, [illegible]nimals were kept in a restricted-access [illegible]th controlled temperature (23°C) and light/dark cycles (12h/12h). Standard rodent chow pellets (Laboratori Piccioni, Gessate, Milan, Italy) and tap water were allowed *ad libitum*. The protocol was approved by the Animal Research Committee of the University of L'Aquila, L'Aquila, Italy.

Induction of Hepatitis

Chronic hepatitis-associated fibrosis was induced by intra-peritoneal administration of DMN (Sigma Aldrich, Milan, Italy) under light ether anaesthesia. Each mouse received 10 μg DMN per g of body weight (1% in saline solution) for the first 3 consecutive days of the week for 6 weeks[28,29]. The amount of DMN administered was adjusted each week to body weight. Animals in the control groups received a sham intra-peritoneal injection of the same volume of 0.9% saline.

Animals were monitored daily, for food and fluid intake, and examined for signs of hepatitis, including weight loss and abdominal distension due to ascites[30], as well as signs of systemic inflammation such as piloerection, lethargy, and periorbital exudates[31]. Animals were weighed at the beginning of the study (pre-treatment), and

thereafter, weekly before each DMN administration. Three days after the last DMN administration (post-treatment), the animals of each group were sacrificed by CO_2 inhalation.

In this protocol, the Authors adhered to the Italian National Research Council criteria for the care and use of laboratory animals.

Experimental Design

Hepatic Fibrosis

The following 5 groups of mice were included in this study: (1) control mice (control group) (n=6); (2) DMN mice (DMN group) without any treatment (n=10); (3) DMN mice treated orally with Boswellia extracts (50 mg/kg/day) (DMN/Boswellia group) (n=10); (4) DMN mice treated orally with Salvia extracts (150 mg/kg/day) (DMN/Salvia group) (n=10); and (5) DMN mice treated orally with both Boswellia (50 mg/kg/day) and Salvia extracts (150 mg/kg/day) (DMN/Boswellia+Salvia group) (n=10). The dose of Boswellia and Salvia extracts used in the study was similar to that r[illegible]ed by other Authors[20,32-36].

As assessed by high performance liqui[illegible]romatography, Boswelic powder extracts [illegible]traceutica Srl, Monterenzio, Bologna, Italy) [illegible] composed of 95% boswellic ac[illegible]hile Sal[illegible] powder extracts (Herbasin [illegible] Lt[illegible]enyang, China) are composed of 40[illegible]anshin[illegible] IIA.

The extracts were dis[illegible]d in di[illegible]d water according to the estab[illegible]hed [illegible]

Mice received or[illegible] either [illegible] of deionized water, Boswelli[illegible]acts (50 mg[illegible]ay) solution, Salvia ex[illegible] mg/kg/day[illegible]ution or Boswellia plus Salvia [illegible]ts solution for a period of 6 w[illegible]s.

Mic[illegible]ere weighed onc[illegible]week to adjust drug[illegible]se to body weight. Daily administration sta[illegible] da[illegible] and continued for 6 weeks. All the m[illegible] sacrifice[illegible] days after the last drug [illegible]inist[illegible]

Sa[illegible]le Reco[illegible]y and Preparation

[illegible]parotomy was performed, the liver was visu[illegible] [illegible]idly excised as a whole. The pres[illegible]ce of ascites (0=absent, 1=mild, 2=severe) and [illegible]sions between the liver and adjacent organs ([illegible]ent, 1=Mild/focal-zonal, 2=Severe/diffuse) was scored on a 0-2 scale[29]. The liver was then weighed, and the right lobe was fixed in 10% buffered formaldehyde and embedded in paraffin for histological studies [Haematoxylin and Eosin (H&E), Sirius Red-Fast Green staining and immunohistochemistry].

Analytical Methods for Serum Sa[illegible]

Blood samples were obtained fro[illegible]e inferior vena cava. Serum was collected f[illegible] blood sample by immediate centrifugation a[illegible]0 g for 8 minutes at 4°C. Serum alani[illegible] ami[illegible]ferase (ALT) and aspartate amin[illegible]nsferase (A[illegible] levels were measured usin[illegible]tomated techniq[illegible]

Assessment of Mac[illegible] and Microscopic L[illegible]ons

Two indepe[illegible]nt observers [illegible]e following param[illegible]ver appeara[illegible]nd colour (0=physio[illegible]gical, [illegible]thological without necrosis, 2=pathological [illegible]necrosis); liver weight, live[illegible]t/body weig[illegible]o, spleen weight and s[illegible]en/body weight ratio[29].

[illegible]pecimens obtained from the right lobe of the [illegible] of all anim[illegible] were washed and immediately [illegible]mersed in [illegible]% buffered formalin in phospha[illegible]ffered [illegible]ne (PBS) (pH 7.4) for 3 h at room t[illegible]re followed by the standard pro[illegible]edure for paraffin embedding. Serial 3 μm sec[illegible]re stained with H&E to assess the degree [illegible]mation and with Sirius Red-Fast Green stain to detect connective tissue and fibrosis. The stained sections were then observed under an Olympus BX51 Light Microscope (Olympus, Optical Co. Ltd., Tokyo, Japan). Two pathologists (A.V. and R.S.), blinded to the study procedures, examined and scored all histological sections of the hepatic samples. Liver fibrosis and necro-inflammatory activity were evaluated semi-quantitatively according to the METAVIR scoring system[37]. Fibrosis was staged on a 0-4 scale as follows: F0=no fibrosis; F1=portal fibrosis without septa; F2=numerous septa without cirrhosis; F4=cirrhosis. Necro-inflammatory activity was graded as follows: A0 = none; A1 = mild; A2 = moderate; A3 = severe.

Colorimetric Evaluation of Collagen Content

Samples (5 mm^3) of the right lobe of the liver parenchyma, not including the Glisson's capsule and larger portal branches, were removed and immediately immersed in 10% buffered formalin for paraffin embedding. Eight 15-mm thick, 100 mm^2 large sections were obtained from each liver and used for colorimetric evaluation. Additional 5-mm thick sections were stained and observed

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at light microscopy together with stained sections for colorimetric evaluation according to Lopez-De Leòn and Rojkind[38].

Sections for colorimetric evaluation were deparaffinized through successive baths in absolute toluol, toluol: ethanol (50:50) and 50% aqueous ethanol and water. Staining procedures with fast green FCF 0.1 (Chroma-Gesellshaft, no. IA30, Stuttgart, Germany) and Sirius Red F3B 0.01% (Atomergic Chemical Corporation, no. 10022; Plainview, NY, USA) were performed according to Gascon-Barrè et al[39]. Colours were eluted in 0.05_M NaOH and 50% aqueous methanol.

The eluted colours were examined in a Lambda 4 B PE spectrophotometer. Correlations between absorbance and protein estimations were assessed according to Gascon-Barrè et al[39].

Non-collagenous protein determination was obtained using the following formula:

$$\text{Non-collagenous protein (mg)} = \frac{\text{Absorbance at 605 nm}}{2.08}$$

Collagenous protein determination wa[illegible] tained using the latter interference facto[illegible] following formula:

$$\text{Collagen } (\mu g) = \frac{\left(\begin{array}{c}\text{Absorbance} \\ \text{at 540 nm}\end{array}\right) - \left(\begin{array}{c}\text{0.26 absorban[illegible]} \\ \text{[illegible]05 nm)}\end{array}\right)}{\text{[illegible].4}}$$

Collagen content ([illegible] collag[illegible] calculated using the [illegible]llowing [illegible]la:

$$\begin{array}{c}\text{Collagen con[illegible]} \\ (\mu g/\text{mg total pr[illegible]ein})\end{array} = \frac{\mu g \text{ colla[illegible]}}{\text{[illegible]g collagen + mg n[illegible]lagenous protein)}}$$

[illegible]bove[illegible]ntioned formulae were applied accor[illegible]ascon-B[illegible]è et al[39].

[illegible]unohi[illegible]istry

[illegible]ples fro[illegible]e right lobe of the liver, obtai[illegible] as described above, were promptly fixed [illegible]ered formalin in PBS (pH 7.4) for 3 [illegible] dehydrated in graded ethanol, and embedded in [illegible]w-temperature-fusion paraffin. Serial 3 μm se[illegible]s were incubated for 40 min in methanol and 3% hydrogen peroxide solution and then rinsed in PBS. Thereafter, sections were incubated overnight at 4°C with polyclonal antibodies to TGF-β1, CD3, α-SMA, collagen I and III, Smad3, Smad7 and CTGF (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), used at a dilution of 1:500, 1:200, 1:500, 1:300+1:300, 1:500, [illegible] and 1:500, respectively, in PBS. Sa[illegible] then rinsed with PBS for 5 min and i[illegible]bated with a labelled streptavidin-biotin-pero[illegible]e conjugate kit (Dako LSAB, cod. K0675, Da[illegible]tomation, Milan, Italy). After rinsing in PBS fo[illegible]n, the sections were incubated [illegible] 3,3-diami[illegible]zidine-tetrahydrochlorid[illegible]DAB, Sigma A[illegible] Milan, Italy) for 1-3 [illegible]or neg[illegible]e controls [illegible]e primary antibody was [illegible] (same d[illegible]tion) with normal seru[illegible]rom the [illegible] specie[illegible]

Finally, the [illegible]nples were [illegible]ned with Mayer's H[illegible]ylin and o[illegible]ed under a photomic[illegible]scop[illegible]lympus BX[illegible]1 Light Microscopy, Olympus [illegible]tical Co. Ltd., Tokyo, Jap[illegible] immunohis[illegible]mistry analysis was e[illegible]aated using a semi-qua[illegible]titative score, as pre[illegible]usly described[40].

[illegible]or *in situ* de[illegible]ion of cell apoptosis, the term[illegible] deoxynu[illegible]otidyltransferase-mediated trip[illegible]ate en[illegible]belling (TUNEL) method was used [illegible] kit; Oncor, Gaithersburg, MD, [illegible]SA).

[illegible]lar proliferation was assessed by prolifer[illegible]lular nuclear antigen (PCNA) staining (Dako, PC10; mouse anti-PCNA, used at dilution of 1:100, Dako-Cytomation, Milan, Italy).

Statistical Analysis

Data are reported as mean ± standard deviation (SD). For ordinal variables (ascites, liver adhesions, liver colour, degree of fibrosis), results in all groups of mice were analyzed using Kruskal-Wallis non parametric ANOVA. Post-hoc comparisons between pairs of groups were assessed by using Wilcoxon rank sum test. For quantitative variables (body weight, liver weight, spleen weigh, aminotransferase levels, collagen content), results in all groups of mice were analyzed using one-way parametric ANOVA. A $p < 0.05$ value was considered to be statistically significant.

Data analysis was performed with SAS, version 9.2, 2008 (SAS Institute, Inc, Cary, NC, USA).

Results

Clinical and Macroscopic Findings

The effects of Boswellia and Salvia administration and the characteristics of DMN-induced he-

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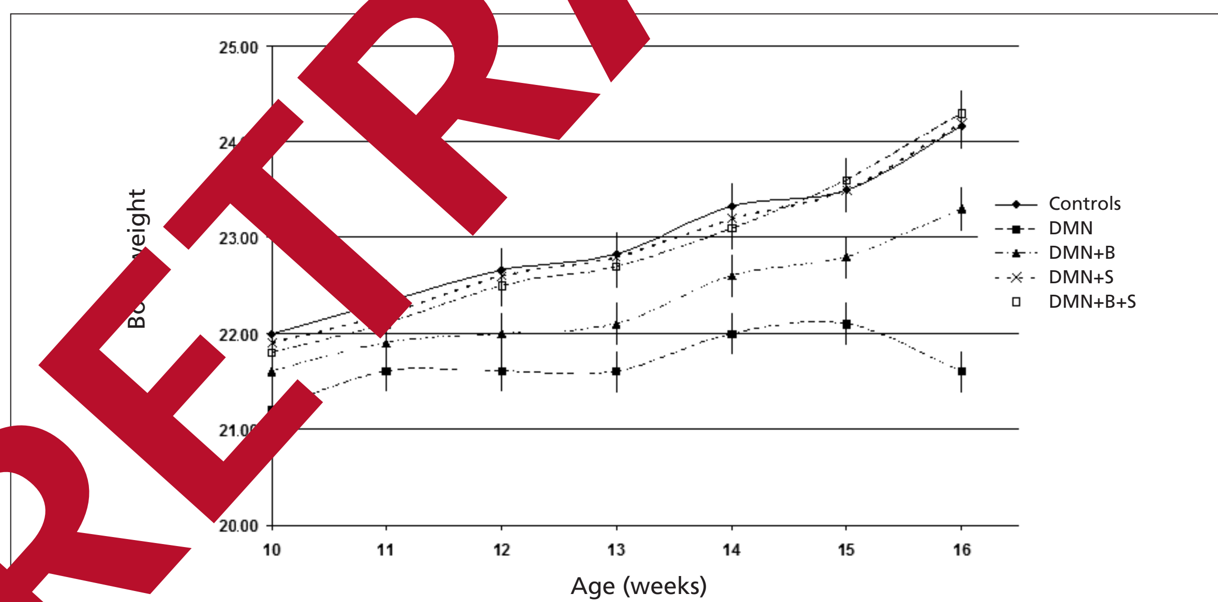
Table I. Effects of Boswellia (B) and Salvia (S) extracts on the course and macroscopic findings of dimethylnitrosamine (DMN)-induced chronic hepatitis in mice.

Parameters	Controls (n = 6)	DMN (n = 10)	DMN+B (n = 10)	DMN+S (n = 10)	DMN+B+S (n [illegible]
Age (weeks)	10	10	10	10	10
Sex (F/M)	4/2	8/2	5/5	5/5	5/5
Duration of DMN treatment	6 weeks	6 weeks	6 weeks	6 weeks	[illegible] weeks
Mortality (%)	0	30	30	10	[illegible]
Body weight (g)	24.17 ± 6.68	21.60 ± 3.72	23.30 ± 4.35	24.20 [illegible]25	24.[illegible]21
Ascites	Np	0.57 ± 0.53*	0.28 ± 0.48	0.[illegible] 0.33	[illegible]
Adhesions	Np	0.42 ± 0.53*	0.28 ± 0.48	Np	Np
Pathologic colouring	Np	1.28 ± 0.48*	0.85 ± 0.37	[illegible]4 ± 0.52	0.30 ± 0.4[illegible]
Liver weight (g)	1.01 ± 0.34	0.82 ± 0.21	0.89 ± 0.26	[illegible]	0.95 ± 0.25
Liver weight/Body weight ratio	0.041 ± 0.003	0.039 ± 0.013	0.037 ± 0.0[illegible]	0.0[illegible]04	0.03[illegible].004
Spleen weight (g)	0.10 ± 0.03	0.11 ± 0.02	0.10 ± [illegible]	0.10 [illegible]	[illegible] ± 0.03
Spleen weight/ Body weight ratio	0.004 ± 0.001	0.005 ± 0.001	0.004 [illegible]1	0.004 ± [illegible]	[illegible]4 ± 0.001

Data are expressed as mean ± SD; Np = not present; *$p < 0.05$ vs controls, DM[illegible]S and [illegible]B+S.

patic fibrosis are outlined in Table I. Age, sex and duration of DMN treatment were comparable in the four groups of mice. The administration of DMN induced weight loss only in the mice of DMN group whereas the groups of mice treated with drugs showed a progressive gain of w[illegible]t (Figure 1). In particular, mice treated wit[illegible] alone or in association with Boswellia sh[illegible]d a trend comparable to that of the control grou[illegible]t the end of the treatment period, DMN-treated [illegible] h[illegible] lower body weight co[illegible]pared to control mice ([illegible].60±3.72 and 2[illegible].17±6.68, respectively) whereas [illegible]e treated [illegible] drugs (DMN/ Boswellia, D[illegible]/Salvia a[illegible] DMN/Boswellia + Salvia) sho[illegible] body [illegible]eight (23.30±4.35, 24.20±5.25 and 24.[illegible], respectively) comparable to con[illegible]ol-group mice (24.17±6.68) (Table I).

[illegible]lity rates in DMN group, DMN/Boswellia [illegible]ou[illegible]MN/Salvia and DMN/Boswellia + Salvia group were 30%, 30%, 10% and 0% respectively.



[illegible]1. Body weight gain curves of the 5 groups of mice at basal and weekly, over a 6 weeks treatment. Plot of weight (g) vs a[illegible] (days). Each point represents mean weight data pooled from 6 controls and 10 treated mice. Standard deviations are indicated. Each point between controls and DMN, as well as between controls and DMN+B, were statistically significant ($p < 0.05$). Comparing controls versus DMN+S and DMN+B+S, the differences were not statistically significant. DMN = dimethylnitrosamine, B = Boswellia extracts, S = Salvia extracts.

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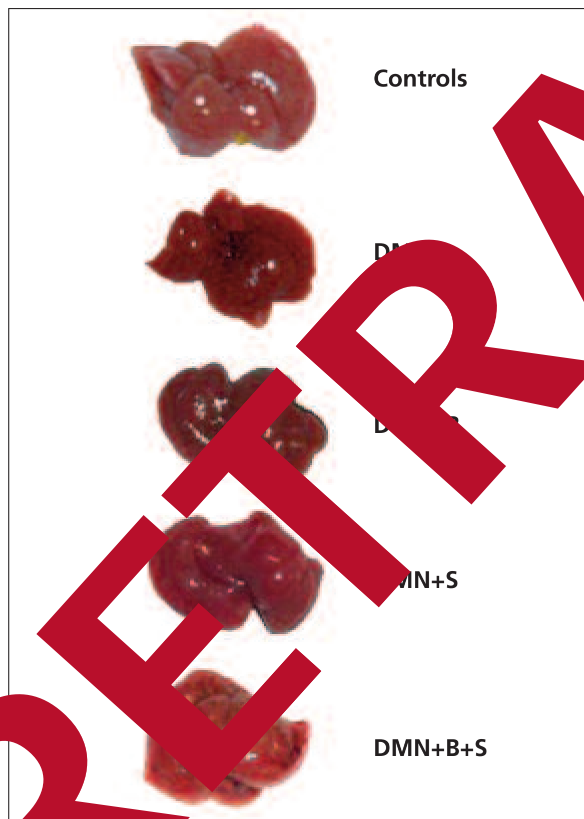
Mild or severe ascites was found in 42.8% of the DMN mice, mild ascites in 28.5% of the DMN/Boswellia mice and in 11% of the DMN/Salvia mice, but was absent in the DMN/Boswellia + Salvia group of mice. Adherences with other organs were present in 42.8% of the DMN mice and in 28.5% of the DMN mice treated with Boswellia alone, but were absent in the DMN mice treated with Salvia alone or in association with Boswellia.

Representative examples of liver taken from each group of mice are shown in Figure 2. The liver of DMN-treated mice was slightly smaller than that of control-mice (0.82±0.21 and 1.01±0.34, respectively),was harder, had a darker colour and a granulose appearance of the surface with the presence, in several cases, of foci of necrosis. The liver of mice treated with Boswellia alone was similar to that of DMN mice and did not show significant differences in the above-mentioned parameters. Instead, livers of mice treated with Salvia alone or in association with Boswellia were similar. In particular, the simultaneous administration of Boswellia and Salvia extracts improved the liver wei[illegible] pathologic colouring, ascites and adhes[illegible] I). Liver weight and liver weight/bod[illegible]eight ratio (%), in all groups of mice, are show[illegible] Figure 3.



[illegible]gure 2. [illegible]acroscopic appearance of liver from the 5 [illegible]s of mice. The liver from DMN-treated mice appeared [illegible] and harder and showed a more brown colouring wit[illegible]crotic areas compared to that from controls. No differences were observed between livers from DMN+B+S and controls. DMN = dimethylnitrosamine, B = Boswellia extracts, S = Salvia extracts.

Microscopic Findings

Representative histologi[illegible] findings ([illegible]nd Sirius Red-Fast Green [illegible]in) of the liver [illegible] controls, DMN mice [illegible]out a[illegible]reatment [illegible]d DMN mice treated w[illegible]lia plus Salvia extracts, are sho[illegible]n Figu[illegible]

A marked i[illegible]ease of con[illegible]e, in the portal tract[illegible]ithin the li[illegible]renchyma, was foun[illegible] mi[illegible]eiving DMN compared to controls. The liver [illegible]N/Boswellia + Salvia trea[illegible]e presented [illegible]t normal parenchym[illegible] architecture and mild [illegible]brosis. The degree of h[illegible]atic fibrosis was significantly higher in DMN m[illegible]e compared [illegible] controls and the DMN mice t[illegible]d with Sal[illegible] alone and those treated with Bo[illegible] + S[illegible]a (Table II). Likewise, colorimetric [illegible]on of collagen content demonstrated statistically higher values of collagen in [illegible] of DMN mice compared to controls and [illegible]M[illegible]ice treated with Salvia alone or with Boswellia + Salvia (Table II).

The degree of hepatic inflammation was increased in DMN mice compared to controls, but did not differ between the three groups of DMN mice treated with Boswellia alone, Salvia alone or Boswellia + Salvia (data not shown). Furthermore, in DMN mice not receiving treatment, the serum levels of ALT and AST were higher compared to those in controls and DMN mice treated with Boswellia alone, Salvia alone or Boswellia + Salvia (Table II).

Immunohistochemical Evaluation

A marked reduction in α-SMA and collagen I-III (Figure 5), CTGF and TGF-β1 (Figure 6), as well as Smad3 and Smad7 (Figure 7) staining was observed in the liver of all DMN mice treated with Boswellia plus Salvia compared to the liver of untreated DMN mice. Compared to untreated DMN mice, a clear reduction in all immunostainings was also found in the majority of DMN mice treated with Salvia alone, but not in those treated with Boswellia alone (data not shown). Overall, the immunostaining pattern of α-SMA, collagen I-III, CTGF, TGF-β1, Smad3 and Smad7 in the DMN mice treated with

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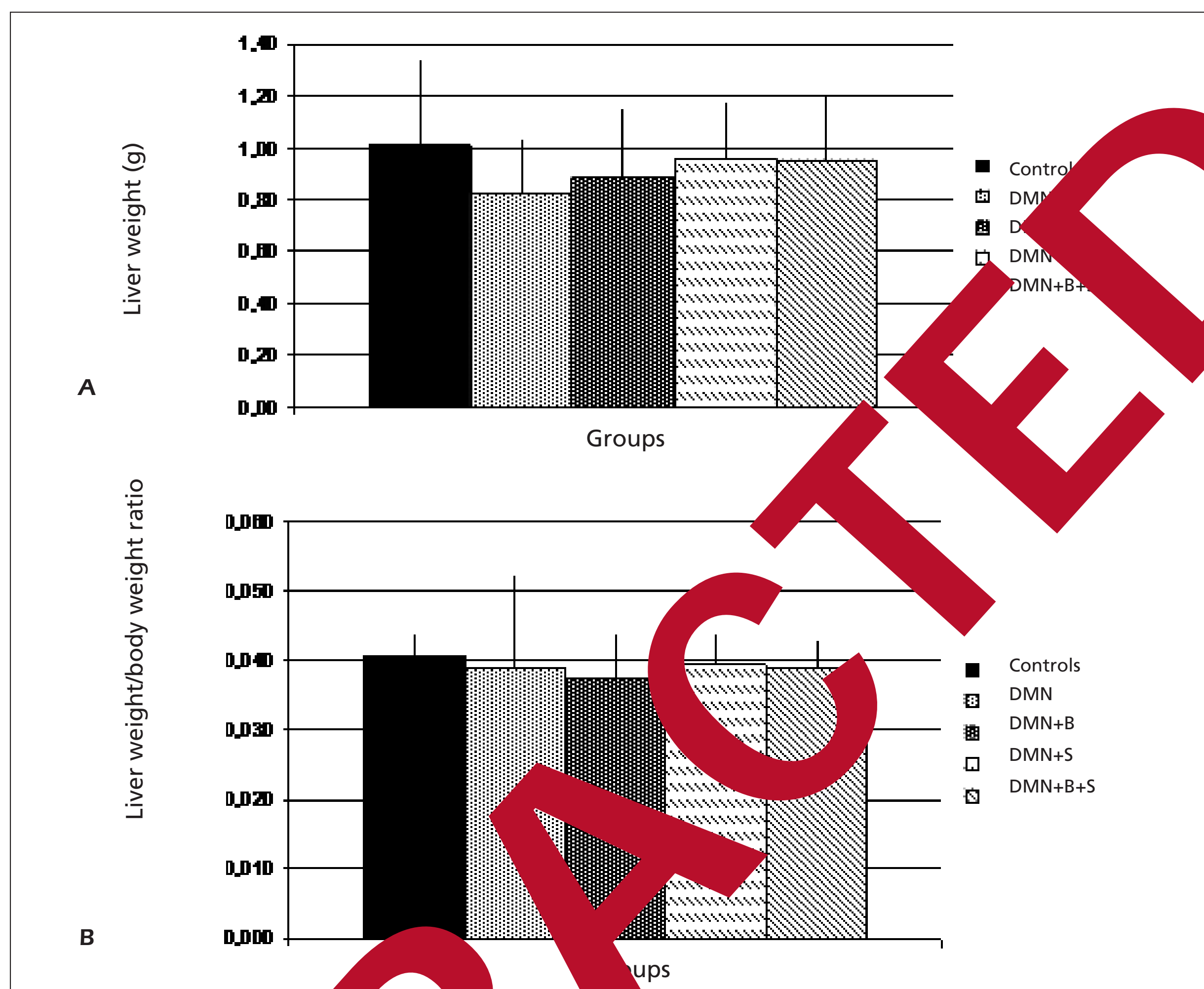

Figure 3. ***A-B,*** Liver weight ... r weig... dy weight ratio (%) in the 5 groups of mice. No significant differences were observed between the vario... gro...

Boswellia plus S... was simila... at in the DMN mice tre... d ... Salvia alone.

The pattern of CD3... lls staining was comparable in ... e three group... DMN mice treated with B... ellia alone, Salvi... ne or Boswellia plus Salvia, but was less intense than that observed in DMN mice not receiving treatment (data not shown).

PCNA and TUNEL staining were both markedly increased in the liver of mice receiving

Tab... Serum ... els of alanine aminotransferase (ALT), aspartate aminotransferase (AST), degree of hepatic fibrosis, and hepatic ... ontent in ... eated dimethylnitrosamine (DMN) mice and in DMN mice treated with Boswellia (B) alone, Salvia (S) ... r with b... Boswellia and Salvia.

	ALT(U/L)	AST (U/L)	Fibrosis total score	Collagen content (μg/mg total protein)
...)	44.33 ± 19.42	66.83 ± 28.08	0	0.0135 ± 0.0006
DMN (n=7)	159.71 ± 89.30*	436.71 ± 123.87*	1.85 ± 0.69#	0.0197 ± 0.0018#
...MN + B (n=7)	60.16 ± 16.79	85.42 ± 23.47	1.42 ± 0.53	0.0186 ± 0.0012
... + S (n=9)	52.50 ± 10.59	80.16 ± 11.40	0.77 ± 0.66	0.0158 ± 0.0007
D...N+ B+S (n=10)	50.16 ± 14.52	78.16 ± 17.47	0.60 ± 0.51	0.0137 ± 0.0011

(n) = Number of mice. *p < 0.05 vs Controls, DMN+B, DMN+S and DMN+B+S; #p < 0.05 vs Controls, DMN+S and DMN+B+S.

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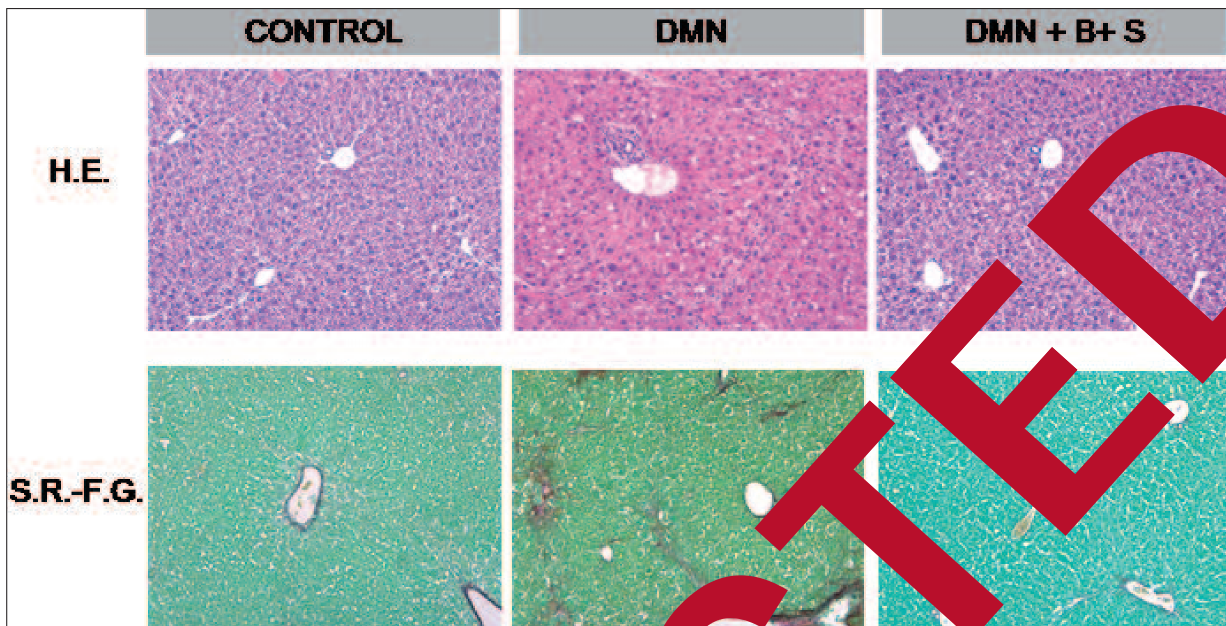

Figure 4. Haematoxilyn-Eosin (H&E) staining: O.M. 4X. An in[illegible]ase of inflammat[illegible]n is present in dimethylnitrosamine (DMN)-treated group of mice, which is non found in DMN mice [illegible]ted with Bosw[illegible] (B) plus Salvia (S) extracts. Sirius Red-Fast Green (SR-FG) staining O.M. 4X. The liver from DMN [illegible]p of mice sh[illegible] an increase in the collagen content compared to that from controls and DMN+B+S group.

DMN, but returned to normal levels of [illegible] following treatment with Boswellia plus [illegible]via extracts (Figure 8). A similar trend was obs[illegible]d in the mice treated with Salvia extracts alone.

Discussion

Th[illegible] present study demonstrated that the combined oral administration of *Boswellia serrata*

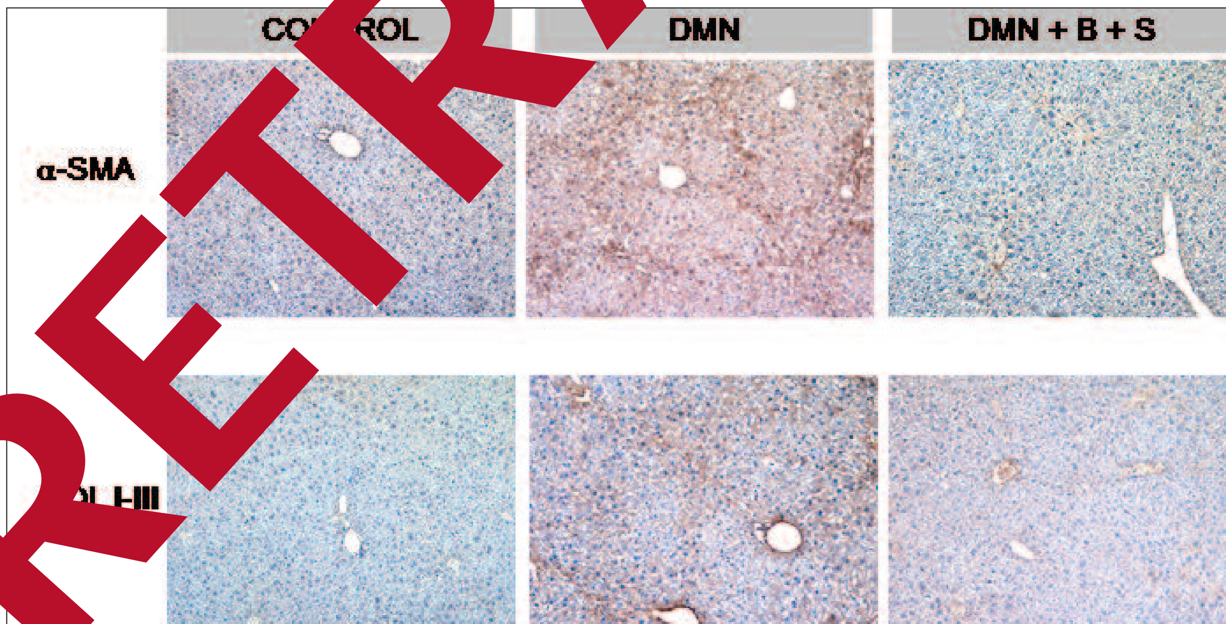

Figure 5. Immunohistochemistry O.M. 4X. α-SMA and Collagen I-III immunostainings are markedly increased in the liver from mice receiving dimethylnitrosamine (DMN) compared to controls. The expression both of α-SMA and Collagen I-III in the DMN mice treated with Boswellia (B) plus Salvia (S) extracts is close to that of controls.

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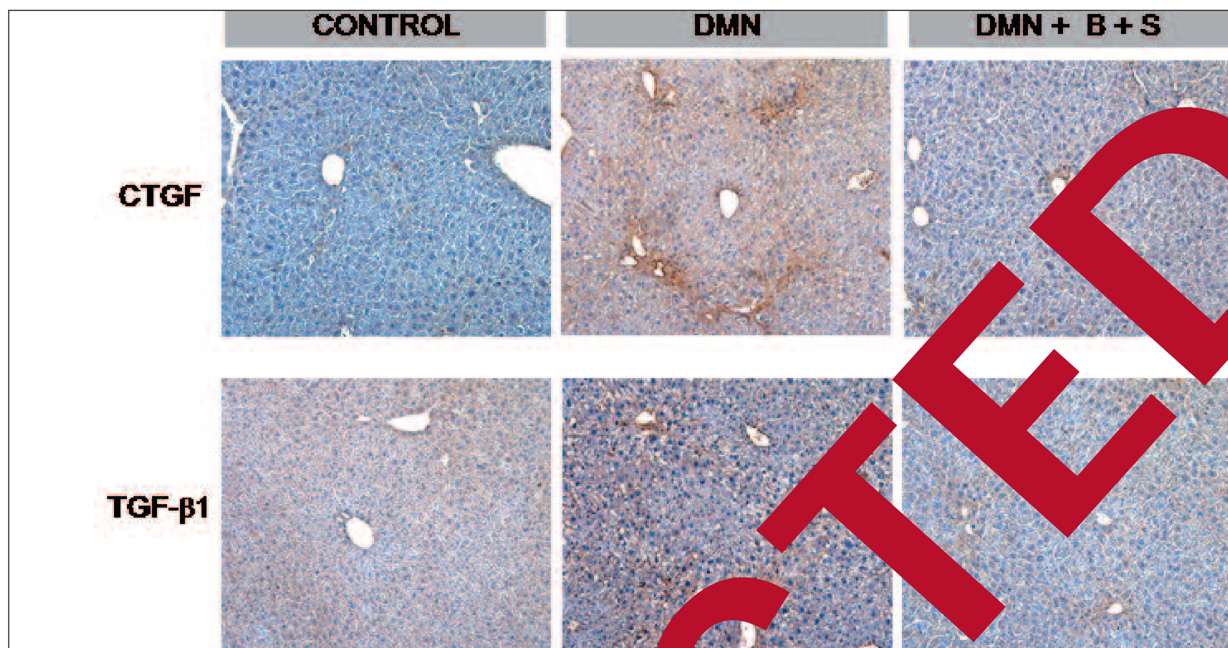

Figure 6. Immunohistochemistry O.M. 4X. Immunostaining both CTGF and TG is most prominent in the liver from dimethylnitrosamine (DMN) group of mice compared to controls, w is markedly r ced by the treatment with Boswellia (B) plus Salvia (S) extracts.

extracts with anti-inflammatory activ a *Salvia Miltiorrhiza* extracts with antifibro ef- fects significantly improved the course d macroscopic findings of DMN-induced chr hepatitis in mice, as well as the of hepa fibrosis and collagen accumulation, compared to N mice treated with Boswellia alone. ow , co-tretment of Boswellia plus Salvia extracts did not significantly increase the antifibrotic effect of salvia extracts.

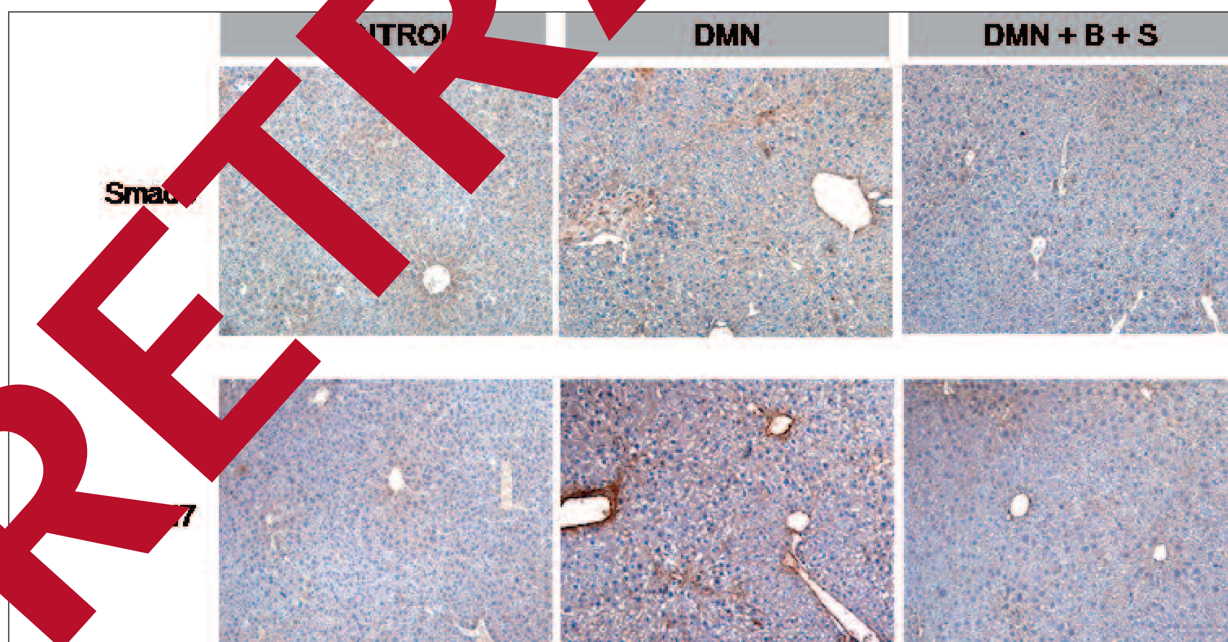

Figure 7. Immunohistochemistry O.M. 4X. Immunostaining both of Smad3 and Smad7 is less prominent in the dimethylnitrosamine (DMN) mice treated with Boswellia (B) plus Salvia (S) extracts compared to DMN mice without any treatment.

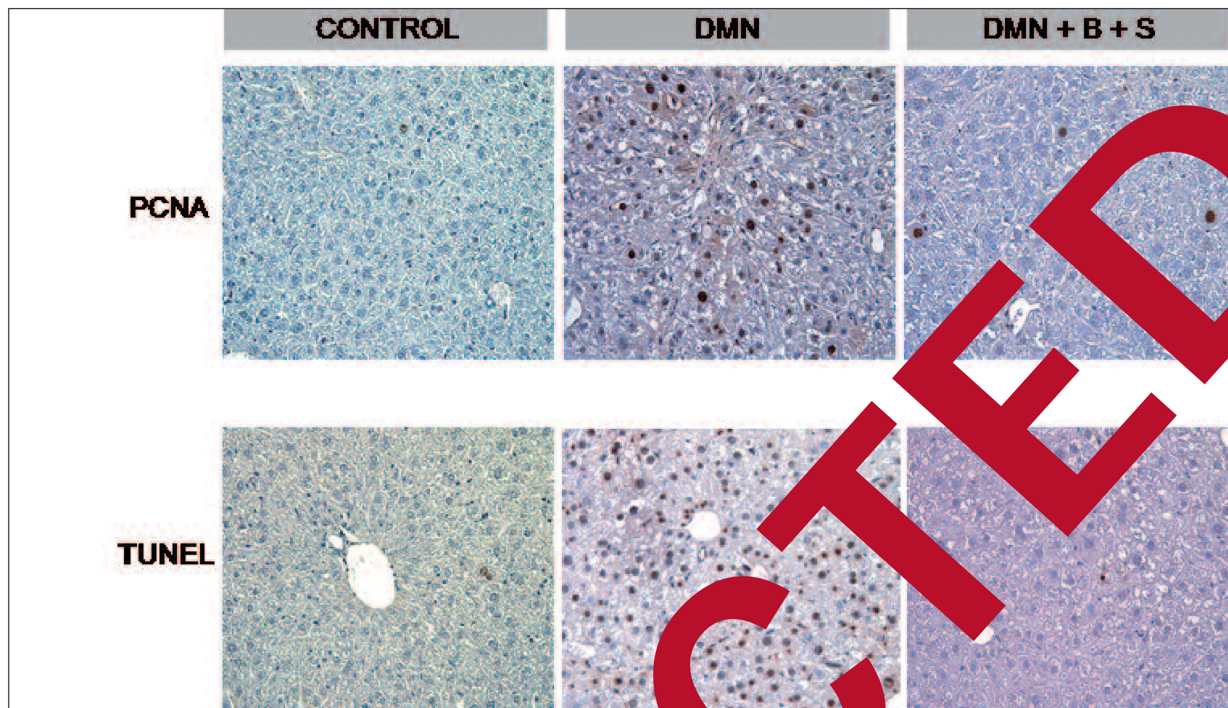

Figure 8. Immunohistochemistry O.M. 4X. Cell proliferation and a[illegible]is were ev[illegible]ated by PCNA and TUNEL staining, respectively. Both PCNA and TUNEL staining are ve[illegible] high in the liv[illegible]ving dimethylnitrosamine (DMN), but they are reverted to control levels by the treatment w[illegible]llia (B) plus Sa[illegible] extracts.

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The main mechanisms of action of the ex[illegible]s of these two botanical plants remain to be defi[illegible] The gum resin extracts from *B[illegible]llia serra[illegible]* have been reported to have a[illegible]tory an[illegible] immunomodulatory effects[illegible]. Bosw[illegible]ic acids, ursane type of pentacycl[illegible]rpenes [illegible]ve been identified as the main [illegible]iolog[illegible]- nents of Boswellia e[illegible]acts[11]. B[illegible]ic acids suppress the synthes[illegible] the leukotr[illegible]y inhibition of 5-lipox[illegible] and decreas[illegible] a dose-dependent ma[illegible]ner, th[illegible]ruitment of adherent leukocyte[illegible]d platelets in[illegible]flamed tissue, primarily [illegible] preventing the upre[illegible]tion of adhesion mole[illegible]es[32,42]. In addition, Boswellic acids inhibit leu[illegible] ela[illegible]se[43] and lipopolysaccharide-induced [illegible]xide pro[illegible]tion[44] and suppress the [illegible]clear [illegible]κB ([illegible]κB) and NFκB-regulated [illegible] expres[illegible]oswellic acids also possess an[illegible]omplem[illegible] activity[46]. In clinical trials, pr[illegible]sing results have been observed in patients [illegible]toid arthritis, ulcerative colitis, [illegible]rohn's disease, and bronchial asthma[9-11,32,47-49].

[illegible]lvia Miltiorrhiza extracts have been widely us[illegible] the treatment of cardiovascular and cerebrovascular diseases as well as diabetic nephropathy, arthritis and hepatitis[12-16,19-23,50-52]. Both the aqueous and lipid soluble fractions of Salvia con[illegible]e components with important pharmacological effects being observed in basic experiments and in clinical studies[12-16,53]. More than 30 diterpene compounds have been separated and identified from Salvia, the most active and potent of which appear to be salvianolic acid and tanshinoses. Tanshinone IIA, a lipophilic compound, is the most abundant and structurally representative of the tanshinones of *Salvia Miltiorrhiza*. Tanshinone IIA has anti-oxidant properties and protects against lipid peroxidation *in vitro* and *in vivo* thus making it a potential antidote for free radical-based disorders[54-57]. In addition, it appears to possess immunoregulatory effects by inhibiting the production of pro-inflammatory cytokines (IL-1β, IL-6, IL-12, TNFα, INFγ)[58-60]. Beside these anti-inflammatory activities, tanshinone IIA has displayed a potent antifibrotic effects in several organs including the liver[15,16,19,20,53,60]. Nevertheless, to date, little is known about the cellular and molecular mechanisms of tanshinone IIA-mediated anti-fibrotic effects in chronic hepatitis. Albeit, several experimental studies have confirmed the effectiveness of salvia extracts in the prevention and treatment of hepatic fibrosis, but, in general, the effects of specific compounds were not evaluated or assessed only *in vitro* studies[13,14,19-23,61-65].

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Hepatic fibrosis is a common characteristic of chronic liver diseases, which is related to the abnormal accumulation of fibrillar ECM proteins. Fibrosis is a chronic and progressive process acting through complex cell/matrix/cytokine and growth factor interactions, but may be a reversible event[2-5]. As a result of chronic tissue damage, ECM-producing cells undergo a process of activation characterized by proliferation, motility, contractility, and synthesis of ECM proteins. ECM-producing cells, namely activated hepatic myofibroblasts, may derive from different cell types, such as fibroblasts and myofibroblasts of the portal tracts, biliary epithelial cells and hepatocytes, bone marrow stem cells, and mainly from HSCs[1-3,66,67]. During an inflammatory process HSCs become phenotypically like a myofibroblast acquiring proliferation, mobility and contractility properties. Once the trans-differentiation process starts, transformed myofibroblasts are capable of autocrine stimulation due not only to a variety of secreted mediators but also to the expression of surface receptors. Myofibroblast activation may, therefore, become progressive and be independent of inflammation suggesting that inflammation is a necessary event to start the fibrogenic response even if it plays a minor role in the progression of the disease[68,69]. The fact that fibrosis, in the various organs, does not respond to anti-inflammatory drugs, would appear to confirm the hypothesis that inflammation is not a key process in the pathogenesis and progression of the disease. In the present study, the administration of anti-inflammatory Boswellia [illegible] did not prevent the development of hepatic fibrosis.

Activation of ECM-producing cells is regulated by several [illegible] factors, including cytokines, chemokines, growth factors, and products of oxidative stress [illegible] these molecules, TGF-β appears to play a pivotal role[7,67]. TGF-β is a member of a large family of pleiotrophic cytokines that includes bone morphogenetic proteins ([illegible]), activins and other related factors. Three different isoforms of TGF-β (β1, β2, β3) [illegible] been [illegible] mammals; TGF-β1 is the most extensively studied and would appear to be the most fibrogenetic. The TGF-β intracellular [illegible]ction pathway is mediated mainly by Smad proteins[70,71].

Smads are a family of 8 related proteins which function as signalling intermediates for the TGF-β superfamily members[70,71]. Upon ligand binding and activation of TGF-β receptors (I, II, and III), phosphorylated Smad2 and 3 bind with the common mediator Smad4. The Smad 2/3- Smad4 complex translocates into the nucleus where it regulates specific TGF-β target genes. The inhibitory Smad7 antagonizes TGF-β signalling by competing with ligation of Smad2/3 to the activated receptor complex Smad4. Specifically, the target genes known to contain Smad-responsive regions and that are directly or indirectly involved in fibrogenesis, include several fibrillar ECM proteins (collagen, fibronectin), matrix degrading enzymes (MMPs) and some specific inhibitors (plasminogen activator inhibitor type 1, TIMP-1), as well as genes regulating epithelial-mesenchymal cell transition, proliferation (cyclin-dependent kinase inhibitor [illegible]) and apoptosis (caspase [illegible]).

Besides the immune regulatory function, TGF-β/Smads signalling plays a central role in hepatic fibrosis by up-regulating ECM expression and suppressing matrix degradation by inhibiting MMPs[1-3,7]. Furthermore, TGF-β stimulates the synthesis of CTGF, a potent enhancer of myofibroblast proliferation, ECM production and TGF-β self-induction[7,69,73-75]. Disruption of the TGF-β/Smad signalling pathway, either through the loss of Smad3 or the increase of Smad7 expression, confers resistance to tissue fibrosis in several organs including skin, kidney, liver, lung and the cardiovascular system[29,76-85].

Hepatic fibrosis is considered a chronic progressive, dynamic and potentially reversibile process[1,2,5]. An important goal of research would be to identify anti-fibrotic agents capable to inhibit either the accumulation of activated EMC-producing cells or their pro-fibrogenic action, or capable to stimulate the degradation of EMC[1-3].

In this study, the prophylactic combined oral administration of *Boswellia serrata* and *Salvia Miltiorrhiza* extracts prevented the development and progression of hepatic fibrosis in DMN-induced chronic hepatitis in mice, which appears to be related to the inhibition in the hepatic expression of α-SMA, collagen I-III, CTGF, TGF-β1, and Smad3. These findings were also observed when the DMN mice were treated with Salvia alone, confirming data of previous studies[13,14,19-23]. The rescue effects of either Boswellia or Salvia on established hepatic fibrosis was not evaluated. Nevertheless, the therapeutic anti-fibrotic effect, on established chronic hepatitis, has been reported elsewere by other Authors[13,14,19,22]. The inhibition of the TGF-β1/Smad3 pathway appears to be linked to the action of Salvia extracts and could be responsible for the reduced activation of the my-

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ofibroblasts, as indicated by the concomitant reduction in the expression of α-SMA, a marker of the activated myofibroblast phenotype. We did not assess, at which level (transcriptional or post-traslational), the TGF-β1/Smad3 pathway was affected by the treatment. However, inhibition of the TGF-β1/Smad3 pathway reduces the synthesis of CTGF, a potent stimulator of myofibroblast proliferation and ECM proteins production, as well as of TGF-β autoinduction[29,69,80]. TGF-β autoinduction is thought to be an important aspect in the maintenance of the fibrotic process over time[69]. On the other hand, the reduction of Smad7 observed in the DMN mice treated with Boswellia plus Salvia is in contrast with the observation that an increase in Smad7 expression confers resistance to tissue fibrosis[81-86]. The reason for these discrepancies is not clear since the mechanisms regulating Smad7 expression remain to be fully elucidated. Smad7 is strongly and rapidly induced by TGF-β itself. The properties of Salvia extracts in modulating the TGFβ/Smads pathway, as well as the expression of α-SMA and CTGF have been described in several studies[20,60,61,64].

Besides the TGF-β/Smad pathway, myofi[illegible]lasts also appear to play a role in liver [illegible]osis through the action of other fibrogenetic mo[illegible]es, such as interleukin-1β, interleukin-6, interle[illegible]-13, insulin-like growth factor-1(IGF-1), TN[illegible] CTGF, platelet-derived growth [illegible] basic [illegible]broblast growth factor, angio[illegible]rting enzyme, angiotensin II, vasc[illegible] endoth[illegible] growth factor, endothelin-1, and [illegible]isome[illegible]oliferate-activated receptor-γ, w[illegible]ch s[illegible]ly with Smad3 or [illegible]r activit[illegible] Smad3-dependent[2-4,7,29,69,71,7...] [illegible]everal obse[illegible]s suggest that tanshinone [illegible] A [illegible]tenuate tiss[illegible]rosis by reducing angio[illegible]ensin I[illegible] 1β-, IL-6-, and TNF-α-induce[illegible]llagen expre[illegible][16,23,53,59,60]. Furthermore, t[illegible]inone IIA possess[illegible]tioxidant action by i[illegible]iting the intracellular generation of ROS wh[illegible]ay a[illegible]portant role in hepatic fibrosis. These [illegible]es and R[illegible]S were not evaluated in [illegible] prese[illegible]ly. *Sa[illegible] miltiorrhiza* extracts may [illegible]orate h[illegible]s oxidative damage and atten[illegible] HSCs a[illegible]tion, potentially conferring hepa[illegible]otective and anti-fibrotic effects.

Conclusions

Combined treatment with *Boswellia serrata* and *Salvia miltiorrhiza* extracts prevents the development of hepatic fibrosis in an experimetal model of DMN-induced chronic hepatitis in mice. The anti-fibrotic properties are mainly related to Salvia extracts and appear to be related, in part, to their effects on the modulation of the TGFβ/Sm[illegible]nalling pathway in the activated myo[illegible]. Long-term studies in chronic hepati[illegible] associated fibrosis, both in animal models a[illegible] human, are needed to establish the safety and [illegible]al potentials of this anti-fibrotic treat[illegible]nt.

Acknowledgements

This work was s[illegible]porte[illegible]t from the [illegible]iversity of L'Aquil[illegible]Aquila, I[illegible]thors a[illegible]ateful to Mrs Marian[illegible]elds for help i[illegible] t[illegible]anuscript.

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