

RESEARCH ARTICLE

Synergistic Effects of Jerusalem Artichoke in Combination with Pegylated Interferon Alfa-2a and Ribavirin Against Hepatic Fibrosis in Rats

Nabil Mohie Abdel-Hamid¹, Ahmed Wahid^{2*}, Maiiada Hassan Nazmy², Marwa Abdel-Moniem Eisa²

Abstract

Background: Complementary and alternative medicine has been highly appreciated as a supportive regimen for classical treatment strategies. Here we offer a nutrition-based adjuvant therapy for liver fibrosis, a major risk factor for cirrhosis and hepatocellular carcinoma. **Aim of the study:** To evaluate the possible hepatoprotective effects of Jerusalem artichoke tubers (JAT) in combination with interferon and ribavirin. **Materials and Methods:** Twelve groups of rats were administered JAT, interferon and ribavirin either separately or in combination from day one of CCL₄ administration until the end of the study. Animals were killed after 8 weeks of CCL₄-induced hepatotoxicity. **Results:** Hepatocytes from rats treated with triple combination of interferon, ribavirin, and JAT showed more less normal architecture compared to CCL₄-treated rats. We also detected significantly higher hepatic protein expression levels of p53, BAX and transforming growth factor-β (TGF-β) in the CCl₄-intoxicated group compared to normal controls, as evidenced by immunohistochemical staining and western blotting analyses. Addition of JAT as a supportive regimen improved response to ribavirin and interferon and effectively participated in retaining normal histopathological and biochemical criteria and significantly lowered protein expression of p53, BAX, and TGF-β. **Conclusions:** We suggest that addition of JAT as a supportive regimen to interferon and ribavirin effectively potentiates their anti-fibrotic effects.

Keywords: Jerusalem artichoke tubers - rat liver model - fibrosis - p53 - BAX - TGF-β

Asian Pac J Cancer Prev, 17 (4), 1979-1985

Introduction

Repeated liver damage over a relatively long period can cause chronic liver injury which usually results in a healing response that finally replaces the normal liver architecture in the form of fibrosis or cirrhosis (Minano and Garcia-Tsao, 2010). Amongst the factors that can lead to liver fibrosis are toxins and drugs (Aithal et al., 2011; Gao and Bataller, 2011), metabolic disorders (Nobili et al., 2011), autoimmune-related diseases (Mayo, 2011), and viral infections (Thursz et al., 2011).

Interferons are endogenous proteins produced by a variety of cells following to viral infection. It has been long used for patients with hepatitis C virus infection together with ribavirin (Fujii et al., 2015; Veillon et al., 2015).

Complementary and alternative medicine has been highly appreciated as a supportive regimen for classical treatment strategies. Identification of a hepatoprotective agents derived from natural sources is an urgent necessity. Indeed, herbs have been used as a remedy for various diseases (Abdel-Hamid et al., 2011). Recently, it has been proposed that some medicinal plant extracts have potential

antifibrotic effects (Schuppan et al., 1999; Gebhardt, 2002) such as silymarin (Boigk et al., 1997) and Inchin-ko-to (TJ-135) (Sakaida et al., 2003).

Here we offer a nutrition-based adjuvant therapy for liver fibrosis, a major risk factor for cirrhosis and hepatocellular carcinoma. Jerusalem Artichoke Tubers (JAT) (*Helianthus tuberosus L.*) is a species of sunflower, native plant to the North American plains cultivated for different purposes in many countries. The plant was brought to Europe by the French explorer Samuel de Champlain. It has other names like, sunroot, sunchoke, or earth apple. It can be eaten both raw and cooked. It was reported to have potential anti-diabetic and anti-oxidant effects but little is known about possible mechanisms by which it exerts these effects (Zawistowski et al., 1986; Chang et al., 2014; Punzi et al., 2014). The root contains dietary fibers, antioxidants, in addition to small proportions of minerals, and vitamins (Roberfroid and Delzenne, 1998; Roberfroid et al., 1998). It is one of the finest sources for dietary fibers which are reported to decrease serum cholesterol and low density lipoproteins. JAT is rich in oligo-fructose inulin, which is a soluble,

¹Biochemistry Department, Faculty of Pharmacy, Kafsr-El-Sheikh University, ²Biochemistry Department, Faculty of Pharmacy, Minia University, Minya, Egypt *For correspondence: ahmed_wahid@mu.edu.eg

zero calorie, non-starch polysaccharide and since it does not metabolize inside the human body, JAT is considered an ideal sweetener for diabetics. Moreover, it was also reported that JAT contains minor amounts of anti-oxidant vitamins such as vitamin C, and other flavonoid containing compounds which together help scavenge harmful free radicals, and therefore protect against liver inflammation (Kucukgergin et al., 2010; Serni et al., 2013).

We noticed that most studies focused on role of JAT against diabetes mellitus or its hepatic manifestation, non-alcoholic fatty liver diseases. No previous study tested its hepatoprotective effect in a CCL_4 fibrosis model. Our preliminary study (Abdel-Hamid et al., 2015) showed that JAT ameliorated CCL_4 -induced hepatotoxicity in rats. Here, we suggest that addition of JAT to the standard interferon-ribavirin anti-fibrotic regimen may effectively improve their curative potential.

Materials and Methods

Chemicals and kits

Diagnostic kits for serum alanine aminotransferase (ALT) and aspartate amino transferase (AST) were purchased from Biodiagnostic CO., Egypt. Diagnostic kit for bilirubin was purchased from Randox Laboratories Co., UK. Carbon tetrachloride was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Interferon and ribavirin were purchased from Virazole, October Pharma, Cairo. Bax, P53 and TGF- β primary antibodies and secondary antibodies for immunohistochemical studies and western blot analysis were purchased from Santa Cruz Biotechnology, CA.

Plant materials & extract preparation

Jerusalem artichoke tubers were kindly gifted from Dr/ Abdel Badea Saleh Ezzat and Dr/ Amal Abou-El Fotouh E-Awady, Assistant Professors of Agriculture, Vegetable Research Department, Horticulture Research Institute, Agricultural Research Centre, Cairo, Egypt. The tubers were cultivated, identified and preserved under their supervision. A voucher specimen was deposited in Agricultural Research Centre Herbarium, Cairo, Egypt.. JAT was washed and sliced individually to a thickness of approximately 1mm. Artichoke slices were then soaked in lemon juice combined with citric acid and ascorbic acid in order to inhibit polyphenol oxidase activity. Thereafter were subjected to dryness process by solar energy, blended, packed and frozen at -80 °C (Zawistowski et al., 1986).

Animals and experimental design

Male albino rats (150-200g) were purchased from the animal house colony, National Research Centre, Giza. The animals were housed under standardized environmental conditions, fed with standard diet and libitum during the entire period of experimentation, and left to acclimatize to the environment for one week prior to inclusion in the experiment at $22^{\circ}\text{C} \pm 2^{\circ}\text{C}$ under a 12/12 hrs light/dark cycle. All animal experiments were submitted to and approved by Faculty of Pharmacy, Minia university research ethics committee and were conducted

in accordance with the guide for the care and use of laboratory animals of the National Institute of Health (NIH publication No. 85-23, revised 1985).

Animals were divided randomly into 12 groups (20 rats each) as follows: **Group I:** untreated normal healthy group received only olive oil for the whole period of experiment (8 weeks). **Group II:** rats received only JAT orally (1g/kg body weight daily)(EA, 2009). **Group III:** rats received only peginterferon-alpha intraperitoneally (1.5 $\mu\text{g}/\text{kg}$ per week)(Tasci et al., 2007). **Group IV:** rats received only ribavirin orally (60 mg/kg daily)(Solbrig et al., 2002). **Group V:** rats received peginterferon-alpha and ribavirin. **Group VI:** rats received triple treatment of JAT, peginterferon-alpha and ribavirin. **Group VII:** hepatic fibrosis was induced by treating rats by gavage with CCl_4 -olive oil (1:1, 2.8 ml/kg followed by 1.4 ml/kg after one week)(Abdel Salam et al., 2007). **Group VIII:** rats received JAT starting from day one of CCl_4 treatment. Group IX: Rats received peginterferon-alpha starting from day one of CCl_4 treatment. **Group X:** Rats received ribavirin starting from day one of CCl_4 treatment. **Group XI:** Rats received peginterferon-alpha and ribavirin starting from day one of CCl_4 treatment. **Group XII:** Rats received triple treatment of JAT, ribavirin and peginterferon-alpha starting from day one of CCl_4 treatment. All animals were sacrificed after 8 weeks of CCl_4 -treatment.

Sample preparations

Blood samples were withdrawn along the experiment after an overnight fast from retro-orbital venus plexus each week. At the end of the experiment, the blood was withdrawn from the carotid vein by cutting with fine scissors. The blood was collected by dripping into non-EDTA containing test tube. Serum was separated by centrifugation for 15 minutes at 3000 xg. Samples were then stored in aliquots in eppendorf tubes and frozen at -80 °C.

Biochemical analysis

Serum ALT and AST activities were measured by the dinitrophenylhydrazene method according to Reitman and Frankel (Reitman and Frankel, 1957). Colorimetric determination of serum bilirubin was done as described previously (Watson and Rogers, 1961).

Histopathological investigation

Liver slices were fixed in 10% formaldehyde and embedded in paraffin wax blocks. Sections of 5 μm thick were stained with hematoxylin and eosin (H&E), and Masson trichrom stain then examined under light microscope for determination of pathological changes. The histological evaluation of the hepatic injury was semi-quantitatively scored by two independent observers who undertook the evaluation using an ocular micrometer by light microscopy. The severity of the hepatic injury was scored viz. (-) no injury; (+) mild; (++) moderate, and (+++) severe. The examination of the hepatic injury consisted of the evaluation of the following: vena centralis, portal area, and sinusoidal congestion, ballooning degeneration of hepatocytes and

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its location, the presence of focal parenchymal necrosis, Polymorphonuclear leukocytes (PMNL) infiltration and mononuclear leukocytes (MNL) infiltration, karyolysis of hepatocyte nuclei, the presence of pyknosis, loss of intercellular borders and disintegration of hepatic cords. Assessment of hepatic fibrosis was performed using semiquantitative fibrosis scores. Healthy liver was classified as 0. Fibrous expansion of the portal areas was scored as 1. Stage 2 denotes septal fibrosis with marked fibrous septa, and stage 3 was characterized by portal-portal septa (bridging fibrosis but intact architecture). The advanced fibrotic stage 4 (i.e., cirrhosis) is characterized by bridging fibrosis with nodules (Roderfeld et al., 2006a; Roderfeld et al., 2006b).

Immunohistochemical analysis of Bax, P53 & TGF-β

Liver tissue sections were subjected to dewaxing, hydration and thermal induction antigen retrieval. Slices were blocked and incubated with anti-Bax antibody (1:200), anti-p53 antibody (1:100), and anti-TGF-β antibody (1:200) which were diluted in phosphate buffered

saline solution and incubated at 4°C overnight. The following day, tissue slices were washed and incubated with secondary antibodies. The slices were then incubated with diaminobenzidine tetrachloride for 5-10 minutes to develop colour, and staining was observed under microscope (CR, 1994).

Western Blot analysis of Bax, P53 and TGF-β

Proteins were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes (Millipore). The membranes were blocked with 5% BSA for 2 h, and then incubated overnight at 4°C with anti-Bax antibody (1:200), anti-p53 antibody (1:100), and anti-TGF-β antibody (1:200). The membranes were then incubated with HRP-conjugated goat anti-rabbit IgG and goat anti-mouse IgG (1:5000) at room temperature for 2 h, and then washed again and detected by the enhanced chemiluminescence reaction (Towbin et al., 1979). The intensities of the bands were then analyzed with Image J software.

Table 1. Liver Function Tests for Different Experimental Groups in the Study

	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	P value
ALT ±SD (IU/L)	30.8± 2.89	32.7± 0.82	30.2± 2.56	32.0± 0.89	32.3± 1.08	30.2± 0.98	141± 1.47	91.7± 5.49	83.7± 1.63	56.2± 1.57	41.9± 2.06	36.2± 1.47	<0.01
AST ±SD (IU/L)	22.5± 2.26	22.6± 1.11	22.6± 1.11	22.7± 1.99	22.8± 1.72	24.7± 1.47	92.5± 1.52	75.2± 1.52	45.8± 1.33	64.8± 1.6	46.0± 1.67	31.7± 1.47	<0.01
Bilirubin ±SD (mg/dl)	0.44± 0.09	0.48± 0.05	0.43± 0.04	0.5± 0.04	0.41± 0.05	0.40± 0.04	1.9± 0.48	1.05± 0.06	1.1± 0.06	1.2± 0.11	1.08± 0.09	0.69± 0.09	<0.01

Data are expressed as X± SD of 20 rats in each group (n=20). Significant difference between groups was analyzed by t-Student test, where P < 0.05 is considered significant.

Table 2. Histopathological Evaluation for Different Experimental Groups in the Study

Group	Congestion			PMNL			MNL		
	Central vein	Portal space	Sinusoid	Central vein	Portal space	Sinu-soid	Central vein	Portal space	Sinusoid
Control	-	-	-	-	-	-	-	-	-
CCl4	+++	+++	+++	-	+	-	+	+++	++
Artichoke	++	+++	++	-	-	-	-	++	+
ribavirin	++	+++	++	-	-	-	+	+++	++
Interferon	+	+	+	-	-	-	+	++	-
Interferon and ribavirin	+	+	+	-	-	-	+	++	-
Interferon, ribavirin and artichoke	-	+	-	-	-	-	-	+	-
	Ballooning degeneration and localisation		Necrosis	Karyolysis and pyknosis		Loss of intercellular borders		Disintegration of hepatic cords	
Control	-	-	-	-	-	-	-	-	-
CCl4	+++CL	++MZ	+++	++	++	+++	+++	+++	+++
Artichoke	+CL	-MZ	+	++	++	+	+	++	++
ribavirin	++CL	++MZ	++	++	++	++	++	++	++
Interferon	+CL	-MZ	+	++	++	+	+	+	+
Interferon and ribavirin	+CL	-MZ	-	+	+	+	+	+	+
Interferon, ribavirin and artichoke	-CL	-MZ	-	+	+	+	+	-	-

The severity of the hepatic injury was scored viz. (-), no injury; (+), mild; (++) moderate, and (+++) severe. The examination of the hepatic injury consisted of the evaluation of the following: vena centralis, portal area, and sinusoidal congestion, ballooning degeneration of hepatocytes and its location, the presence of focal parenchymal necrosis, Polymorphonuclear leukocytes (PMNL) infiltration and mononuclear leukocytes (MNL)

Statistical analysis

Data were estimated using analysis of variance and all values were expressed as mean \pm SD. P-value < 0.05 was considered significant. All analyses were implemented by SPSS 20 software for windows (Chicago IL, United States).

Results

Liver toxicity studies for JAT, interferon and ribavirin

Six groups of rats were administered the same dose of JAT, interferon, and ribavirin without intoxication with CCl₄. Liver biomarkers were then measured (Table 1). Our data showed that the used doses in our experiment were safe and comparable to control healthy group.

Effect of JAT and other combinations on serum biomarkers of hepatic injury induced by CCl₄

ALT,AST activities and bilirubin levels were markedly increased in CCl₄-intoxicated group compared to the normal healthy group, which were significantly lowered by administration of JAT in combination with interferon and ribavirin ($p < 0.01$).

JAT in combination with interferon and ribavirin attenuates hepatic fibrosis

Following 8 weeks of CCl₄ administration, liver histopathology was significantly changed in the CCl₄ group. The livers showed moderate to marked congestion

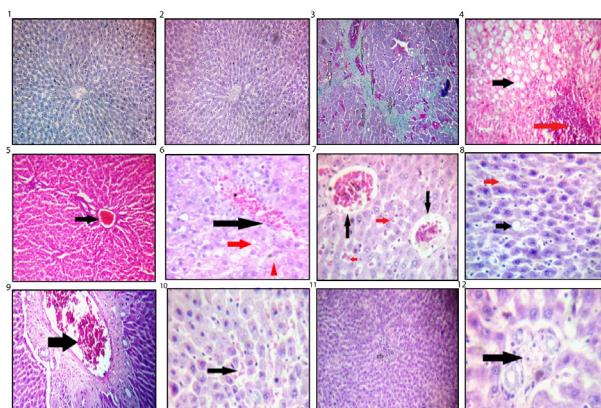


Figure 1. Histopathological Analysis for the Effect of Different Treatment Combinations on Hepatic Damage using H&E and Masson Trichrome Stains. A. Normal untreated control 1.Normalarchitecture. 2. No fibrosis, no fatty change B. CCl4 group: 3. Black arrow showing marked fibrosis.4. Marked fatty change (black arrow), marked inflammation (red arrow). H&E stain.5. Showing marked sinusoidal congestion (black arrow). 6. Marked congestion (black arrow), marked desentigration of hepatic cords (red arrow). Hepatocyte necrosis (red arrow head; H&E stain).C. CCl4 + Interferon group: 7. Mild central venous congestion (black arrow). Mild sinusoidal congestion (red arrow). H&E stain. 8. Mild fatty change (black arrow). Mild sinusoidal congestion (red arrow). H&E stain.D. CCl4 and Ribavirin group:9. Marked congestion in portal tract (black arrow). H&E stain. E. Interferon and ribavirin group. 10. Mild sinusoidal congestion (black arrow). H&E stain. F triple 11. Mild portal tract inflammation (black arrow), preserved hepatic cords. H&E stain. 12. Mild inflammatory cellular infiltrate of portal tract. H&E stain

in the central vein and severe congestion in the portal area and sinusoids. Parenchymal necrosis was observed.

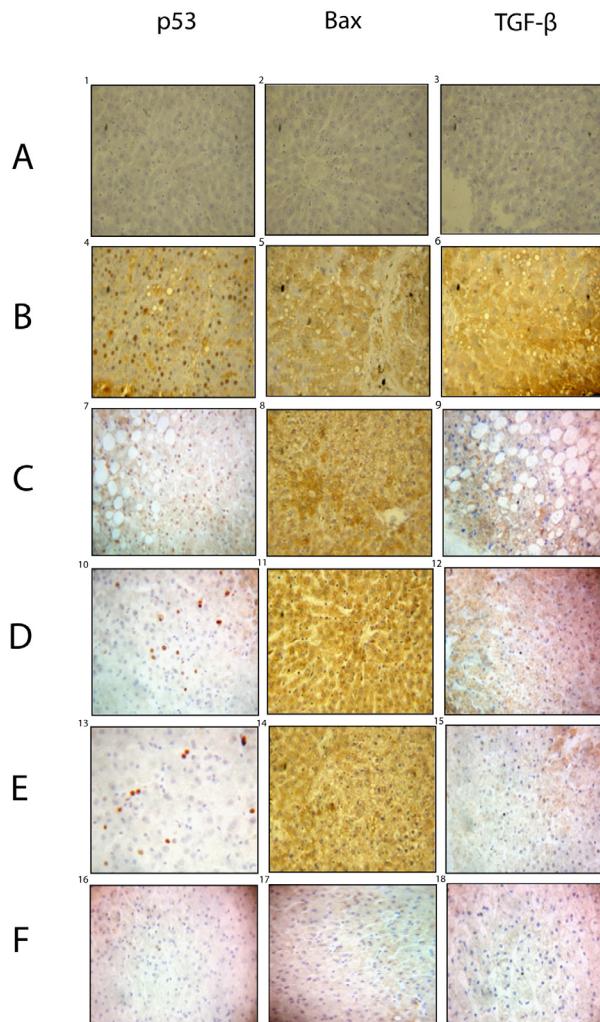


Figure 2. Immunohistochemical Analysis of TGF-β, Bax, P53 in liver tissue of different experimental Groups. A. Normal untreated control B. CCl4 group C.CCl4 intoxicated group treated with Interferon D.CCl4 intoxicated group treated with Ribavirin E. CCl4 intoxicated group treated

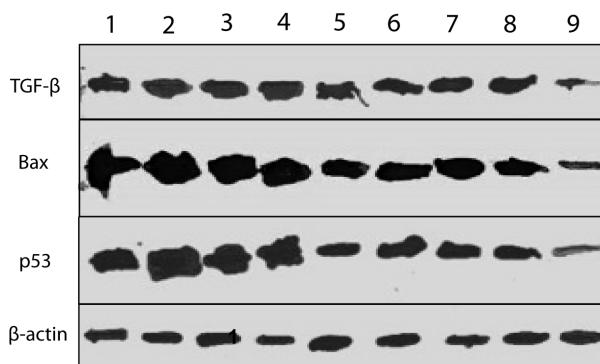


Figure 3. Western Blotting forP53, Bax, andTGF-β Proteins in Liver Homogenate. 1. Normal untreated control 2,3, and 4. CCl4 group 5. CCl4 intoxicated group treated with Interferon 6. CCl4 intoxicated group treated with Artichoke7. CCl4 intoxicated group treated with Ribavirin 8. CCl4 intoxicated group treated with Interferon and Ribavirin. 9. CCl4 intoxicated group treated with Interferon, Ribavirin and JAT. β -actin acted as loading control to ensure even loading

Polymorphonuclear leukocytes (PMNL) infiltration was mild whereas mononuclear leukocytes (MNL) infiltration was mild in the central vein, severe in the portal area and moderate in the sinusoids. Moderate karyolysis and pyknosis, and severe loss of intracellular borders and disintegration of hepatic cords were observed. In the groups treated by single agent; JAT, interferon or ribavirin alone with CCl_4 , livers appeared to exhibit some liver protection against CCl_4 as evident by the presence of normal hepatic cords, absence of necrosis and lesser fatty infiltration. However, hepatocytes from rats treated with triple combination of interferon, ribavirin, and JAT showed more or less normal appearance.

Effect of JAT on hepatic protein expression of P53, TGF- β and Bax

We further examined effect of various agents on TGF- β and Bax protein expression , as shown in Figure 2 and 3, the expression levels of the pro-apoptotic protein, Bax, and TGF- β were increased in CCl_4 group. In contrast, groups treated with JAT, Peg-interferon and ribavirin separately or in combination showed reduction in the expression of TGF- β and Bax. The lowest level of expression was observed in the triplet combination. Hepatic protein expression of p53 was also significantly higher in CCl_4 -intoxicated group compared to other groups as detected by immunohistochemical staining. P53 immunopositive cells reached up to 70% of the stained area compared to 5% observed in the negative control group. Moreover, CCl_4 treatment caused cytoplasmic translocation of p53 protein. In the group treated by triple combination of interferon, ribavirin, and JAT, the expression level of p53 was markedly reduced. This is consistent with our western blotting analysis which showed that p53 protein expression was enhanced in CCl_4 -treated group and significantly reduced with the triplet-treatment.

Discussion

Most chronic liver disorders are associated with fibrosis which is a process of excessive accumulation of extracellular matrix proteins in the liver (Bataller and Brenner, 2005). This process may lead to damage of the original hepatic architecture. Severe form of liver fibrosis may eventually progress to hepatocellular carcinoma (Castilla et al., 1991; Baldo et al., 2008; Schuppan and Afdhal, 2008).

Several studies have demonstrated the anti-fibrogenic effect of interferon (Muriel, 1996) via inhibiting TGF- β expression (Castilla et al., 1991; Tsushima et al., 1999; Chang et al., 2005), which decreases hepatic stellate cell activation and stimulates apoptosis (Chang et al., 2005). In the current study we demonstrate that the co-administration of JAT along with interferon and ribavirin can revert liver fibrosis more effectively.

CCl_4 intoxication is the best known experimental model for liver injury so far. CCl_4 administration can induce chronic liver damage in rats. It is metabolized by cytochrome P450 system to highly reactive trichloromethyl free radicals, which initiate liver cell destruction (McCay et al., 1984; Brattin et al., 1985). As previously reported,

administration of CCl_4 for 8 weeks resulted in a significant increase in serum ALT, AST activities as well as total bilirubin levels (Mehmetcik et al., 2008; Arici and Cetin, 2011). Damaged liver architecture can lead to an increase in the serum activities of liver enzymes due to their localization in the cytoplasmic area of the cell (Althnaian et al., 2013). Theses biomarkers showed major improvement to near normal values with triplet combination, which is an indication of the stabilization of the plasma membrane and the repair of hepatic tissue damage caused by CCl_4 intoxication. A previous study showed a significant decrease in serum ALT activities of diabetic rats fed on JAT (Aslan et al., 2010).

In the present study, Liver sections of animals intoxicated with CCl_4 showed centrilobular congestion and ballooning degeneration, vacuolization and necrosis with loss of hepatocytes, leading to disintegration of hepatic cords with fibrosis as well as massive necrosis, accompanied with microvesicular steatosis and mononuclear cellular infiltration. Moreover, Masson's trichrome stain of liver sections showed congestion and thickening of the portal tract with dense fibrous tissue. Similar observations of the liver tissues in CCl_4 intoxicated rats were reported previously (Domitrovic et al., 2009; Althnaian et al., 2013).

P53 gene is a major well studied tumor suppressor gene. It has been reported before to be inactivated in most cancers (Helm et al., 1999; Vogelstein et al., 2000). P53 protein was reported to inhibit cellular division in stressed cells. Alternatively, p53 causes these stressed cells to undergo apoptosis (Helm et al., 1999). It is still unclear what other roles are played by p53. Importantly, it was shown before that p53 is involved in the molecular mechanisms leading to liver injuries (Blight et al., 2003; Schafer et al., 2003). Indeed, patients with various inflammatory liver diseases were found to accumulate p53 protein (Akyol et al., 1999; Panasiuk et al., 2006; Attallah et al., 2009). It has been also reported that p53 serum level was elevated in HBV-related cirrhosis patients (Shahnazari et al., 2014). In line with these reports, we could show that the level of p53 was significantly higher in the livers of rats intoxicated with CCl_4 compared to normal control as detected by immunohistochemical staining. The triple combination was able to successfully diminish the elevated expression level of p53 protein. One possible explanation for the elevated p53 level may be the reduction of p53 protein breakdown, leading to the accumulation of p53 protein. Another possibility is that p53 gene is modulated enhancing p53 synthesis (Papakyriakou et al., 2002; Panasiuk et al., 2006).

P53 protein can cause apoptosis through different p53-regulated pathways such as p21, and Bax for example (Vousden and Lu, 2002). BAX is a pro-apoptotic protein. Its expression level was previously shown to elevate following to an apoptotic stimulus (Green, 2000). The increment in the BAX expression level leads to caspase-3 activation, with consequent cell death (Hengartner, 2000; Panasiuk et al., 2006; Ramalho et al., 2006). We were able to show that the expression level of the pro-apoptotic protein, Bax, was increased in CCl_4 group. This was in accordance with previous reports (Tsamandas et

al., 2003; Guo et al., 2013) which reported that Bax was over regulated in advanced fibrotic stages. Our results demonstrated that the triple combination was more effective in suppression of p53 and Bax expression levels compared to any single treatment. This finding suggests that the triple combination can attenuate CCl_4 -induced hepatocyte apoptosis via a p53-mediated pathway and can retard the progression of liver fibrosis induced by CCl_4 in rats.

Our results also showed similar hepatic expression patterns for TGF- β . Liver fibrosis usually leads to the development of irreversible cirrhosis if untreated (Riley and Bhatti, 2001). It was reported before that hepatocellular damage stimulates inflammatory cells which eventually lead to activation of hepatic stellate cells. Activated hepatic stellate cells produce large amounts of extracellular matrix proteins and thereby enhance fibrosis. This happens due to secreting a broad spectrum of cytokines such as TGF- $\beta 1$ which exerts pro-fibrotic actions (Xu et al., 2004). Indeed, one of the key mediators in liver fibrogenesis is the growth factor, TGF- β . It has been reported before that the inhibition of TGF- β synthesis significantly suppresses liver fibrosis development (Lotersztajn et al., 2005).

In conclusion, addition of JAT to the classical anti-fibrogenic regimen (interferon and/or ribavirin) can effectively improve their curative potential.

Acknowledgements

Authors are grateful to Prof. Salwa Gaber and Dr. Mona Osman Department of Pathology, Faculty of Medicine, Minia University for their assistance in the histopathological study. The authors declare that no conflict of interests exists. Nabil Mohie Abdel-Hamid, proposed the idea of the study and participated in paper revision. Ahmed Wahid participated in selection of study design, animal handling, biochemical analysis, western blotting analysis, paper writing and submission. Maiada Hassan Nazmy participated in selection of study design, animal handling, biochemical analysis, western blotting analysis and paper revision. Marwa Abdel-Moniem Eisa participated in animal handling, biochemical analysis and western blotting analysis.

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