Indigofera irsute ethanol extract, attenuates oxidative stress, and down regulates gene expression of pro inflammatory factors; NF-κB, TGF-β and IL-6 during fibrogenesis in rat.

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Abstract: Liver fibrosis refers to wound healing in response to chronic inflammation. This study aimed to investigate the hepatoprotective effect of *Indigofera irsute* extract against CCl_4- induced fibrogenesis. Fibrosis was induced in rat model and several biomarkers of oxidative stress, inflammation, and fibrogenesis were determined. *I irsute* significantly protected the liver by decreasing the level of serum aspartate amino transferase (AST), alanine amino transferase (ALT) and hydroxyproline level. In addition, *I irsute* attenuated oxidative stress by inhibiting malondialdehyde (MDA) formation and restoring the level of reduced glutathione in liver homogenate. Similarly, activities of superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione transferase (GST) as well as glutathione (GSH) level were increased in rat treated with *I. irsute.* Finally, *I irsute* suppressed inflammation through down regulation of mRNA levels of some proinflammatory and fibrosis markers (NF-κB, IL-6 and TGF-β). Histological analysis closely tallied with all the biomarkers tested; showing inhibition of fibrogenesis by *I hirsute* extract. This result indicates that *I irsute* attenuated oxidative stress, inflammation and inhibited liver fibrogenesis. Therefore, it could be used as a source of therapeutic antifibrotic agent.
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Key words: *Indigofera irsute* extract; oxidative stress; pro inflammatory factors; Fibrogenesis

1. Introduction:

Hepatic fibrosis denotes wound healing in response to chronic hepatitis injury, which is characterized by excessive synthesis and accumulation of extracellular matrix, accompanied by derangement in sinusoidal structure [1]. Oxidative stress and inflammation are closely related and act simultaneously to create a Malicious cycle, that derives fibrogenesis; by activating hepatic stellate cells (HSC) [2]. HSC can undergo trans differentiation from quiescence (a vitamin A storing cells) into myofibroblastic cells which lead to activation of nuclear factor kappa B (NF- κB) and in turn stimulates the production of various proinflammatory mediators such as IL-1, IL-6, TNF-α and TGF-β [2]. NF-κB activation can thus contribute to hepatocyte injury through subsequent inflammation causing massive hepatocyte death, with concomitant accumulation of ECM [3]. On the other hand, when NF-κB activation is inhibited, HSCs will undergo apoptosis and the condition would be attenuated [3].

Currently, there is no standard treatment for liver fibrosis, but a great percentage of interest for treatment is now focused on herbal product [4]. *Indigofera (Hausa /Fulani name: Hakwoi/kaikayi; H)* is a large genus of about 700 species of flowering plants belonging to the family *Fabaceae.* It is commonly known as hairy Indigo, in Africa, it leaves are used to boost immunity in infant and for urinary tract and chest complains [5]. The Whole plant extract is used to treat injury of eye ball and inflammation of eye lids; root decoction is used in Nigeria as poison antidote [6].

In this study, we examined how *Indigofera irsute* whole plant (the leaves, stem and root) ethanol extract, attenuates oxidative stress and down regulate gene expression of proinflammatory factors; NF-κB, TGF-β and IL-6 in carbon tetrachloride induced hepatotoxicity in rats.

2. Materials and methods

2.1 Chemicals and reagents

Chemicals used were, purchased from Sigma Chemical Co. (St. Louis, Mo, USA). All

chemicals and reagents were of analytical grade. Other reagents were purchased from Biodiagnostis (Egypt) and Bio vision company (USA).

2.2 Collection and extraction of plant sample

Plant sample (which include the root, leaves and fruit) was collected in January 2015 from Keffi, Nasarawa State, Nigeria. The plant was authenticated at the Department of plant science and Biotechnology, Nasarawa State University, Keffi and Voucher specimen was deposited at the herbarium (NSUKH 158). The lyophilized extract was obtained and kept at -20^{0}C until used.

2.3 Characterization of Plant extract

2.3.1 Determination of Total Phenolic Content Reagents:

The Folin-Ciocalteu reagent was used to determine the total phenolic contents of the extracts [7]. Folin-Ciocalteu reagent (10 % v / v) Sodium carbonate Na_2CO_3 (7.5 % w/v) Extract (2 mg / ml in 95 % ethanol). To 0.5 ml of extract solution, 2.5 ml of Folin-Ciocalteu reagent and 2.0 ml of Na_2CO_3 solution were added. The mixture was incubated for 20 minutes at 25 °C, then the absorbance was measured at 760 nm. The total phenolic content (mg / g) was calculated in the equation below; as gallic acid equivalent (GAE / g of extract), using the concentration of gallic acid established from the calibration curve (mg /ml).

2.3.2 Determination of Total Flavonoid Content

The total flavonoid content of each extract was determined colorimetrically using the aluminum chloride method [8]. Reagent include: Aluminium chloride ($AlCl_3$:10 %) Sodium hydroxide (NaOH: 5 %) NaOH (1M) Extract (1mg / ml 95 % ethanol). Fifty microliters of the extract were made up to 1 ml with ethanol, mixed with 4 ml of distilled water and then 0.3 ml of $NaNO_2$ solution; 0.3 ml of $AlCl_3$ solution was added after 5 min of incubation. Then, 2 ml of NaOH solution were added, and the final volume of the mixture was brought to 10 ml with double-distilled water. After 15 min, absorbance was measured at 510 nm. The total flavonoid content (mg / g) in the extracts was expressed as quercetin equivalent (QUE / g of extract) using the concentration of quercetin established from the calibration curve (mg /ml).

2.3.3 HPLC Identification of Polyphenolic Compounds.

Reagents include; Extracts (20 mg / ml ethanol) Formic acid (1 v/v %) Acetonitrile (99.93 v/v %) 2-propanol (99.5 v/v %). Chromatographic separation of 20 µl of extract was carried out on 150 mm x 4.6, 5µm Elipse XDB-C18 column at 320nm and at a flow rate of 0.75 ml min. The mobile phase was formic acid: acetonitrile: 2-propanol (70:22:8), pH 2.5. A MerckHitachi (LaChrom, Tokyo, Japan) instrument equipped with an L-7100 pump, an L-7455 UV diode array detector, a D-7000 chromato-integrator and a column compartment was used for analyses. The sample was separated on a Purospher star-C18 column (250 mm 5 mm, 4.6 mm i.d., Merck, Germany). The mobile phase consisted of 10% formic acid in water (A) and acetonitrile (B). A gradient program was used for HPLC–DAD and ESI-MS as follows: 90 % A in the first 4 min, linear gradient to 75 % A over 25 min, then linear gradient back to initial conditions for other 15 min. The mobile phase flow rate was 1 mL/min. The column temperature was set at 25 °C; the detector was monitored at 320–254 nm for phenolics and flavonoids compounds, respectively [9].

2.4 Animals

Experimental procedure was approved by Alexandria University Animal Ethics Committee (AEC), and animals received tender care as contained in the guidelines of National Health and Medical Research Council (NHMRC), Arab Republic of Egypt. Thirty (35) Male and female Sprague Dawley rats (167-210g) were purchased and housed in polypropylene cage at the Alexandria Medical Research Institute, Alexandria, Egypt. All animals were maintained under standard husbandry conditions (12:12 hour light-dark cycle at a constant temperature of 25^{oc} and humidity 55 %±10%) with standard lab chow and water ad libitum. The 5 rats were used for acute toxicity (LD_{50} determination) and the remaining for hepatotoxicity studies.

2.5 Acute toxicity study

Preliminary experiment for the determination of lethal dose (LD_{50}) of the extract was carried out by the Acute Oral -fixed Dose Procedure. One female rat was administered 300 mg / kg of the

extract and left for 24 hours to check for sign of toxicity. In the following day, same animal received 2000 mg/ kg of the extract and left for 24 hrs. On the third day 5000 mg / kg was administered to the same rat. On the fourth day, a confirmatory test of the highest dose (5000 mg/ kg) was carried out by the administration of 5000 mg / kg to four rats and observed for any toxicity or death according OECD, 2001 [10] .

2.6 Establishment of rat model with hepatic injury and fibrosis by CCl_4

A mixture of CCl_4: the toxicant (0.1ml/100g b.wt.) and olive oil: the vehicle (1:1 v/v) was injected to the rats interperitoneally, to induce fibrosis. Group 1 vehicle control; received (olive oil only). Group 2 (CCl_4 + olive oil). Group 3 received (CCl_4 + olive oil) and extract (200 mg/kg P.O). Group 4 received (CCl_4 + olive oil) and extract (400 mg/kg P.O.). Group 5 Standard control; received (CCl_4 +olive oil) and silymarin (50 mg/kg P.O). Rat in groups 2-5 were injected with (CCl_4 + olive oil) every other day for 8 weeks while extract (treatment) was administered orally with gavage [11]. At the end of the experiment rats were weighed, anaesthetized using ether and dissected. Blood was collected from abdominal aorta and liver was isolated, washed in normal saline and divided into three portions: one fixed in neutral 10 % formalin, one used to prepare homogenate while the other stored at -80°C for RNA extraction.

2.7 Liver homogenate preparation

Five gram (5g) of liver was cut into pieces and homogenized in 45ml phosphate buffer (pH 7.40) using Ultra -Turrax® T25 homogenizer at 700g in ice for 10 min and stored at -20°C for further use.

2.8 Determination of Hydroxyproline Level

The content of hydroxy proline in the liver samples was determined based on the protocol described below; with modifications [12]. Hydroxyproline is oxidized into pyrrole followed by coupling with Ehrlich's Reagent (Dimethyl-amino-benzaldehyde (DMAB), to give a red colour which absorbs at 540-560 nm. Reagents include; Oxidant solution; 0.01M $CuSO_4$ + 2.5N NaOH + 6 % H_2O_2, 3 N H_2SO_4, HCl. Hydroxyproline standard; 10 mg / ml. Ehrlich's reagent; (Dimethyl-amino-benzaldehyde (DMAB) and 5 % in propanol or 95 % ethanol). To 100 µl of standard and sample (10 % liver homogenate in saline), 100 µl of oxidant solution was added and shook for 5min at 80 °C then chilled on ice. One hundred micro litre of H_2SO_4 were added with agitation then 200 µl of Ehrlich's reagent were added. The mixture was heated at 70 °C for 16 min and the absorbance against blank was taken after cooling. The Concentration of hydroxyproline was calculated using the standard curve.

2.8 Determination of Biochemical indexes of liver injury

Serum aspartate amino transferase (AST), alanine amino transferase (ALT) and total albumin (Bio diagnostics, Egypt and Bio vision company, USA) manufacturer's instructions was followed. The levels of liver lipid peroxidation (MDA), total glutathione (GSH), superoxide dismutase (SOD) glutathione peroxidase (GPx) glutathione transferase (GST) were analysed using kits obtained from Solarbio company, China.

2.9 Quantitative real-time polymerase chain reaction (qRTPCR)

Total RNA was isolated from liver tissue using spin column RNA mini-preps kit (Bio basic Canada). The concentration and purity of the extracted RNAs from all the samples were determined using Biodrop Spectrophotometer. Finally, total RNAs samples were standardized. Gene expression for NF-κB, TGF-β and IL-6 and β-actin (reference gene) were quantified by real time PCR system (Thermo Scientific PikoReal) using SYBER Green 1-step qRTPCR Kit (Enzynomics korea). The gene primers used for the PCR include:
NF-κB (F: CTGTATCCCGCCCTGCTGGTG. F: TTGCGTTGATGGTGGCTGTCTT) [5] IL-6, (F: CGAGCCCACCAGGAACGAAAGTC; R: CTGGCTGGAAGTCTCTTGCGGAG. [5], TGF-β (F: TGGCGTTACCTTGGTAACC; R: GGTGTTGAGCCCTTTCCAG.[5]. β-actin (F: GTGGGCCGCTCTAGGCACCAA; R: TCTTTGATGTCACGCACGATTTC [13].

2.10 Histological Study of liver tissue

The histological study was carried out based on the method explained below. The Reagents include**;** Ethanol, 70, 80, 95 % and absolute, Formalin, 10 %. Ehrlich's acid Hematoxylin. Xylene, Acid fuchsin 1 %. The liver tissues of all animal groups were fixed in 10 % formalin for 48 h, and then liver tissues were transferred to 95 % ethanol for 24 h. The liver sections were washed for 24 h under running tap water. The tissues were transferred to 70 %, 80 %, 95 % and absolute alcohol at least 72 h each. After that, sections of liver were done using a microtome. Sections were stained in Ehrlich's acid hematoxylin for 5 min and were washed under running tap water for 30 min. The sections were then counter stained in 1 % acid fuchsin for 15 min and were washed not more than 5 min with distilled water then passed on alcohol 70, 95, 100 % ethanol and

xylene. The slides were seen under a light microscope [14].

2.11. Statistical analysis

All data were expressed as mean± standard deviation (SD). The differences were statistically significant at P< 0.05. Statistical analyses were carried out using SPSS for analysis of unpaired student T- Test and one way (ANOVA).

3. Results

3.1 Extraction yields, and Characterization of the plants extracts

The extraction yield of the plants extracts was 20 g/100g of plant's dry weight, total phenolic content 54.38 mg/gallic acid equivalent) and total flavonoids (116.03 mg/quercetin equivalent). HPLC analysis revealed the presence of Caffeic acid (0.5 mg), 3,4-Dicafeoylquinic acid (0.46 mg), 4,5-Dicaffeoyl quinic acid (0.44 mg), Vanillic acid (0.067 mg), Epicatechin (0.0018 mg) and Salicylic acid (0.0004 mg).

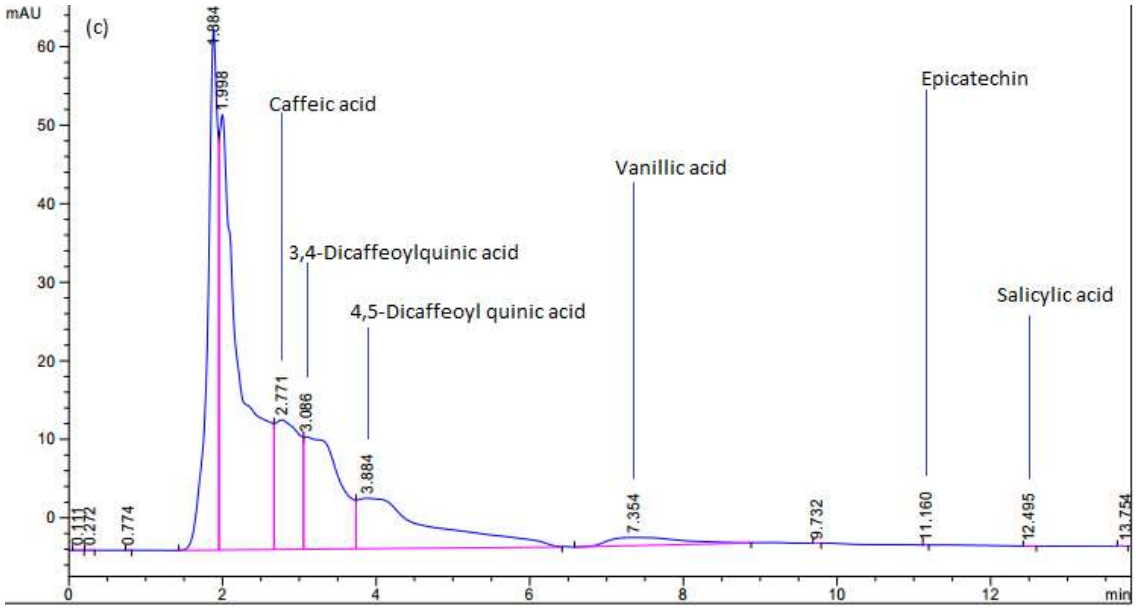

Fig. 1. HPLC analysis of polyphenolic compounds present in *Indigofera hirsuta* ethanol extract.

3.2 *Indigofera hirsuta* protected the Liver against injury upon CCl_4 administration and Suppressed Fibrosis in Rat Model.

The acute toxicity revealed no death at 5000 mg/kg thus the extract was confirmed to be practically nontoxic. From the commencement of the experiment and at intervals we measured the weight of each rat. At the end of induction period (two months), the weight of each rat was taken and percentage gain in weight was determined. The weight of the rats in group2 was significantly (p< 0.05) less than the control. Treatment with Indigofera *hirsuta* significantly increased body weight in concentration dependent manner compared to group2. (table1). Since hydroxyproline is almost exclusively found in collagens, its Determination would give an insight to the extent of liver injury/ fibrosis and to test efficacy of new anti-fibrotic agents. The level of hydroxyproline was significantly higher in the CCl_4 control (group2) compared to the vehicle control (group1). Treatment with 200mg and 400mg/kg b.wt. respectively of Indigofera *hirsuta* (groups 3 and 4) significantly decreased the level of hydroxyproline, though not in concentration dependent manner. Similarly, administration of silymarin 5mg / kg significantly (P< 0.05) decreased the level of hydroxyproline compared to the CCl_4 control group2 but not significantly different (p> 0.05) from the treatment groups 3 and 4. These results suggest that Indigofera *hirsuta* ethanol extract suppressed fibrosis.

Furthermore, additional Biochemical markers of liver injury were assayed. These include Serum total protein (TP), total albumin (TA), AST and ALT. Serum concentrations of TP and TA which are pointers of liver synthetic function, decreased significantly (p< 0.05) compared to the vehicle control (group1). However, treatment with *I. hirsuta* and reference drug (silymarin) significantly improved the levels of TP and TA compared to the CCl_4

induced group but still significantly less than the vehicle control. These results suggest the protective effect of *I. hirsuta* extract on liver.

Table 1. Determination of percentage weight gain and Biochemical indexes of liver injury in rats.

Groups	Percentage Weight gain	Total protein(g/dl)	Total albumin(g/dl)	AST(U/L)	ALT(U/L)	Hydroxyproline level (µmol/g tissue)
1. Vehicle control	43.10±2.69^{b}	7.48 ± 1.0^{c}	2.03±0.14^{b}	171.67±26.25^{c}	77.66±8.50^{b}	7.51±0.83^{b}
2. CCl_4 control	25.10±1.20^{a}	5.73± 0.73^{a}	1.58±0.15^{a}	247.22±29.60^{d}	114.33±13.39^{c}	8.75±0.28^{c}
3.I. *hirsuta* (200 mg/kg)	44.21±2.84^{b}	8.16± 0.72^{d}	1.62±0.19^{a}	164.00±22.53^{c}	80.83±11.45^{b}	7.12±0.64^{a}
4. I. *hirsuta* (400mg/kg)	45.85±3.09^{b}	8.23± 0.01^{d}	2.10±0.10^{b}	144.00±19.47^{b}	65.50±15.68^{a}	7.04±0.08^{a}
5. Silymarin forte (5mg/kg)	43.80±2.84^{b}	6.56± 0.92^{b}	1.93±0.18^{b}	115.83±18.63^{a}	60.83±10.75^{a}	6.84±0.48^{a}

Values are presented as Mean ± SD (n = 6), Values with different letters are significantly different (P < 0.05). letter (a) represent the lowest value while (d) the highest one.

3.4 *Indigofera hirsuta* attenuated oxidative stress and inhibit lipid peroxidation in CCl_4 Rats.

Oxidative stress is considered one of the most important hallmarks in liver injury and fibrosis. We assessed some markers of oxidative stress which include GSH, GST, SOD and MDA. The level of GSH and the activity of GST, GPx and SOD decreased significantly in group2 compared to group1(Fig.2). Administration of Indigofera *hirsuta* extract and silymarin (groups 3,4 &5) normalised the level of GSH and increased the activities of GST, GPx and SOD significantly (P<0.05), in concentration dependent manner compared to group1 and 2. Similarly lipid peroxidation induced by CCl_4 (group2); indicated by the high level of MDA was decreased in groups 3, 4 and 5 compared to the 2. These indicate the role of *Indigofera hirsuta* in attenuating oxidative stress.

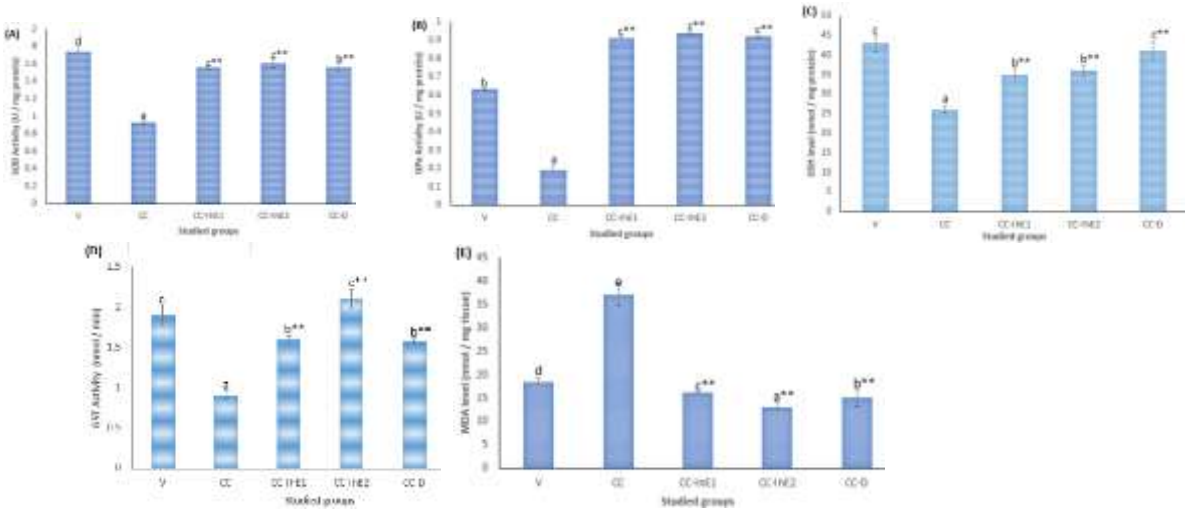

Fig. 1. Effect of *Indigofera hirsuta* on some oxidative stress markers (A) Superoxide dismutase (SOD) (B) Glutathione peroxidase (GPx) (C) Reduced glutathione (GSH) (D) Glutathione transferase (GST) (E) malondialdehyde (MDA). Values are presented as Mean ± SD (n = 6), Values with different letters are significantly different (P < 0.05). letter (a) represent the lowest value while (d) the highest one. ** indicate significant difference from CCl_4 group.

3.5 Indigofera hirsuta down regulates NF-ƙB mRNA level in CCl_4 Rat and decreased mRNA levels of IL-6 and TGF-β in CCl_4 Rats.

Inflammation is tightly associated with hepatic injury and fibrosis during liver diseases. The level of NF-ƙB mRNA, pro-inflammatory cytokine; IL-6 mRNA and TGF-β quantified by qPCR were significantly increased in CCl_4 control group compared to the vehicle control. Treatment with Indigofera *hirsuta* significantly ($P< 0.05$) modulates this increase even better than the reference drug; silymarin.

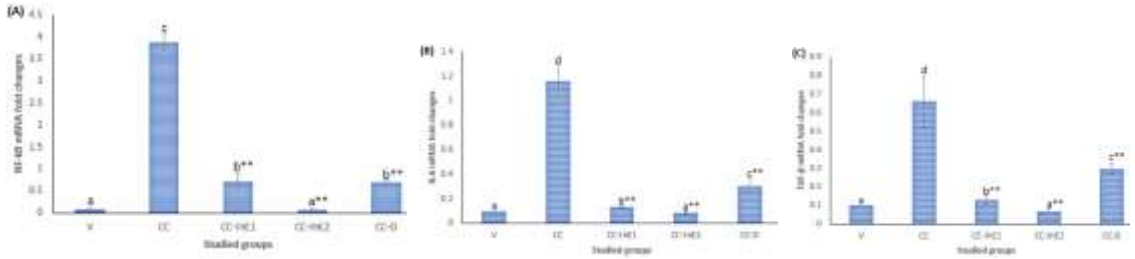

Fig. 2. Effect of *Indigofera hirsuta* on mRNA levels of some fibrogenic markers (A) Nuclear factor kappa B (NF-ƙB) (B) Interleukine-6 (IL-6) (C) transforming growth factor beta (TGF-β). Values are presented as Mean ± SD (n = 6), Values with different letter are significantly different ($P < 0.05$). letter (a) represent the lowest value while (d) the highest one. ** indicate significant difference from CCl_4 control group.

3.6 Histological Analysis

The histology of the vehicle control group, showed normal hepatic architecture with hepatocytes displayed in trabeculae formed of two -cell thick and in between sinusoids with no remarkable pathological changes. Hepatocytes are polygonal with distinct cell borders radiating from central veins. Portal tracts are identified at the periphery of the lobules containing a branch of the hepatic artery, a tributary of hepatic vein and bile duct. (Fig.3). The rats injected with CCl_4 revealed a section in the liver showing widened portal tracts, by a cellular inflammatory infiltrate, composed of lymphocytes, histiocytes and plasma cells and fibrosis. Micro vesicular fatty change is discerned as well as foci of lytic necrosis (Fig.3). However, rat treated with different doses of *I. hirsuta* 200 mg and 400 mg / kg b.wt. reveals a section of liver showing well preserved hepatic tissue, arranged in lobules. Very minimal inflammatory cellular infiltrate is noted. (Fig.3). Similarly, silymarin forte treated group revealed mild infiltrations of lymphocytes and plasma cells, some of which extend into the adjacent parenchyma (Fig.3).

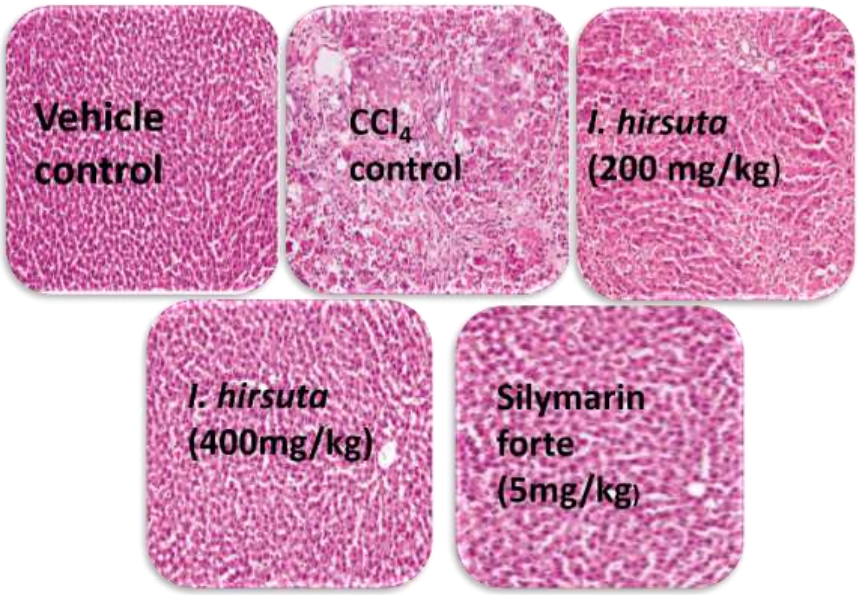

Fig.3. *I hirsuta* liver from CCl_4 induced hepatic injury and fibrosis in rats. Rats were divided into vehicle control, CCl_4 control, *I. hirsuta* (200 mg/kg and 400 mg/kg) and silymarin forte groups respectively. A piece of liver tissue from each rat was fixed with formalin and then embedded in paraffin, then thin section was cut and stained with H&E Microscopic.

4. Discussion

The toxicity of CCl_4 in inducing liver fibrosis in rodent has been well established. The metabolism of CCl_4 in the liver stimulates lipid peroxidation and generation of free radicals [15], which cause necrosis of the hepatocytes and inflammation. During liver Inflammation, the hepatocytes, kuffer cells, platelets and leukocytes are stimulated to produce reactive oxygen species (ROS) and inflammatory mediators such as plate late derived growth factor (PDGF) and transforming growth factor beta (TGF-β) [16]. These factors activate quiescent HSCs resulting in development of fibrogenesis [17].

Antioxidants have been known to protect hepatocyte against lipid peroxidation induced by CCl_4 or DMN in rats [9]. Indigofera *hirsuta* is one of the medicinal plants used in Nigerian traditional medicine to treat various ailments associated with inflammation and oxidative stress. *I hirsuta* has been shown to have antioxidant and anti-inflammatory activities [6]. In this study, we examined the protective effect of this herb against liver fibrogenesis. We established that *I hirsuta* significantly reduced the biochemical indexes of liver injury, attenuated hepatic oxidative stress and suppressed inflammation and necrosis triggered by CCl_4 in rat model. Furthermore, *I hirsuta* down regulated the expression of genes associated with HSC activation in rats. It is essential to mention that, most of the biochemical parameters revealed that *I hirsuta* administration at the concentration of 200 mg/kg had significantly better protection to the liver than 400m/kg. However, in some cases no significant difference was observed between the two doses.

The link between oxidative stress and hepatic fibrosis has been well established [18]. Administration of antioxidants of natural origin such as silymarin and curcumin has been shown to suppressed lipid peroxidation and necrosis of hepatocytes in rats [19]. In this research, we presumed that *I. hirsuta* could protect liver against CCl4-induced liver injury. Daily oral administration of *I hirsuta* ethanol extract for 8 weeks ameliorated CCl_4-induced liver injury as reflected by the decrease in AST, ALT and hydroxyproline levels with corresponding increase in total serum protein and albumin. Similarly, the activities SOD, GPx, GST and level of GSH were increased, while MDA and hydroxy proline levels were decreased in the treatment groups compared to CCl_4 control. These activities could be attributed to the presence of different polyphenolic compounds in the extract. Vanillic acid found in the extract was reported to decrease ALT, AST, suppressed collagen accumulation and hydroxyproline in CCl_4 induced rat [20]. Epicatechin and caffeic acid was found to stimulate SOD and GPx activities and inhibit lipid peroxidation [9]. Similarly, the polysaccharide fractions found in the extract could have contributed to its antioxidant properties [21].

Overwhelming evidences from Previous studies have shown that oxidative stress and inflammation are closely related and act simultaneously to create a malicious cycle, which derives fibrogenesis by activating HSCs [22]. The activation of HSC induces NF-ƙB expression and signalling, which in turn stimulates the generation of various proinflammatory factors including IL-1, IL-6, TNFα and TGF-β [23]. These indicate that drug that can down regulate NF-ƘB and inhibits release of proinflammatory cytokines production may perhaps be a promising anti-fibrotic drug. In this study administration of *I hirsuta* for 8 weeks down regulated the mRNA levels of NF-ƘB, IL-6 and TGF-β.

In summary, this study has confirmed our hypothesis and showed that *I hirsuta* ethanol extract offered protection to rat liver against CCl_4 induced fibrogenesis through; attenuation of oxidative stress, suppression of liver inflammation and inhibition of HSC activation. However, the therapeutic role of *I hirsuta* as anti-fibrotic agent fibrosis need to be further investigated.

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Conflict of interest

Authors declared no conflict of interest.

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