Liver Protective Activity of *Indoneesiella Echioides* Against Carbon Tetrachloride (CCl_4)-Induced Hepatotoxicity In Rats

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Summary

Indoneesiella echioides (False Waterwillow) (L) Nees. (Acanthaceae) is an herb used in the treatment of liver diseases. This study evaluated the hepatoprotective activity of the ethanol extract of *Indoneesiella echioides* using a carbon tetrachloride (CCl_4)-induced liver injury in rats. *Indoneesiella echioides* was extracted by continuous hot percolation using ethanol. *In vitro* antioxidant activity determined using DPPH. The extract at doses of 1000, 2000 and 3000 mg/kg and silymarin 25 mg/kg were administered for 7 days and on 7th day all rats were challenged with CCl_4 except control group animals. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total bilirubin (TB) and total protein levels were determined to prove the hepatoprotective effect. Catalase (CAT), superoxide dismutase (SOD), and thiobarbituric acid reactive substances (TBARS) estimated using liver homogenate. Dose dependent antioxidant activity was obtained against DPPH radical. Levels of serum markers AST, ALT, ALP, TB and total protein were significantly increased in CCl_4 treated rats. All three doses of extract and silymarin exhibited significant ($P<0.01$) protective effect against CCl_4-induced hepatoxocity by decreasing the level of serum markers, bilirubin and total protein. Increase in antioxidant enzymes CAT and SOD and a decrease in the level of TBARS was observed. The histopathology studies further revealed liver-protective effect of the extract. The present study confirms the liver protection of ethanol extract of *Indoneesiella echioides* in rats and the results were in comparison with standard drug, silymarin justifying folkloric claim in treatment of liver diseases.

Keywords: Liver disorders, hepatoprotective activity, serum markers, silymarin.

Introduction

Liver diseases, such as jaundice, cirrhosis and fatty liver have become one of the major causes of morbidity and mortality worldwide [1]. Despite its frequent occurrence, high morbidity and mortality, its medical management is currently in adequate; no therapy has successfully prevented the progression of hepatic diseases [2]. The use of natural remedies for the treatment of liver disorders has a long history and medicinal plants are still used all over the world in one form or the other for this purpose [3]. It has been reported that 160 phytoconstituents from 101 plants have hepatoprotective activity [4]. Plants which offer liver protection contain a variety of chemical constituents like phenols, coumarins, lignans, essential oil, monoterpenes, carotinoids, glycosides, flavanoids, organic acids, lipids, alkaloids and xanthenes [5]. However, modern drugs hardly provide protection to the liver from damage or regeneration of hepatic cells.

Indoneesiella echioides (L) Nees. (Acanthaceae), also known as *Andrographis echioides* (L) Nees. which is commonly known as false waterwillow, is an herb commonly found throughout India and Sri Lanka. The genus *Andrographis* is native of India and Sri Lanka contains 28 species of small annual shrubs essentially distributed in tropical Asia. Some of them are medicinally important. The plants from genus *Andrographis* used in goiter, liver diseases [6], fever, fertility problems, bacterial [7], malarial, helmintic, fungal, diarrhea and larvicidal disorders [8, 9]. Leaf juice boiled with coconut oil used to control falling and graying of hair [10]. On the basis of its traditional uses the present study is aimed at evaluating the hepatoprotective effect of ethanol extract of whole plant of *Indoneesiella echioides*. No concrete scientific work has been reported to prove the folklore claim in the utility of *Indoneesiella echioides* in the treatment of liver diseases and hence the objectives of the present study was to investigate the hepatoprotective activity afforded by the ethanol extract of this plant on CCl_4-induced hepatotoxicity in rats.

Methods

Drugs and Chemicals

Silymarin was purchased from Micro Labs. India. ALT, AST, ALP, bilirubin and total protein kits were procured from Span Diagnostics, India. All other chemicals were of analytical grade and purchased from local suppliers.

Collection and authentication of plant

The whole plant of *Indoneesiella echioides* was collected in January 2006 from Kolli hills, Tamil Nadu, identified by Dr. Kumareshan, Botanist, authenticated by Dr. Marimuthu and the voucher specimen of *Indoneesiella echioides* was deposited at the herbarium of Vinayaka Mission's Deemed University, Salem (Ref. No: 006/COL/219) for future reference.

Preparation of extract

The whole plant was dried under shade at room temperature and then powdered with a mechanical grinder. The powdered material was passed through sieve No 40 and stored in an airtight container for future use. About 350 g of powdered leaves of *Indoneesiella echioides* was defatted with petroleum ether (60-80°C) and extracted with ethanol in a Soxhlet apparatus for 72 h. The extracted material was filtered and the filtrate was concentrated under reduced pressure using a rotary flash evaporator to obtain a semisolid mass.

Preliminary phytochemical screening of *Indoneesiella echioides*

Preliminary phytochemical tests has been conducted for the presence of alkaloids, carbohydrates, glycosides, phenolic compounds, tannins, protein and amino acids, saponins, gums and mucilage, sterols, fixed oils, fats and flavonoids [11].

Experimental Animals

Wistar male albino rats weighing 150-200 g were used for the study. The rats were procured from Sri Venkateswara Enterprises, Bangalore, India. The animals were grouped and housed in polyacrylic cages (38 x 23 x 10 cm) with not more than six animals per cage and maintained under standard laboratory conditions (temperature 25 ± 20°C) with dark and light cycle (12/12 h). The animals were fed with standard pellet diet supplied by Hindustan Lever Ltd., Bangalore, India and fresh water *ad libitum*. All the animals were acclimatized to laboratory condition for a week before commencement of experiment. All procedures described were reviewed and approved by the University Animals Ethical Committee (UAEC).

Acute toxicity studies

For determination of lethal dose (LD_{50}) 5 groups of mice (n=6) were administered orally with 2.5, 5, 7.5, 10 and 12.5 g/kg of ethanol extract of *Indoneesiella echioides* and the groups were observed for mortality for 24 h [12].

***In vitro* antioxidant activity using DPPH method**

The hydrogen donating ability of ethanol extract of *Indoneesiella echioides* was examined in the presence of DPPH stable radical [13]. An aliquot of 1 mL 0.3 mM DPPH ethanol solution was added to 2.5 mL sample solution of different concentrations and allowed to react at room temperature. After 30 min the absorbance values were measured at 517 nm. Ethanol (1 mL) plus plant extract solution (2.5 mL) was used as a blank. DPPH solution (1 mL, 0.3 mM) plus ethanol (2.5 mL) served as negative control. Ascorbic acid was used as positive control. The concentration (mg/mL) of the extract required to scavenge 50% of the radicals was calculated by using the percentage scavenging activities at five different concentrations of the extract. Percentage inhibition (I%) was calculated using the formula

$$I\% = \frac{(Ac - As)}{Ac} \times 100$$

Where Ac is the absorbance of the control and As is the absorbance of the sample [14].

Carbon tetrachloride (CCl_4)-induced hepatotoxicity

Animals were randomly divided into five groups (n= 6 animals). Group I served as untreated control and fed orally with normal saline 10 ml/kg body weight. Group II rats were similarly treated as group I and served as intoxicated control. Group III were fed with standard drug Silymarin 25 mg/kg, *p.o* [15]. Group IV, V and VI were treated with 1000, 2000 and 3000 mg/kg body weight of ethanol extract of *Indoneesiella echioides* respectively. The ethanol extract and silymarin were prepared by suspending in 0.5% carboxy-methyl cellulose and were given orally daily for seven days [16]. On the 7^{th} day, all animals received CCl_4 1.5 ml/kg (1:1 of CCl_4 in olive oil) orally except the rats in group I. The biochemical parameters were estimated after 18 h of the last dose administration by fasting the experimental animals. The blood was obtained from all rats under light ether anesthesia by retro-orbital sinus puncture. The blood was allowed to clot at room temperature. Serum was separated by centrifugation at 2500 rpm for 15 min and used for the estimation of AST, ALT, ALP, serum bilirubin and total protein were estimated as per the standard procedures prescribed by the manufacturer (Span Diagnostics, India) using auto analyzer.

The rats were sacrificed and liver was removed and washed immediately with ice cold saline to remove as much as blood possible. It was homogenized (5% w/v) in cold potassium

phosphate buffer (50 mM, pH 7.4) using a Remi homogenizer. The unbroken cells and cell debris was removed by centrifugation at 3000 rpm for 10 min, using Remi C-24 refrigerated centrifuge. The supernatant obtained was used for the estimation of catalase (CAT), superoxide dismutase (SOD), and thiobarbituric acid reactive substances (TBARS).

Estimation of catalase (CAT)

The catalysis of H_2O_2 to H_2O in an incubation mixture adjusted to pH 7.0 was recorded at 254 nm. The reaction mixture contained 2.6 ml of 25 mM potassium phosphate buffer pH 7.0 and 0.1ml of tissue homogenate and was incubated at 37°C for 15 min and the reaction was started with the addition of 0.1 ml of 10 mM H_2O_2. The time required for the decrease in absorbance from 0.45 to 0.4 representing the linear portion of the curve was used for the calculation of enzyme activity. One unit of catalase activity was defined as the amount of enzyme causing the decomposition of μmol H_2O_2/mg protein/min at pH 7.0 at 25°C [17].

Estimation of superoxide dismutase (SOD)

SOD activity was determined by the inhibition of auto catalyzed adrenochrome formation in the presence of homogenate at 480 nm. The reaction mixture contained 150 μl of homogenate, 1.8 ml of carbonate buffer (30 mM, pH 10.2), and 0.7 ml of distilled water and 400 μl of epinephrine (45 mM). Auto oxidation of epinephrine to adrenochrome was performed in a control tube without the homogenate. Activity was expressed as μmoles/min/mg protein [18].

Determination of lipid peroxidation (TBARS)

For TBARS, 0.1 ml of tissue homogenate (Tris-HCl buffer, pH 7.4) was mixed with 2.0 ml of TBA-TCA-HCl reagent (Thiobarbituric acid 0.37%, 0.25 N HCl and 15% TCA mixed in 1:1:1 ratio). The resultant solution was placed in water bath for 15 min, cooled and centrifuged at 1000 rpm at room temperature for 10 min. The absorbance of the clear supernatant was measured against reference blank at 535 nm. The results were expressed as nM/min/mg tissue protein [19].

Histopathological studies

Small pieces of liver tissues were taken from each group and placed in 10% formalin for proper fixation. The tissues were processed and embedded in paraffin wax. Sections of 5-6 μm in thickness were cut and stained with hematoxylin and eosin (H&E) and histological observations were made under light microscope**.**

Statistical analysis

The experimental results were expressed as the Mean ± SEM for six animals in each group. The results were analyzed statistically using one way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. P value of < 0.05 was considered as statistically significant**.**

Results and Discussion

The ethanol extract of the *Indoneesiella echioides* revealed the presence of carbohydrates, glycosides, phenolic compounds, tannins, sterols, fixed oils and fats and flavonoids. The phytochemicals isolated from *Indoneesiella echioides* were found to be dihydroechioidinin, along with four known flavones, echioidinin, echioidin, skullcapflavone I 2'-*O*-methyl ether and skullcapflavone I 2'-*O*-glucoside [20].

Acute toxicity studies revealed that the ethanol extract of *Indoneesiella echioides* was toxic at dose level of 10 g/kg. Based on the toxic dose three dose levels 1000, 2000 and 3000 mg/kg has been fixed for the study.

Treatment of rats with CCl_4 produced an increase in liver weight when compared with untreated control group. Administration of ethanol extract of *Indoneesiella echioides* for 7 days significantly reduced ($P < 0.01$) the weight of liver in all three groups of rats when compared with intoxicated (CCl_4) control group. Administration of silymarin reduced the liver weight and the reduction in weight is more than the extract treated group (Table 1). The increase in liver weight in intoxicated control (CCl_4) may be due to blocking of the secretion of hepatic triglycerides into the plasma and accumulation of lipids and proteins in hepatocytes with an impaired protein secretion by hepatocytes [21, 22]. Water is retained in the cytoplasm of hepatocytes leading to enlargement of liver cells resulting in increased total liver mass as observed in the present study [23].

The efficacy of any hepatoprotective drug is essentially dependent on its ability in reducing the harmful effects or maintaining the normal hepatic physiology that has been disturbed by a hepatotoxin. CCl_4 intoxication in normal rats elevated the levels of AST, ALT, ALP, total bilirubin and total protein were observed significantly indicating acute hepato cellular damage and biliary obstruction. The rats that have received 1000, 2000 and 3000 mg/kg of ethanol extract of *Indoneesiella echioides* showed a significant ($P < 0.05$; $P < 0.01$; $P < 0.001$) decrease in all the elevated AST, ALT, ALP, total bilirubin and total protein levels as compared

to silymarin in a dose dependent fashion (Table 2). Liver is considered to be highly sensitive to toxic agents. The study of serum marker enzymes AST, ALT, ALP, total bilirubin and total protein levels have been found to be of great value in the assessment of experimental liver damage [24, 25]. This change in the marker levels reflects in hepatic structural integrity. The rise in the AST is usually accompanied by an elevation in the levels of ALT, which play a vital role in the conversion of amino acids to ketoacids [26]. CCl_4 induces hepatotoxicity by metabolic activation and it selectively causes toxicity in liver cells maintaining semi-normal metabolic function. CCl_4 is metabolized by the cytochrome *P*450 system in the endoplasmic reticulum to produce trichloromethyl radical ($\bullet CCl_3$). This free radical combines with cellular lipids and proteins in the presence of oxygen to form a trichloromethyl peroxyl radical, which may attack lipids on the membrane of endoplasmic reticulum faster than $\bullet CCl_3$ free radical. This leads to lipid peroxidation, destruction of Ca^{2+} homeostasis, and finally, results in cell death [27].

Treatment with ethanol extract of *Indoneesiella echioides* decreased the elevated levels of the serum marker enzymes. The normalization of serum markers by ethanol extract of *Indoneesiella echioides* suggests that the extract is able to condition the hepatocytes and protect the membrane integrity against CCl_4-induced leakage of marker enzymes into the circulation. However the protection offered by silymarin was significantly higher when compared with the extract treated groups as the level of these enzymes were found retrieving towards normal (Table 2).

Ethanol extract of *Indoneesiella echioides* demonstrated DPPH radical scavenging activity in a concentration-dependent manner and the IC_{50} was found to be 139 mg/ml. A positive DPPH test suggests that the extract is a potential free radical scavenger. However, the activity was less when compared with the standard, ascorbic acid (Table 3). DPPH is a proton free radical that shows a maximum absorption at 517 nm. When DPPH encounters proton radical scavengers its purple color fades rapidly. Antioxidants, by providing a hydrogen atom or by donation of electrons, can quench $DPPH^{\bullet}$ free radicals and convert them to a colorless bleached product resulting in a reduction in absorbance. The antioxidant activity was confirmed by a decrease in absorbance band upon increasing concentrations of the ethanol extract of *Indoneesiella echioides* [28].

A significant decrease was observed in the activities of catalase and superoxide dismutase in liver homogenate of CCl_4 alone treated rats. Administration of ethanol extract of *Indoneesiella*

echioides at doses of 1000, 2000 and 3000 mg/kg *b.w*, significantly elevated the levels of catalase and superoxide dismutase when compared to CCl_4 control group Table 4. The result was found to be statistically significant ($P<0.05$). Administration of curcumin increased the enzymatic antioxidant levels. There was significant ($P<0.05$) elevation in tissue TBARS in CCl_4 control rats. Administration of *Indoneesiella echioides* and curcumin to CCl_4 treated rats for 7 days decreased the levels of tissue TBARS in the liver tissue.

Catalase and superoxide dismutase are enzymatic antioxidants. Catalase and superoxide dismutase are considered primary enzymes since they are involved in the direct elimination of ROS. Superoxide dismutase, is an important defence enzyme which catalyses the dismutation of superoxide radicals and catalase is a haemoprotein which catalyses the reduction of H_2O and protects the tissue from hydroxyl radicals. The reduced activity of superoxide and catalase in the liver during hepatotoxicity is a result of deleterious effects which results in the accumulation of, superoxide anion radicals and H_2O_2 [29]. The activity of enzymatic antioxidants are increased significantly in extract treated animals ($P<0.05$). Marked increase in the concentration of TBARS was observed in the liver of CCl_4 control rats. Administration of ethanol extract of *Indoneesiella echioides* and curcumin tends to bring the increased concentration of lipid peroxidation products to near normal level.

The ethanol extract obtained from the plant *Indoneesiella echioides* possess remarkable hepatoprotective activity and this action is comparable to that of the standard drug silymarin used in the study. Since flavonoids have hepatoprotective activities it may be speculated that the constituents of *Indoneesiella echioides* especially the favonones may be responsible for the observed liver protective effects [20, 30].

Histopathological studies also provided supportive evidence for biochemical analysis. The liver section of normal control animals (group 1) showed normal lobular architecture with well preserved cytoplasm, prominent nucleus, nucleolus and central vein (Fig 1). The liver sections of CCl_4-intoxicated rats (group II) showed massive fatty changes, necrosis, ballooning degeneration, infiltration of the lymphocytes and Kupffer cells around the central vein with loss of cellular boundaries (Fig 2). Treatment with silymarin (group III) and ethanol extract of *Indoneesiella echioides* (group V and VI), showed normal lobular pattern with a mild degree of fatty changes, necrosis and lymphocyte infiltration indicating the hepatoprotective effect of the extract which is almost comparable to the normal control group I (Fig 3-5). However, the ethanol extract at the

dose of 1000 mg/kg offered liver protection but when compared with higher doses it was found to be less significant ($P < 0.05$). All the histological changes observed were in correlation with the physical, biochemical and functional parameters of the liver.

In conclusion, the present study demonstrated that ethanol extract of *Indoneesiella echioides* has hepatoprotective effect in CCl_4-induced liver damage. However, it is necessary to determine other parameters such as oxidative stress markers and molecular biology assays to confirm our findings. Works are in progress in our institute to isolate and purify the active principle involved in the hepatoprotective activity of this plant and to determine its mechanism of action.

Table 1: Effect of ethanol extract of *Indoneesiella echioides* on liver weight variation of CCl_4-induced hepatotoxicity in rats

Groups	Design of Treatment	Dose	Liver weight (gm)
I	Control (Saline)	10 ml/kg	4.25 ± 0.15
II	Intoxicated control (CCl_4)	1.5 ml/kg	18.15 ± 0.5[a]
III	Silymarin + CCl_4	25 mg/kg	5.10 ± 0.2[a]
IV	Alcohol extract + CCl_4	1000 mg/kg	7.45 ± 0.4[a]
V	Alcohol extract + CCl_4	2000 mg/kg	5.30 ± 0.3[a]
VI	Alcohol extract + CCl_4	3000 mg/kg	5.90 ± 0.4[a]

Values are expressed as mean ± SEM for 6 animals.
Control group compared with intoxicated control (*P<0.01); Treatment groups compared with intoxicated control (*P<0.01). Data were analyzed by using One-way ANOVA followed by Dunnett's test.

Table 2: Effect of ethanol extract of *Indoneesiella echioides* on CCl_4-induced hepatotoxicity in rats

Drugs	Dose	AST U/L	ALT U/L	ALP U/L	Total Billirubin mg%	Total Protein mg%
Control (Saline)	5 ml/kg	53.9 ± 0.94	47.0 ± 6.75	19.8 ± 2.17	1.6 ± 0.01	5.2 ± 0.02
CCl_4	1.5 ml/kg	129.9 ± 4.88 [b]	80.2 ± 10.50 [b]	38.6 ± 5.05 [b]	6.3 ± 0.08 [b]	7.0 ± 0.06 [b]
Silymarin + CCl_4	25 mg/kg	72.9 ± 0.91[b]	56.7 ± 6.4[c]	22.3 ± 0.78[d]	1.5 ± 0.008[d]	5.6 ± 0.06[a]
Alcohol Extract + CCl_4	1000 mg/kg	91.6 ± 5.08[c]	40.0 ± 8.66[b]	26.4 ± 7.08[c]	4.8 ± 0.01	5.8 ± 0.14[a]
Alcohol Extract + CCl_4	2000 mg/kg	76.9 ± 2.54[b]	48.3 ± 6.80[a]	24.1 ± 4.51[b]	1.9 ± 0.03[d]	5.5 ± 0.12[a]
Alcohol Extract + CCl_4	3000 mg/kg	88.0 ± 2.67[c]	40.7 ± 5.45[a]	27.3 ± 3.98[d]	4.9 ± 1.01	6.1 ± 0.19[d]

Values were expressed as Mean ± SEM for 6 animals. Control group compared with intoxicated control ($P < 0.05$); Treatment groups compared with intoxicated control ([a]$P < 0.001$; [b]$P < 0.05$; [c]$P < 0.01$; [d]$P < 0.02$) Data were analyzed by using One-way ANOVA followed by Dunnett's test.

Table 3: *In vitro* antioxidant activity using DPPH method

Extract /Standard	DPPH		
	Concentration (µg/ml)	% Inhibition	IC_{50} µg/ml
	1000	72.66±2.78	
Indoneesiella	500	70.0±1.78	139±0.85
***echioides* ethanol**	250	64.50±2.39	
extract	125	49.25±1.26	
	62.5	30.15±0.16	
Ascorbic acid			2.69 ± 0.05

*Average of three determinations, values were mean ± S.E.M (n = 3). The results were found to be statistically significant P<0.05.

Table 4: Effect of ethanol extract of *Indoneesiella echioides* on antioxidant enzymes and lipid peroxidation

Treatment	Dose	Catalase (U/mg protein)	Superoxide dismutase (U/mg protein)	Lipid peroxidation µmole of MDA/mg protein)
Control (Saline)	5 ml/kg	354.51± 22.07	91.85±7.39	0.98±0.04
CCl_4	1.5 ml/kg	266.82±24.07 [a]	57.35±4.22 [a]	1.71±0.04 [a]
Silymarin + CCl_4	25 mg/kg	353.63±15.25 [a]	90.56±4.43 [a]	1.13±0.05 [a]
Alcohol Extract + CCl_4	1000 mg/kg	282.17± 24.76[b]	60.21±5.21 [b]	1.66±0.06 [b]
Alcohol Extract + CCl_4	2000 mg/kg	305.25± 20.09 [b]	67.42±6.24 [b]	1.42±0.09 [b]
Alcohol Extract + CCl_4	3000 mg/kg	334.35± 31.05 [b]	85.35±7.24 [b]	1.22±0.08 [b]

Values are mean ± S.E.M. (n=6) Data were analyzed by using One-way ANOVA followed by Dunnett's test.
Control group compared with intoxicated control ([a]$P < 0.01$)
Treatment groups compared with intoxicated control ([a]$P < 0.01$;[b]$P < 0.05$)

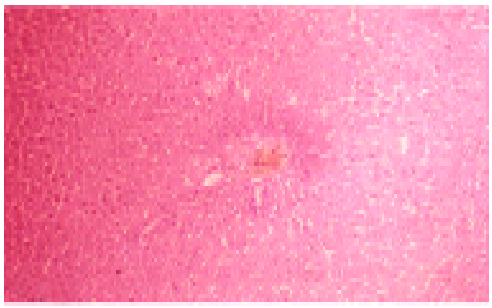

Fig. 1. Normal control

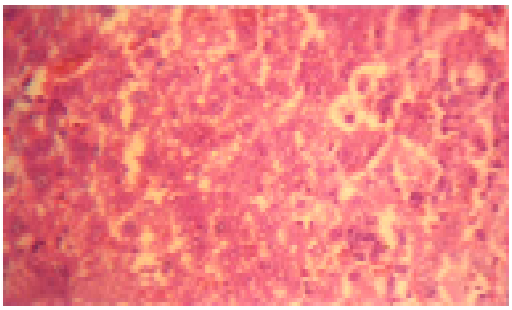

Fig. 2. Intoxicated control (CCl_4))

Fig. 3. Silymarin treated

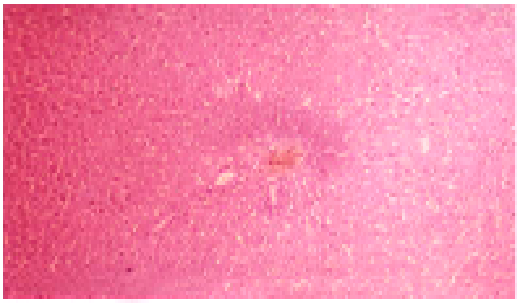

Fig. 4. Ethanol extract (2000 mg/kg)

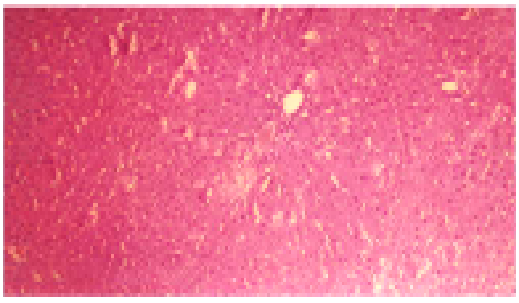

Fig. 5. Ethanol extract (3000 mg/kg)

Histopathology of liver tissue after exposure to CCl_4 and the effect of silymarin and ethanol extract of *Indoneesiella echioides* (H & E, 100x)

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