World Journal of Pharmaceutical Sciences
ISSN (Print): 2321-3310; ISSN (Online): 2321-3086
Published by Atom and Cell Publishers
Available online at: http://www.wjpsonline.org/
Original Article

Hepatoprotective activity of Tagetes *erecta* Linn. in carbon tetrachloride induced hepatotoxicity in rats

Gauri Karwani and Siddhraj S. Sisodia

Bhupal Nobles' College of Pharmacy, Udaipur, Rajasthan, 313 001, India

Received: 13-03-2015 / Revised: 23-05-2015 / Accepted: 27-05-2015

ABSTRACT

Many plants have been claimed to possess liver protecting activity. But, due to scarcity of potent modern medicine to treat severe liver diseases, many folk remedies of plant origin have been scientifically evaluated for their potential hepatoprotective activity in experimental animal models. In the present study, hepatoprotective activity of Tagetes *erecta linn. w*as investigated in CCl_4 intoxicated Albino Wistar rats and the results were compared with, Silymarin. Subcutaneous injection of CCl_4, produced a marked elevation in the level of biochemical markers. Oral administration of *Tagetes erecta linn* at dose 200 mg/kg, 400mg/kg and 600mg/kg in CCl_4 intoxicated rats showed marked decrease in the level of biochemical markers and results were at par with the effect shown by Silymarin. The results of histopathological analysis were in compliance with the findings of blood biochemical parameter analysis. This study confirms that *Tagetes erecta linn.* hydroalcoholic extract has phytocomponents with hepatoprotective potential.

Keywords: *Tagetes erecta* Linn. , Hepatotoxicity, Hepatoprotective, Carbon tetrachloride

INTRODUCTION

Liver plays a vital role in detoxification and excretion of many endogenous and exogenous substances. Continuous exposure and intoxication of liver to different types of exogenous compounds on a daily basis may lead to hepatic dysfunction. Hepatic dysfunction due to exposure to environmental toxic agents is increasing worldwide. Toxins and drugs are among the basic etiopathogenetic agents of acute liver failure in Western countries [1].In view of this, the present study was preceded as CCl_4 induced hepatic study in wistar albino rats. The ethanolic extract of *Tagetes erecta* Linn. (Family, Asteraceae) commonly known as Genda was used for the liver protecting herb in this study. This herb is found throughout the Asian countries.

The *Tagetes erecta* Linn. (Family, Asteraceae) widely used in olden days for the treatment of wounds. It is commonly known as aromatic annual herb reaches 0.4-1 m height. It is very popular as a garden plant and yields a strongly aromatic essential oil (tagetes oil), which is mainly used for the compounding of high-grade perfumes. Different parts of this plant including flower are used in folk medicine to cure various diseases. The leaves are reported to be effective against piles, kidney troubles, muscularpain, ulcers, and wounds. The pounded leaves are used as an external application to boils and carbuncles. The flower is useful in fevers, epileptic fits (Ayurveda), astringent, carminative, stomachic, scabies and liver complaints and is also employed in diseases of the eyes. They are said to purify blood and flower juice is given as a remedy for bleeding piles and also used in rheumatism, colds and bronchitis [2].

MATERIALS AND METHODS

***Extraction of Tagetes erecta Linn*.:** The leaves plant of *Tagetes erecta* Linn. was dried under shade and then powdered with a mechanical grinder to obtain a fine powder (500 gm), the fine powder of whole plant was packed in high quality filter paper, which was then subjected to successive extraction in a soxhlet apparatus using 50% ethanol for about 72 hour, solvent was recovered. Extractive yield of *Tagetes erecta* Linn. was 35 %. After vacuo evaporation the crude extract was dissolved in distilled water freshly as required.

***Animals*:** Albino rats (*Wistar* strain) weighing 125 - 150 gm of either sex were used for the present

Corresponding Author Address: Gauri Karwani, Bhupal Nobles' College of Pharmacy, Udaipur, Rajasthan, 313 001, India

study. The animals were housed in polypropylene cages at control temperature (26 ± 2° C) relative humidity (60 ± 5%) and light. Rats were fed with standard laboratory diet and drinking water was given through drinking bottle throughout the experiment. The animals were maintained as per CPCSEA regulation and cleared by IAEC at Bhupal Nobles' College of Pharmacy, Udaipur (Rajasthan), India.

***Drug Formulation*:** The extract of plant fully dissolves in distilled water. The solution of the whole plant extract (300 mg/ml) was freshly prepared in distilled water.

***Experimental Induction of Hepatotoxicity*:** Liver toxicity was induced in rats by administrating carbon tetrachloride (CCl_4) subcutaneously (sc) in the lower abdomen, in a suspension of liquid paraffin (LP; 1: 2 v/v) at the dose of 1 ml/kg body weight (BW) on alternate days for one week [3].

***Experimental Design*:** After acclimatization the rats were divided in to 6 groups of 6 rats each in equal number of males and females.

Group 1: Rats served as control and received subcutaneous administration of liquid Paraffin (LP) only 1 ml/ kg on alternate day for one week and vehicle for three weeks orally.

Group 2: Rats were given carbon tetrachloride (CCl_4) subcutaneously (sc) in the lower abdomen, in a suspension of liquid paraffin (1: 2 v/v) at the dose of 1 ml/kg BW on alternate days for a week and vehicle for three weeks orally.

Group 3: Rats were given carbon tetrachloride (CCl_4) sc, in a suspension of liquid paraffin (1: 2 v/v) at the dose of 1 ml/kg BW on alternate days for a week and after one hour Silymarin (50 mg/kg BW/ d)) for three weeks orally.

Group 4-6: Rats were given carbon tetrachloride (CCl_4) sc, in a suspension of liquid paraffin (1: 2 v/v) at the dose of 1 ml/kg BW on alternate days for a week and after one hour *tagetes erecta* Linn. (200 mg/kg, 400 mg/kg, 600 mg/kg BW/d) respectively for three weeks orally.

Different doses of above mentioned drug, LP and CCl_4 were administered to rats daily "between" 10.00 to 11.00 am.

Biochemical studies: The blood was obtained from all animals by puncturing retro-orbital plexus. The blood samples were allowed to clot for 45 min at room temperature. Serum was separated by centrifugation at 2500 rpm for 15 min and utilized for the estimation of various biochemical parameters namely SGOT [4], SGPT [4], SALP [5], serum bilirubin [6] total cholesterol [7] and total protein [8]. After collection of blood samples the rats in different groups were sacrificed and their livers were excised immediately and washed in ice cold normal saline, followed by 0.15 M Tris-Hcl (pH 7.4) blotted dry and weighed. A 10%w/v of homogenate was prepared in 0.15 M Tris-Hcl buffer and processed for the estimation of **lipid** peroxidation [9]. A part of homogenate after precipitating proteins with Trichloroacetic acid (TCA) was used for estimation of glutathione [10]. The rest of the homogenate was centrifuged at 1500 rpm for 15 min at 40 C. The supernatant thus obtained was used for estimation of SOD and CAT activities [11,12].

***Serum hepatospecific markers*:** Activities of serum glutamate oxaloacetate transaminase (SGOT) and serum glutamate pyruvate transaminase (SGPT) were estimated by the method of Reitman and Frankel. 0.05 ml of serum with 0.25 ml of substrate (aspartate and α-ketoglutarate for SGOT; alanine and α - keto glutarate for SGPT, in phosphate buffer pH 7.4) was incubated for an hour in case of SGOT and 30 min. for SGPT. 0.25 ml of DNPH solution was added to arrest the reaction and kept for 20 min in room temperature. After incubation 1 ml of 0.4 N NaOH was added and absorbance was read at 505 nm in *uv-vis* spectrophotometer. Activities were expressed as IU/dl.

Based on the method of King and Armstrong alkaline phosphatase activity was assayed using disodium phenyl phosphate as substrate. The colour developed was read at 510 nm in *uv-vis* spectrophotometer after 10 min. Activities of ALP was expressed as IU/dl. Serum total bilirubin level was estimated based on the method of Malloy and Evelyn Diazotised sulphonilic acid (0.25 ml) reacts with bilirubin in diluted serum (0.1 ml serum + 0.9 ml distilled water) and forms purple colored azobilirubin, which was measured at 540 nm in *uv-vis* spectrophotometer. Activities of total bilirubin were expressed as mg/dl. Total cholesterol was determined by method of Richmond .Serum total protein level was estimated based on the method of Gornall *et al* . Biuret reagent (1.0 ml) reacts with serum (10 µL) and the colour developed was read at 578 nm in *uv-vis* spectrophotometer. Activities of total protein were expressed as mg/dl.

Determination of Thiobarbituric Acid Reactive Substances (TBARS): Lipid peroxidations in liver tissues were estimated colorimetrically by measuring **thiobarbituric acid reactive substances (TBARS)** by the method of **Ohkawa** *et al* . To 0.2ml of sample, 0.2ml of 8.1% Sodium dodecyl sulfate, 1.5 ml of 20% acetic acid and 1.5 ml of 0.8% TBA were added. The volume of the mixture was made up to 4 ml with distilled water and then heated at 950 °C in a water bath for 60 min. After incubation the tubes were cooled to

room temperature and the final volume was made upto 5 ml in each tube. Then 5 ml of n-butanol: Pyridine mixture was added and the contents were vortexed thoroughly for 2 min. After centrifugation at 3000 rpm for 10 min the upper organic layer was taken and its OD was read at 532 nm against an appropriate blank without the sample.

Determination of reduced glutathione (GSH):
Reduced glutathione (GSH) was determined by the method of **Ellman**. To 0.1 ml of different tissue homogenate 2.4 ml of 0.02 M EDTA solution was added and kept on ice bath for 10 min. Then 2 ml of distilled water and 0.5 ml of 50 % TCA were added. This mixture was kept on ice for 10-15 min and then centrifuged at 3000 rpm for 15 min. 1 ml of supernatant was taken and 2ml of Tris-Hcl buffer was added. Then 0.05 ml of DTNB solution (Ellman's reagent) was added and vortexed thoroughly. OD was read (within 2-3 min after the addition of DTNB) at 412 nm against a reagent blank. Absorbance values were compared with a standard curve generated from known GSH.

Assay of super oxide dismutase (SOD): Superoxide dismutase (SOD) activity was determined by the reported method. Prepared 10 % w/v tissue homogenate in 0.15 M Tris HCl .Centrifuged at 15000 rpm for 15 min at 4 °C. Supernatant (0.1 ml) was taken consider it as sample and 1.2 ml sodium pyrophosphate buffer (pH 8.3, 0.052 M) + 0.1 ml phenazine methosulphate (186 µM) + 0.3 ml of 300 µM Nitroblutetrazolium + 0.2 ml NADH (750 µM) were added. Incubated at 30°C for 90 s .0.1 ml glacial acetic acid was added. Stirred with 4.0 ml n-butanol.Allowed to stand for 10 min Centrifuged and separated butanol layer. OD at 560 nm was taken (taken butanol as blank) and concentration of SOD was expressed as units/g of liver tissue. Absorbance values were compared with a standard curve generated from known SOD.

Assay of Catalase (CAT): Catalase was assayed according to the method of Aebi. The estimation was done spectrophotometrically following the decrease in absorbance at 240 nm. The liver tissue was homogenized in M/150 phosphate buffer (pH 7.0) at 1-40 C and centrifuged at 5000 rpm. The reaction mixture contained 0.01 M phosphate buffer (pH 7.0), 2 mM H_2O_2 and the enzyme extract. The specific activity of catalase was expressed in terms of units/gram of liver tissue. Absorbance values were compared with a standard curve generated from known CAT.

Histology: The tissues of liver were removed from animals, washed with normal saline to remove blood, fixed in 10% formalin and embedded in paraffin wax. Sections of 5 µm thickness were made using rotary microtome and stained with haematoxylin-eosin and histological observations were made under light microscope [13,14].

Statistical analyses: The experimental results were expressed as the Mean ± S.D for six animals in each group. Statistical analyses were performed using the unpaired t test. A p value of 0.05 or less was considered to indicate a significant difference between groups.

RESULTS

Effect of hydroalcholic extract of tagetes erecta Linn. on biochemical parameters.
Effects of extract on SGPT, SGOT, ALP and total bilirubin levels: Results presented in Table 1 indicate that the levels of serum enzymes namely SGPT, SGOT, ALP and total bilirubin levels were significantly ($p<0.0001$) increased in carbon tetrachloride treated group rats when compared with normal rats. However, treatments of rats with hydroalcoholic extract *tagetes erecta* Linn. (HATE) and silymarin, serum enzymes like SGPT, SGOT, ALP and total bilirubin levels were significantly ($p<0.05$) decreased when compared to carbontetrachloride treated rats only.

Effects of extract on MDA, GSH, SOD, CAT levels: To evaluate the protective effect of HATE on the liver oxidative damage in rats induced by CCl_4, we determined the primary antioxidant enzymes in liver. CCl_4 significantly ($P < 0.0001$) increased the concentrations of liver malondialdehyde (MDA) in CCl_4 treated group (Table 2), which was product of lipid peroxidation, compared to that in normal control group and the concentrations of liver MDA were dose-dependently reduced by the administration of HATE As shown in Table 2, Malondialdehyde (MDA) is the product of lipid peroxidation and is a common marker of lipid peroxidation. The content of MDA was significantly ($p<0.0001$) increased in the liver of CCl_4 (**6.82±0.085** U/mg tissue) treated rats as compared with the normal (**1.65±0.098** U/mg tissue) control group. Treatment with HATE at the doses 200, 400 and 600 mg/kg significantly ($p<0.05$) suppressed the formation of TBARS in the liver, suggestive of less oxidative damage of liver.

The effect of HATE on hepatic SOD activity is shown in table 2. SOD activity of the CCl_4 treated control group (**3.25±0.285** U/mg tissue) treated group was found to be significantly lower than the normal group (**12.85±0.696** U/mg tissue). However, a significant dose dependent reversal of

the SOD level was observed *tagetes erecta* extract treated groups.

TABLE 2 shows the effect of HATE on the content of GSH in CCl_4 induced hepatotoxicity in rats. The administration of CCl_4 significantly decreases the total glutathione content of the liver homogenate. CCl_4 administration with the tested compound significantly ($p<0.05$) inhibited the depletion of GSH compared to the control group.

Catalase activity in the liver homogenate is shown in table 2. CAT activity of the CCl_4 treated control group (**2.58±0.428** U/mg tissue) treated group was significantly ($p<0.0001$) lower than the normal group. The CAT activities in HATE and silymarin treated rats were significantly ($p<0.05$) higher compared to control group.

Effects of extract on total protein and total cholesterol: The rats that received CCl_4 showed a significant ($P<0.0001$) decrease total protein and significant increase in total cholestrol ($P<0.0001$) compared to control group. The administration of carbon tetrachloride induced a marked significant increase total cholesterol and significant decrease total protein levels in the blood as compared to normal control. However, the treatment of rats with HATE co dministration with hepatotoxicants caused a significant reduction in the levels of these markers in a dose dependent manner. The administration of the extract (200, 400 and 600 mg/ kg b.w) and silymarin (200 mg/kg b. w.) restored the levels of these parameters to normal levels in the blood.

DISCUSSION

CCl_4-induced hepatic injury is an experimental model widely used for the screening of hepatoprotective drugs. CCl_4 undergoes a biotransformation by hepatic microsomal cytochrome p450, to produce trichloromethyl free radicals. These hepatotoxic metabolites can react with protein and lipid in the membrane of cells or organelles leading to necrosis of hepatocytes. As a result of hepatic injury, the altered permeability of the membrane causes the enzymes from the cells to be released into the circulation. The magnitude of hepatic damage is usually assessed by measuring the level of released cytosolic transaminases including SGPT and SGOT in the circulation. The rise in the serum levels of ALP, SGPT and SGOT as observed in the present study could be attributed to the damaged structural integrity of the liver, because these are cytoplasmic in location and are released into circulation after cellular damage. Other researchers had reported that increased level of SGPT,SGOT ALP, bilirubin, cholesterol and decreased level of protein are due to CCl_4 hepatotoxicity [15]. In the present study, *tagetes erecta* extract when administered orally exhibited hepatoprotective actions. The evidence of hepatic damage was noted by the level of increased serum enzymes (SGPT, SGOT and ALP), serum bilirubin content, cholesterol, MDA and decrease protein, SOD, CAT GSH and by histopathological studies. The results of the present experiment are in agreement with results obtained in other experimental studies. The increase in LPO level in liver induced by CCl_4 suggests enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanism to prevent formation of excessive free radicals. Treatment with *tagetes erecta* Linn. significantly reverses these changes.

Decrease in enzyme activity of superoxide dismutase (SOD) is a sensitive index in hepatocellular damage and is the most sensitive enzymatic index in live injury. Curtis and Mortiz [16], has been reported SOD as one of the most important enzymes in the enzymatic antioxidant defense system. It scavenges the superoxide anion to form hydrogen peroxide and thus diminishing the toxic effect caused by this radical. In *tagetes erecta* Linn. causes a significant increase in hepatic SOD activity and thus reduces reactive free radical induced oxidative damage to liver.

Catalase (CAT) is an enzymatic antioxidant widely distributed in all animal tissues, and the highest activity is found in the red cells and liver. CAT decomposes hydrogen peroxide and protects the tissues from highly reactive hydroxyl radicals [17]. Therefore reduction in the activity of CAT may result in a number of deleterious effects due to the assimilation of superoxide radical and hydrogen peroxide.

Glutathione is one of the most abundant tripeptide, non-enzymatic biological antioxidant present in the liver. It removes free radical species such as hydrogen peroxide, superoxide radicals and maintains membrane protein thiols. Also it is substrate for glutathione [18]. Decreased level of GSH is associated with an enhanced lipid peroxidation in CCl_4 treated rats. Administration of *Tagetes erecta* Linn. significantly ($P<0.0001$) increased the level of GSH in a dose dependent manner.

Extensive vascular degenerative changes and centrilobular necrosis in hepatocytes was produced by CCl_4. Treatment with different doses of ethanolic extract of leaves of *Tagetes erecta* Linn. produced only mild degenerative changes and

absence of centrilobular necrosis, indicating its hepatoprotective efficiency.

CONCLUSION

In conclusion, our results of this study reported that hydoalcoholic extract of *tagetes erecta* was effective treatment for the control of hepatotoxicity induced by carbon terachloride. The degree of protection was measured by using biochemical parameters like serum transaminases, alkaline phosphatase, total protein, total cholestrol, bilirubin and antioxidant characters. The hydroalcoholic extracts showed the significant hepatoprotective activity comparable with standard drug silymarin. From this investigation, phenolic compounds of plant leaves may be responsible for the most hepatoprotective activity. Our results demonstrated that the plant derived drugs is the best alternative drug for synthetic or chemical drug.

Table : 1 Effect of hydroalcholic extract of *tagetes erecta* Linn. on serum, SGPT, SGOT, ALT and Total bilirubin on carbon tetrachloride induced hepatotoxicity in rats:

GROUPS	Treatment	SGPT(IU/dl)	SGOT(IU/dl)	ALP(IU/dl)	TOTAL BILIRUBIN (mg/dl)
I	Vehicle	4 1.2±2.789	56.2±3.856	105.8±4.985	0.58±0.029
II	CCl_4 control	97.2±6.986***	129.9±10.856***	228.5±8.982***	1.90±0.021***
III	Silymerin_200mg/kg	$55.4\pm9.286^{+++}$	$68.7\pm7.828^{+++}$	$150.4\pm9.186^{+++}$	$0.95\pm0.030^{+++}$
IV	HATE-200mg/kg	93.8 ± 8.209^{ns}	$108.3\pm9.920^{+}$	220.2 ± 7.280^{ns}	1.84 ± 0.081^{ns} 0
V	HATE-400mg/kg	$87.2\pm7.108^{+}$	$110.4\pm6.280^{+}$	$200.9\pm8.568^{++}$	$1.78\pm0.091^{+}$
VI	HATE-600mg/kg	$75.0\pm6.249^{++}$	$98.5\pm8.920^{++}$	$198.6\pm9.982^{++}$	$1.69\pm0.097^{++}$

All values are represents mean ± SD; n = 6 animals.
P values: ***‹ 0.0001 when compared with control untreated rats; +++ ‹ 0.0001; ++ ‹ 0.001; +<0.05 when compared with carbon tetrachloride treated rats.

Table: 2 Effect of hydroalcholic extract of *tagetes erecta* Linn. on SOD, GSH, LPO and Catalase in carbon tetrachloride induced hepatotoxicity in rats:

GROUPS	Treatment	SOD (unit/mg tissue)	GSH (mmol/mgtissue)	LPO (nmolMDA/mgtissue)	CATALASE (unit/mgtissue)
I	Vehicle	12.85±0.696	5.02±0.289	1.65±0.098	15.01±0.698
II	CCl_4 control	3.25±0.285***	1.20±0.382***	6.82±0.085***	2.58±0.428***
III	Silymerin(200mg/kg)	$11.02\pm0.386^{+++}$	$4.85\pm0.308^{+++}$	$1.98\pm0.213^{+++}$	$14.52\pm0.490^{+++}$
IV	HATE-200mg/kg	3.45 ± 0.156^{ns}	$1.65\pm0.208^{+}$	6.75 ± 0.290^{ns}	$3.25\pm0.258^{+}$
V	HATE-400mg/kg	$3.86\pm0.405^{+}$	$1.95\pm0.286^{+}$	$6.50\pm0.180^{++}$	$4.08\pm0.785^{+}$
VI	HATE-600mg/kg	$4.01\pm0.508^{+}$	$2.01\pm0.256^{++}$	$6.35\pm0.220^{++}$	$5.25\pm0.565^{+++}$

All values are represents mean ± SD; n = 6 animals.
P values: ***‹ 0.0001 when compared with control untreated rats; +++ ‹ 0.0001; ++ ‹ 0.001; +<0.05 when compared with carbon tetrachloride treated rats.

Table: 3 Effect of hydroalcholic extract of *Tagetes erecta* Linn. on serum Total Protein and Total Cholesterol on carbon tetrachloride induced hepatotoxicity in rats:

GROUPS	Treatment	Total Protein (µg/mg)	Total Cholestrol(mg/dl)
I	Vehicle	95.8±3.281	95.2±5.686
II	CCl_4 control	50.2±4.185***	185.6±10.980***
III	Silymerin(200mg/kg)	$89.1\pm5.198^{+++}$	$109.2\pm7.890^{+++}$
IV	HATE-200mg/kg	52.2 ± 4.102^{ns}	$173.1\pm6.108^{+}$
V	HATE-400mg/kg	$58.1\pm3.280^{+}$	$169.4\pm5.947^{+}$
VI	HATE-600mg/kg	$62.8\pm3.954^{++}$	$154.6\pm6.809^{++}$

All values are represents mean ± SD; n = 6 animals.

P values: ***‹ 0.0001 when compared with control untreated rats; +++ ‹ 0.0001; ++ ‹ 0.001; +<0.05 when compared with carbon tetrachloride treated rats.

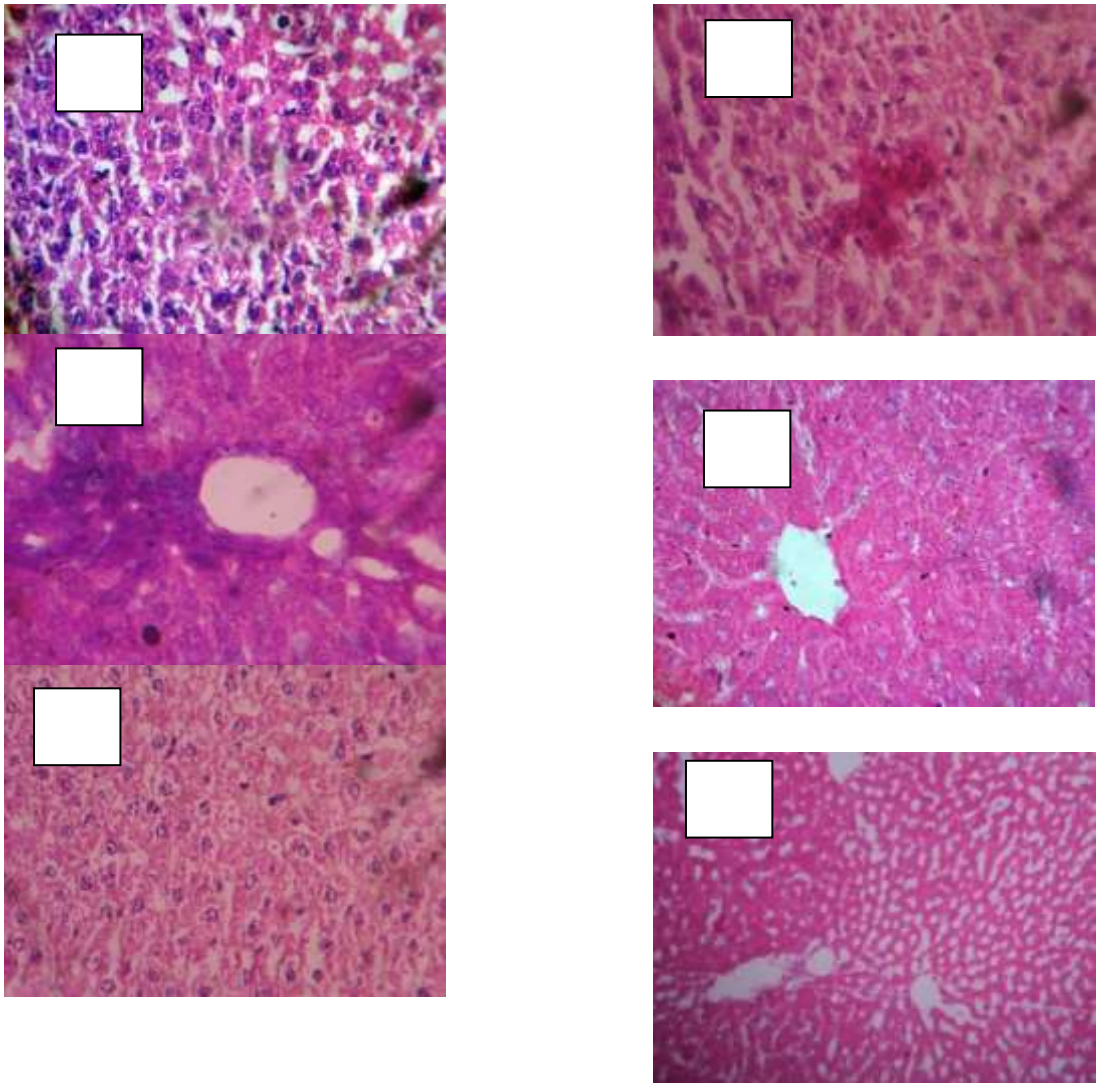

Fig. 1: Histopathalogical monograph of extract and standard. a: Control; b: CCl_4 (1 ml/kg) alone; c: CCl_4+ *Tagetes erecta* (1ml/kg +200 mg/kg); d: CCl_4 + *Tagetes erecta (* 1 ml/kg+400 mg/kg); e: CCl_4+ *Tagetes erecta* (1ml/kg+600 mg/kg); f: CCl_4+Silymarin (1ml/kg+ 200 mg/kg).

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