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Acute, Sub-Acute and Genotoxicity Assessment of *Cocculus hirsutus* Hydroalcoholic Leaf Extract in Wistar Rats

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Abstract

Acute (0, 500, 1000 and 2000 mg/kg) and 28-day (0, 100, 200 and 400 mg/kg) toxicity assessments of *Cocculus hirsutus* hydroalcoholic leaf extract (CH-Ex) were performed in Wistar rats consistent with OECD guidelines 423 and 407 respectively. Average body weights were recorded, blood and biochemical parameters were analyzed and histopathological studies were performed. At doses (12.5–100 µg/ml), genotoxicity assessments were conducted on pUC 19 plasmid by evaluating DNA damage protection. Acute toxicity assessment categorized CH-Ex as safe and non-toxic (category 5). The 28-day toxicity assessments showed no significant ($p>0.05$) changes in body weights, hematology and histopathology. At 400 mg/kg females exhibited a slight decline in SGPT, creatinine and BUN levels, while male rats exhibited a slight enhancement of SGOT and ALP levels ($p<0.05$). CH-Ex exhibited strong ability to protect DNA damage from oxidative stress in a dose-dependent manner.

Keywords: Biochemical, *Cocculus hirsutus*, DNA Damage, Histopathological, Oxidative Stress, Toxicity

1. Introduction

The *Cocculus hirsutus* (Menispermaceae), usually referred to as "Patalgarudi", grows in India and many other countries in South Asia, that has been a long-standing medicinal creeper to treat many ailments[1]. The shrub climbs up to 3 meters with simple, alternate, sub deltoid and mucronate leaves[2]. The phytochemicals of *C. hirsutus* are pharmacologically active materials with considerable medicinal and preventive uses[3]. Biologically active phytochemical constituents present in *C. hirsutus* include alkaloids, flavonoids, phenols, glycosides, sterols, resins and carbohydrates[4]. The leaves of *C. hirsutus* confirmed to have D-trilobine, isotrilobine, shaheenine, jamtinine, (+) syringaresinol, hirsutine, protoquericitol, jamatine-N-oxide and haiderine[5–7].

The leaves and roots of *C. hirsute* sare primarily used in traditional medicine for the treatment of diabetes, kidney problems, hepatic disorders, leprosy, malaria, acute gonorrhea, eczema, dyspepsia, rheumatism and syphilitic cachexia[8,9]. In vivo assessments of various parts of *C. hirsutus* have been reported for antioxidant, anti-tumor[10], antidiabetic[11], hepatoprotective[12], diuretic[13], spermatogenic[14], nephroprotective[15], immunostimulant[16], antimalarial[17], analgesic and anti-inflammatory[18]. The parts of this medicinal plant have a constrained safety assessment. Therefore, the safety and quality need to be standardized. The goal of our current pharmacological research was to analyze acute and 28-day oral toxicity of *C. hirsutus* leaf hydroalcoholic extract in Wistar rats and assessed genotoxicity using pUC 19 plasmid DNA.

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2. Material and Methods

2.1 Collection of Leaves and Preparation of Extract

The fresh leaves of *C. hirsutus* were procured from Visakhapatnam district of Andhra Pradesh, India in February 2020, identified and authenticated by Department of Botany, Andhra University, Visakhapatnam, Andhra Pradesh, India. The fresh leaves were collected, dried under shade conditions and powdered mechanically. CH-Ex was extracted with (30:70) water and ethanol by using Soxhlet extraction for 48 hr. The extracted liquid was concentrated by placing on water bath, desiccated overnight at room temperature and the final CH-Ex was kept in the refrigerator at 3 ± 1^{0}C. The yield of the hydroalcoholic extract was 11.40% W/W.

2.2 Proximate Analysis

For present study healthy Wistar albino rats of both genders weighing 120 ± 20 g was used. The animals were held in typical propylene cages and were maintained in regulated environmental conditions at 25 ± 2°C temperature, 30-60%. Humidity and 12 hr cycles of light/dark. With typical pellet diet and water *ad libitum*, they were accustomed to hygienic laboratory environment for 2 weeks. The Institutional Animal Ethical Committee (IAEC) of Vignan Institute of Pharmaceutical Technology under registration number No. 2003/PO/Re/S/18/CPCSEA, dated 09/02/2018 approved the research protocol and all the studies were performed with the IAEC and CPCSEA guidelines and regulations.

2.3 Acute Oral Toxicity Assessment

The acute oral toxicity assessments were performed in compliance with OECD guidelines 423[19,20]. Briefly, 20 healthy, nulliparous and non-pregnant young female rats were randomly selected and convened into four groups (n = 5). Prior to the treatment, all the rats were maintained at room temperature 22 ± 3 and acclimatized for 7 days to the laboratory environment. All the animals were fasted overnight prior to the treatment and the doses were calculated according to their body weights.

The predetermined dose levels of 0 (Group I), 500 (Group II), 1000 (Group III) and 2000 (Group IV) mg/kg CH-Ex was administered orally in a unit dose to the rats with the help of oral intubation canula. The treated animals were assessed individually for mortality, toxicity, physiological, behavioral and neurological changes continuously for 3 h, periodically throughout the first 24 h and subsequently daily for a total period of 2 weeks. During the assessment period, body weights were recorded on day 1, day 7 and day 14 for physical examination. Using diethyl ether, all the treated rats were anesthetized, sacrificed and the vital organs were assessed for any histopathological changes through gross pathological examination.

2.4 Subacute Oral Toxicity Assessment

The subacute oral toxicity assessments were performed for 28 days in accordance with OECD guidelines 407 with minor modifications[21,22]. For this assessment study, 24 healthy young rats of either sex (12 males, 12 females) weighing around 120 ± 8 g were randomly selected and convened into four groups (n = 6), comprising 3 males and 3 females each. The first group was treated with distilled water (10 ml/kg) for four weeks and served as the control group. Remaining three groups (Groups II, III and IV) were treated daily with fixed dose levels of 100, 200 and 400 mg/kg CH-Exby oral gavage for a period of four weeks (28 days).

All the treated groups were kept under clinical observation two times a day for any signs of toxicity and the body weights were monitored for every 7 days interval for four weeks. At the end of assessment period, overnight fasted rats were anesthetized by means of diethyl ether and the blood samples were collected from the carotid artery in both heparin-coated and uncoated microcentrifuge tubes for hematological and biochemical estimations. Further the animals were sacrificed by decapitation and the vital organs such as liver and kidneys were dissected out for histopathological examination.

2.5 Hematological Assessment

Blood samples collected in the heparin-coated tubes were used to assess various hematological parameters[23] such as erythrocyte count (RBC), leucocyte count (WBC), Differential Leucocyte Count (DLC), hematocrit, Red Cell Distribution Width (RCDW), Mean Corpuscular Volume (MCV), hemoglobin estimation, Mean Corpuscular Hemoglobin (MCH), platelet count, Mean Corpuscular Hemoglobin Concentration (MCHC) and Mean Platelet Volume (MPV) using hematology analyzer (Priecount, Robonik, India).

2.6 Biochemical Assessment

Blood samples collected in the uncoated tubes were centrifuged at 3000 rpm at 5 for 10 min and the obtained plasma was used to assess biochemical parameters[24] such as total protein, Blood Urea Nitrogen (BUN), glucose, creatinine, Serum Glutamate-pyruvate Transaminase (SGPT), Serum Glutamic-oxaloacetic Transaminase (SGOT), total bilirubin, conjugated bilirubin, Alkaline Phosphatase (ALP), urea, uric acid, triglycerides and total

cholesterol using automated biochemistry analyzer (Prietest touch plus, Robonik, India).

2.7 Histopathological Assessment

Subsequently, the dissected vital organs were fixed overnight in 10% solution of neutral buffered formalin at 37. After fixation, they were washed, dehydrated overnight and mounted in paraffin wax blocks. The tissue blocks were sectioned into 5-7 µm ribbons and were stained with hematoxylin and eosin (H and E). The properly stained sections were used for assessment of histopathological changes by using an optical microscope[25].

2.8 Genotoxicity Assessment

Genotoxicity assessments of CH-Ex were conducted on pUC 19 plasmid by evaluating DNA damage protection from oxidative stress[26]. The 20 µL reaction mixture contains different concentration of CH-Ex (12.5, 25, 50 and 100 µg/ml), 3 µL of pUC 19 plasmid DNA, 10 µL of Fenton's reagent and distilled water to adjust final volume. After 30 min of incubation at 37, 0.25% bromophenol in 50% glycerol was added, subjected to 0.8% agarose gel electrophoresis at 90 V for 1 hr followed by ethidium bromide staining and photos of DNA bands were collected under gel analyzer.

2.9 Statistical Data Analysis

The results were stated as mean±SEM. Statistical difference was calculated by using one-way ANOVA followed by post hoc Dunnett's multiple comparison test at p>0.05. The statistical analysis was carried out using GraphPad Prism version 5.0.

3. Results

3.1 Acute Toxicity Study of CH-Ex

The acute toxicity study of CH-Ex at graded doses of 500, 1000 and 2000 mg/kg body weight in treated rats showed no signs of toxicity and mortality. No noticeable physiological, behavioral and neurological changes were observed in tested groups compared with the control (Group I) during the study period of 14 days. No gross variation in pathological findings was observed in treated groups. As a result, the CH-Ex (LD_{50}>2000 mg/kg) can be graded in the class of lower toxic substances.

Steady increase in the body weight was recorded in both control and treated groups. The data indicated that there was no significant difference in the mean body weights of CH-Ex treated groups when compared with each other and with the control (p>0.05). Average weight gain in control (Group I) and treated (Groups II, III and IV) groups recorded as 19.74 g, 18.23 g, 17.59 g and 19.45 g respectively (Table 1). The gross pathological examination on the vital organs (liver and kidneys) of the treated rats revealed that there was no substantial change in the physical parameters such as size, shape, color and texture (morphological analysis) when compared with each other and with the control (p>0.05). At the end of the study period, the mean weights of the liver were 5.81 ± 0.10 g (500 mg/kg), 5.99 ± 0.03 g (1000 mg/kg) and 5.92 ± 0.30 g (2000 mg/kg) compared with the control group (5.39 ± 0.05 g). The average weight of the kidneys was 1.53 ± 0.12 g (500 mg/kg), 1.71 ± 0.24 g (1000 mg/kg) and 1.63 ± 0.26 g (2000 mg/kg) compared with the control group (1.35 ± 0.08 g) (Figure 1).

Table 1. Effect of CH-Ex on body weights of female rats during acute oral toxicity study

Group	Treatment	Body weight (g)		
		Day 1	Day 7	Day 14
Group I	Control	132.37 ± 1.79[a]	140.07 ± 0.50[a]	152.11 ± 2.65[a]
Group II	500 mg/kg	139.01 ± 2.55[a]	148.00 ± 3.05[a]	157.24 ± 3.40[a]
Group III	1000 mg/kg	137.48 ± 2.01[a]	144.96 ± 3.50[a]	155.07 ± 2.68[a]
Group IV	2000 mg/kg	138.80 ± 2.53[a]	147.92 ± 3.28[a]	158.25 ± 2.70[a]

Result data are expressed as Mean±SEM, n = 5 rats/group.
Mean separation by Dunnett's multiple comparison test (P>0.05).
Means within the column followed by same superscript are not statistically different.

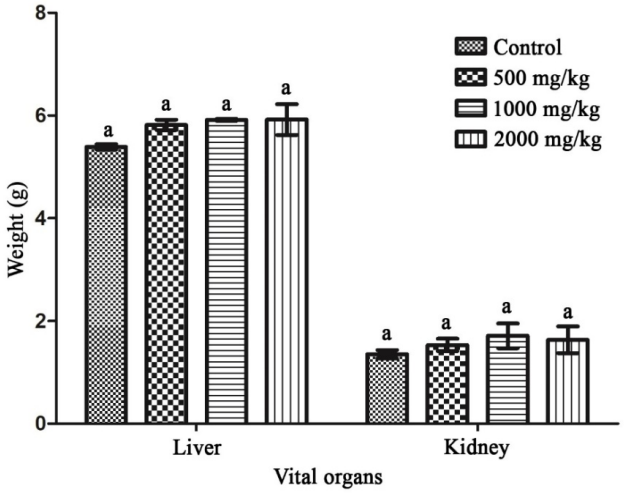


Figure 1. Effect of CH-Ex on organ weights of rats during acute oral toxicity study. Result data are mean ± standard error of the mean of quintuplet measurements. [a]Differences within-groups (Means followed the same letter are not significantly different) (p>0.05).

3.2 Subacute Toxicity Study of CH-Ex

3.2.1 *Effect of CH-Ex on Body Weights*

Oral administration of CH-Ex at predetermined dose levels of 100, 200 and 400 mg/kg body weight did not show any toxic signs and death in CH-Ex treated rats throughout the assessment period. However, no significant difference (p>0.05) recorded in the average body weight of CH-Ex treated rats (both sexes) when compared with the control (Table 2).

3.2.2 *Effect of CH-Ex on Hematological Parameters*

In the assessment of CH-Ex (100, 200 and 400 mg/kg) daily administration for 28 days, various hematological parameters have been in the normal range in rats. The result shows no significant difference (p>0.05) between the CH-Ex treated and control groups of both sexes (Table 3).

Table 2. Effect of CH-Ex on body weights of rats during subacute oral toxicity study

Treatment groups	Body weight (g)				
	Day 1	Day 7	Day 14	Day 21	Day 28
Male					
Control	117.80 ± 4.46[a]	128.15 ± 3.26[a]	168.38 ± 2.10[a]	188.55 ± 1.89[a]	217.82 ± 2.97[a]
100 mg/kg	115.53 ± 6.53[a]	134.27 ± 2.52[a]	164.71 ± 2.64[a]	194.25 ± 1.99[a]	229.16 ± 3.14[a]
200 mg/kg	122.36 ± 3.56[a]	144.49 ± 2.83[a]	159.89 ± 1.05[a]	193.32 ± 2.35[a]	225.12 ± 1.85[a]
400 mg/kg	119.78 ± 3.16[a]	139.94 ± 3.01[a]	165.70 ± 0.41[a]	190.30 ± 2.11[a]	219.91 ± 3.29[a]
Female					
Control	124.79 ± 2.86[b]	137.53 ± 1.47[b]	155.48 ± 1.56[b]	161.46 ± 4.33[b]	172.85 ± 2.06[b]
100 mg/kg	118.50 ± 1.29[b]	133.90 ± 0.17[b]	159.16 ± 3.96[b]	169.60 ± 3.65[b]	180.10 ± 3.34[b]
200 mg/kg	125.73 ± 2.53[b]	138.68 ± 0.98[b]	158.41 ± 5.14[b]	179.34 ± 3.31[b]	189.78 ± 2.03[b]
400 mg/kg	122.25 ± 3.19[b]	141.93 ± 1.85[b]	160.73 ± 1.93[b]	174.91 ± 3.35[b]	184.54 ± 2.09[b]

Outcomes are expressed as Mean±SEM, n = 3 rats/group.
Mean separation by Dunnett's multiple comparison test (P>0.05).
Means within the column followed by same superscript are not statistically different.

3.2.3 *Effects of CH-Ex on Biochemical Parameters*

In toxicological assessment of CH-Ex (100, 200 and 400 mg/kg) daily administration for 28 days, serum biochemical estimations have substantial role as indicator of toxic effects in tissues of various vital organs. The outcomes of all biochemical assessments showed no significant difference (p>0.05) between the treated and control groups of both sexes except, at 400 mg/kg females exhibited a slight decline in SGPT, creatinine and BUN levels (p<0.05), while male rats exhibited a slight increase in SGOT and ALP levels (p<0.05) (Table 4).

Table 3. Effect of CH-Ex on hematological parameters in rats during subacute oral toxicity study

Hematological parameters	Treatment groups			
	Control	100 mg/kg	200 mg/kg	400 mg/kg
Male				
RBC ($x10^{12}$/L)	7.80 ± 0.25[a]	7.48 ± 0.08[a]	8.01 ± 0.13[a]	7.86 ± 0.15[a]
WBC ($x10^{9}$/L)	10.77 ± 0.23[a]	9.88 ± 0.29[a]	10.27 ± 0.72[a]	10.23 ± 0.60[a]
Platelet count ($x10^{9}$/L)	884.48 ± 82.08[a]	816.45 ± 162.25[a]	995.40 ± 175.70[a]	978.03 ± 153.77[a]
Mean platelet volume (fL)	9.67 ± 0.02[a]	9.74 ± 0.01[a]	9.63 ± 0.04[a]	9.74 ± 0.01[a]
Hematocrit (%)	48.38 ± 0.36[a]	47.93 ± 0.56[a]	48.67 ± 0.24[a]	49.45 ± 0.60[a]
RCDW (%)	13.78 ± 0.15[a]	13.38 ± 0.49[a]	14.36 ± 0.34[a]	13.83 ± 0.15[a]
MCV (fL)	58.89 ± 1.76[a]	61.59 ± 1.48[a]	63.52 ± 1.64[a]	59.47 ± 1.47[a]
Hemoglobin (g/dL)	15.52 ± 0.21[a]	15.59 ± 0.58[a]	15.49 ± 0.26[a]	15.84 ± 0.10[a]
MCH (pg)	19.70 ± 0.24[a]	18.91 ± 0.54[a]	19.88 ± 0.36[a]	18.87 ± 0.12[a]
MCHC (g/dL)	31.73 ± 0.18[a]	31.03 ± 0.33[a]	31.81 ± 0.13[a]	32.70 ± 0.30[a]
Basophils (%)	0.48 ± 0.07[a]	0.53 ± 0.04[a]	0.57 ± 0.08[a]	0.47 ± 0.02[a]
Eosinophils (%)	0.89 ± 0.03[a]	0.98 ± 0.06[a]	0.84 ± 0.05[a]	0.95 ± 0.01[a]
Neutrophils (%)	11.71 ± 2.05[a]	14.80 ± 0.79[a]	13.02 ± 1.53[a]	13.60 ± 1.78[a]
Monocytes (%)	1.52 ± 0.07[a]	1.73 ± 0.16[a]	1.42 ± 0.16[a]	1.38 ± 0.20[a]
Lymphocytes (%)	85.07 ± 1.31[a]	82.02 ± 1.73[a]	84.15 ± 2.36[a]	83.62±3.00[a]
Female				
RBC ($x10^{12}$/L)	7.91 ± 0.10[b]	8.11 ± 0.14[b]	8.10 ± 0.24[b]	7.97 ± 0.32[b]
WBC ($x10^{9}$/L)	8.46 ± 0.42[b]	7.98 ± 0.50[b]	8.61 ± 0.31[b]	8.37 ± 0.88[b]
Platelet count ($x10^{9}$/L)	850.08 ± 116.29[b]	838.32 ± 84.52[b]	844.75 ± 121.07[b]	921.70 ± 28.46[b]
Mean platelet volume (fL)	8.68 ± 0.02[b]	8.67 ± 0.02[b]	8.76 ± 0.02[b]	8.73 ± 0.02[b]
Hematocrit (%)	46.73 ± 0.28[b]	45.78 ± 0.47[b]	47.64 ± 0.32[b]	46.92 ± 0.10[b]
RCDW (%)	13.63 ± 0.26[b]	13.89 ± 0.14[b]	14.00 ± 0.12[b]	13.57 ± 0.06[b]
MCV (fL)	57.21 ± 1.49[b]	59.03 ± 0.83[b]	56.05 ± 1.15[b]	59.85 ± 0.80[b]
Hemoglobin (g/dL)	14.74 ± 0.19[b]	13.89 ± 0.18[b]	14.87 ± 0.11[b]	13.78 ± 0.68[b]
MCH (pg)	17.86 ± 0.11[b]	18.50 ± 0.29[b]	18.01 ± 0.10[b]	17.74 ± 0.25[b]
MCHC (g/dL)	28.63 ± 0.52[b]	29.72 ± 0.20[b]	29.39 ± 0.83[b]	29.79 ± 0.20[b]
Basophils (%)	0.38 ± 0.01[b]	0.29 ± 0.02[b]	0.46 ± 0.02[b]	0.39 ± 0.04[b]
Eosinophils (%)	1.25 ± 0.04[b]	1.04 ± 0.05[b]	1.13 ± 0.17[b]	1.11 ± 0.10[b]
Neutrophils (%)	11.86 ± 1.69[b]	10.87 ± 2.09[b]	12.13 ± 1.72[b]	12.12 ± 2.40[b]
Monocytes (%)	1.79 ± 0.09[b]	1.71 ± 0.37[b]	1.63 ± 0.23[b]	1.88 ± 0.13[b]
Lymphocytes (%)	84.72 ± 0.69[b]	86.26 ± 1.32[b]	85.72 ± 1.44[b]	84.87 ± 1.17[b]

Outcomes are expressed as Mean±SEM, n = 3 rats/group. Mean separation by Dunnett's multiple comparison test (P>0.05).
Means within the row followed by same superscript are not statistically different.
RBC: Red blood cells, WBC: White blood cells, RCDW: Red cell distribution width, MCV: Mean corpuscular volume, MCHC: Mean corpuscular hemoglobin concentration.

Table 4. Effect of CH-Ex on serum biochemical parameters in rats during subacute oral toxicity study

Serum biochemical parameters	Treatment groups			
	Control	100 mg/kg	200 mg/kg	400 mg/kg
Male				
SGOT (U/L)	175.78 ± 17.42[a]	176.76 ± 22.50[a]	173.31 ± 14.78[a]	246.12 ± 10.87[c]
SGPT (U/L)	55.25 ± 4.36[a]	64.25 ± 7.28[a]	68.84 ± 4.79[a]	65.39 ± 4.46[a]
ALP (U/L)	251.56 ± 9.14[a]	255.73 ± 22.93[a]	267.11 ± 24.26[a]	322.74 ± 18.30[c]
Total protein (g/dL)	6.28 ± 0.12[a]	6.68 ± 0.49[a]	6.29 ± 0.24[a]	6.59 ± 0.25[a]
Total bilirubin (µmol/L)	15.08 ± 1.85[a]	16.43 ± 2.24[a]	13.65 ± 1.38[a]	17.42 ± 0.93[a]
Conj. bilirubin (µmol/L)	6.39 ± 1.04[a]	6.13 ± 0.13[a]	7.30 ± 0.59[a]	6.54 ± 0.61[a]
Urea (mmol/L)	4.82 ± 0.21[a]	6.17 ± 0.43[a]	5.41 ± 0.78[a]	5.33 ± 0.30[a]
Creatinine (mg/dL)	0.42 ± 0.02[a]	0.48 ± 0.01[a]	0.46 ± 0.01[a]	0.43 ± 0.02[a]
Uric acid (mmol/L)	0.15 ± 0.01[a]	0.17 ± 0.02[a]	0.13 ± 0.03[a]	0.18 ± 0.03[a]
BUN (mg/dL)	13.92 ± 0.38[a]	12.85 ± 1.19[a]	14.25 ± 1.00[a]	14.65 ± 1.54[a]
Glucose (mg/dL)	110.02 ± 10.67[a]	123.26 ± 10.38[a]	106.29 ± 12.46[a]	115.68 ± 16.23[a]
Triglycerides (mg/dL)	21.51 ± 3.06[a]	22.35 ± 5.58[a]	19.94 ± 5.14[a]	24.01 ± 7.61[a]
Total cholesterol (mg/dL)	62.41 ± 7.35[a]	68.41 ± 10.36[a]	57.69 ± 8.39[a]	65.13 ± 5.80[a]
Female				
SGOT (U/L)	234.26 ± 23.71[b]	250.93 ± 18.10[b]	272.49 ± 12.54[b]	266.78 ± 12.21[b]
SGPT (U/L)	70.30 ± 3.25[b]	69.82 ± 3.02[b]	78.96 ± 4.04[b]	52.61 ± 2.30[c]
ALP (U/L)	157.36 ± 11.87[b]	149.92 ± 15.40[b]	155.40 ± 12.47[b]	145.19 ± 10.94[b]
Total protein (g/dL)	6.19 ± 0.25[b]	6.59 ± 0.21[b]	6.37 ± 0.40[b]	6.49 ± 0.25[b]
Total bilirubin (µmol/L)	22.02 ± 1.71[b]	18.70 ± 2.16[b]	25.02 ± 2.83[b]	23.24 ± 1.40[b]
Conj. bilirubin (µmol/L)	8.17 ± 0.56[b]	7.48 ± 0.80[b]	8.17 ± 0.75[b]	7.66 ± 0.99[b]
Urea (mmol/L)	6.78 ± 0.33[b]	5.90 ± 0.26[b]	6.43 ± 0.36[b]	5.74 ± 0.63[b]
Creatinine (mg/dL)	0.42 ± 0.02[b]	0.47 ± 0.02[b]	0.49 ± 0.02[b]	0.34 ± 0.01[c]
Uric acid (mmol/L)	0.22 ± 0.03[b]	0.17 ± 0.04[b]	0.23 ± 0.02[b]	0.19 ± 0.02[b]
BUN (mg/dL)	18.22 ± 2.21[b]	19.59 ± 3.09[b]	18.77 ± 2.02[b]	11.55 ± 0.50c
Glucose (mg/dL)	117.12 ± 16.63[b]	108.31 ± 8.11[b]	99.59 ± 14.12[b]	111.96 ± 7.17[b]
Triglycerides (mg/dL)	12.69 ± 2.35[b]	11.47 ± 3.54[b]	16.89 ± 4.26[b]	14.42 ± 2.24[b]
Total cholesterol (mg/dL)	70.29 ± 4.35[b]	68.13 ± 4.73[b]	73.35 ± 4.99[b]	65.22 ± 6.65[b]

Result data represented as Mean±SEM, n = 3 rats/group.
Mean separation by Dunnett's multiple comparison test (P>0.05).
Means within the row followed by same superscript are not statistically different.
SGOT: Serum glutamic-oxaloacetic transaminase, SGPT: Serum glutamate-pyruvate transaminase, ALP: Alkaline phosphatase, BUN: Blood urea nitrogen.

3.2.4 *Effects of CH-Ex on Histopathology of Vital Organs*

Histopathological optical microscopic assessment of rats (both sexes) treated with CH-Ex (400 mg/kg body weight) showed no substantial histological and pathological changes in the HE stained sections of liver and kidney when compared with the control. No morphological variations, abnormalities or lesions were seen in the liver and kidney sections under light microscope (Figure 2).

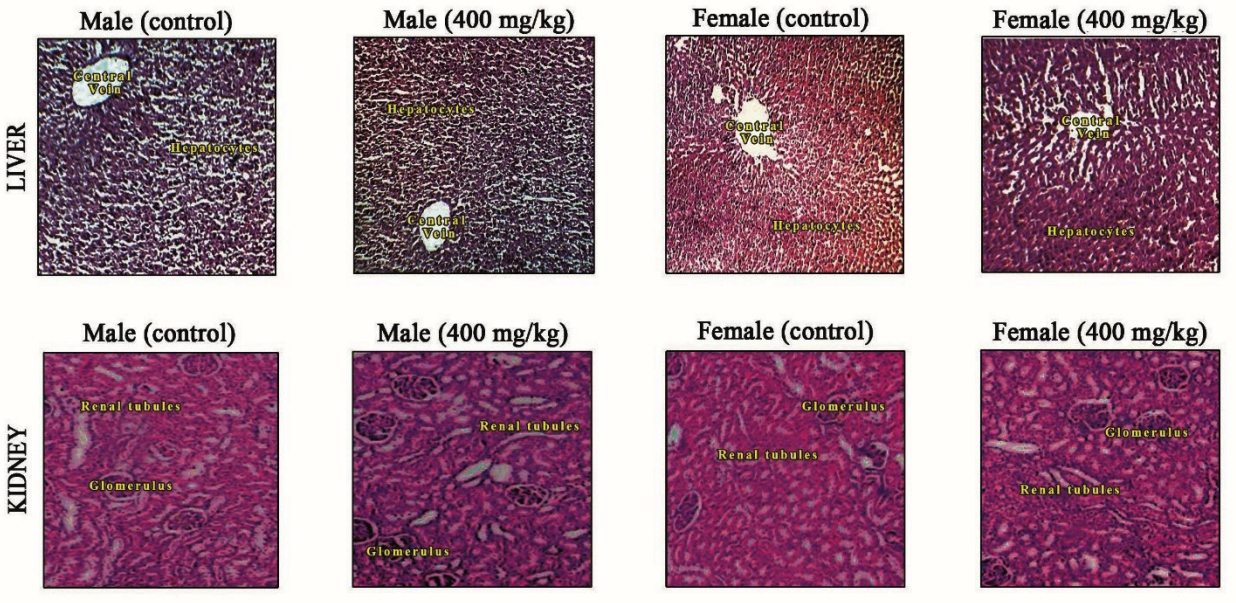


Figure 2. Photomicrographs of the sections liver and kidney of rats treated with control and CH-Ex over a period of 28 days (H-E staining, original magnification 100x).

3.3 Genotoxicity Assessment of CH-Ex

Genotoxicity assessments were based on the ability of CH-Ex to protect strand breaks in plasmid DNA due to generation of ROS-hydroxyl radical by Fenton's reagent. Studies on pUC 19 plasmid treated with CH-Ex (12.5, 25, 50 and 100 µg/ml) protect DNA damage in the dose-dependent manner (Figure 3).

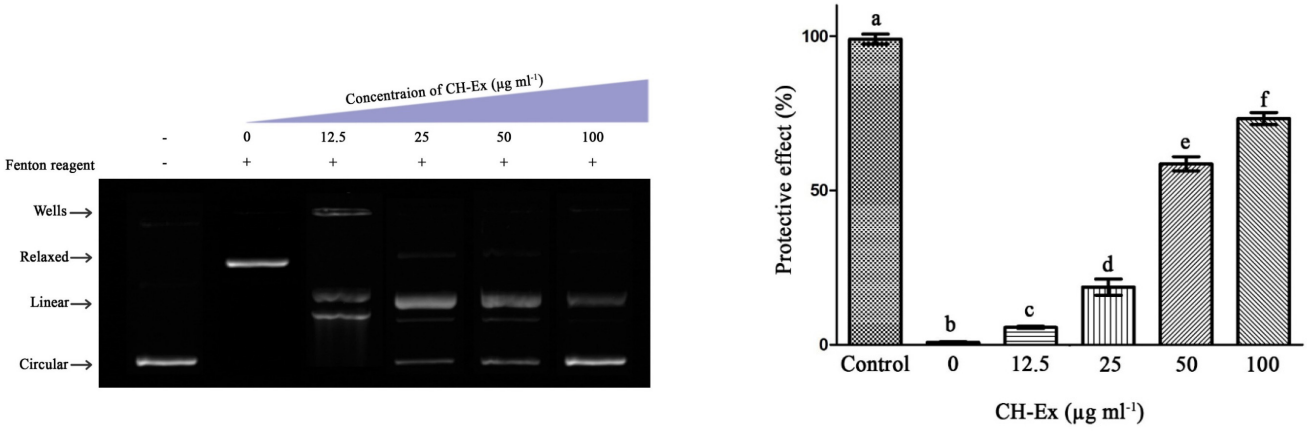


Figure 3. Protective effect of CH-Ex against pUC 19 plasmid DNA damage induced by Fenton reaction. Data are Mean±SEM, n = 3 rats/group. [a-f]Differences within-samples (Means not followed by the same letter are significantly different) ($p<0.05$).

4. Discussion

Remedial plants have been gaining courtesy for decades as an alternative source of medication to cure a multitude of diseases around the world[27]. Even in developed nations, the use of natural remedies has enormously increased[28]. Preclinical toxicity screening of natural compounds is very important for the drug development process[29]. Animal model toxicity tests were carried out to assess the safety and to determine the optimal dose of a drug for disease treatment[30]. Toxicological studies in animals, rodents as well as non-rodents are extremely important to determine the protection and effectiveness of biological extract, according to OECD guidelines. To evaluate the safety of broad spectrum of natural materials for medical use, there is continuing assessment of acute and 28-day toxic effects of natural remedies[31]. In present study, oral toxicity of hydroalcoholic extract of *C. hirsutus* leaf studied for acute and 28 days oral toxicity in Wistar rat and assessed genotoxicity using pUC 19 plasmid DNA.

Acute toxicity assessments are performed to assess the immediate toxic properties of the extract when given in a single dose in rodents by individually assessing mortality, toxicity, physiological, behavioral, neurological and gross histological changes[32]. The assessment of acute toxicity by oral administration of CH-Ex up to 2000 mg/kg exhibited no significant ($p>0.05$) toxic effects in the rats. In accordance with OECD guidelines 423, the CH-Ex (LD_{50}>2000 mg/kg) can be graded in the class of lower toxic substances. Acute treatment did not have any significant ($p>0.05$) impact on body weight and organ weights of both sexes. Therefore, CH-Ex can be indicated to be non-toxic via oral route.

The subacute toxicity tests are intended to evaluate the lethal effects of an extract when given repeatedly in rodents for 28 days by evaluating body weights, blood and biochemical parameters, histological and pathological changes[33]. The 28-day toxicity assessment presented that the growth pattern of rats was stable with consistent growth in their body weight. The monitored body weights for every 7 days exhibited no significant ($p>0.05$) change compared to the control in both sexes. Hematological parameters played a vital role in the evaluation of toxicity[34]. The CH-Ex at given doses (100, 200 and 400 mg/kg) was proved to be non-hemotoxic. The serum biochemical parameters play a significant role as biomarkers in sub-acute toxicological evaluation[35]. SGOT, SGPT, ALP and total protein estimations have been used as biomarkers in assessment of hepatotoxicity[36], whereas the measurement of BUN, creatinine, urea, uric acid and bilirubin can be used to assess nephrotoxicity[37]. Serum triglycerides and total cholesterol were totally controlled by liver synthesis and changes in the lipid levels can be used to assess cardiovascular risk. No significant difference in the levels of biochemical parameters were observed at dose levels of 100 and 200 mg/kg extract when compared with the control group ($p>0.05$). At 400 mg/kg females exhibited a slight decline in SGPT, creatinine and BUN levels ($p<0.05$), while male rats exhibited a slight enhancement of SGOT and ALP levels ($p<0.05$). Liver and kidney play a crucial role in the metabolism and excretion of many drugs[38]. No morphological disparities or lesions were seen in the liver and kidney sections under optical microscope.

Research evidence presented that ROS generated from oxidative stress can cause a variety of diseases by damaging cellular biochemicals including DNA[39]. Mutagenesis caused by genotoxicity (DNA damage) can cause chronic neurodegenerative diseases[40]. Experiments on DNA damage protection are a valuable initial step in assessing the potential toxicity of the test material[41]. Increased quantities of Reactive Oxygen Species (ROS) can cause oxidative stress induced damage to DNA and other cellular biochemicals. The protective effects of CH-Ex were evaluated against oxidative damage to DNA induced by Fenton reagent[42]. CH-Ex at various concentrations 12.5, 25, 50 and 100 µg/ml prevents DNA damage induced due to oxidative stress in a dose-dependent manner. Therefore, the outcomes obtained encourage studies on its additional toxicological properties. In conclusion, CH-Ex was found to be safe and non-toxic at the given doses and ancillary clinical toxicological assessment must be conducted. In addition, CH-Ex could be an indirect preventive therapy against neurodegenerative diseases associated with DNA damage from oxidative stress.

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6. Conflict of Interest

The authors declare no conflict of interest.

7. References

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