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ORIGINAL ARTICLE

Evaluation of CYP2D, CYP1A2 and Distribution of Tetrandrine, Fangchinoline in the Brain, Liver, and Kidney of Wistar Rats after Short-Term Exposure to *Cyclea peltata*

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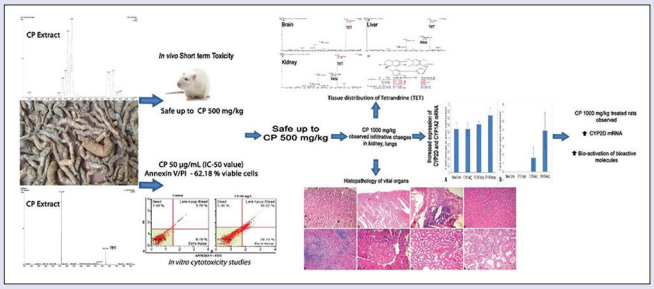
ABSTRACT

Background: *Cyclea peltata* (CP) roots are used in Indian traditional medicine to treat various diseases. However, short-term toxic effects of CP are completely unknown. **Objectives:** The aim of this study is to evaluate short-term toxic effects of CP, tissue distribution of bioactive alkaloids tetrandrine, fangchinoline in the brain, liver, kidney, and mRNA expression of CYP2D, CYP1A2 after CP administration. **Materials and Methods:** *In vitro* toxicity evaluation of CP was carried out using annexin 5/propidium iodide assay with or without CP treatment in L 929 cell line. And in vivo short term toxicity of CP was evaluated in Wistar rats for 28 days. Liquid chromatography quadrupole time-of-flight mass spectrometry (LC-Q-TOF-MS) based tissue distribution of bioactive molecules was assessed and mRNA expression of CYP1A2, CYP2D, CYP2C6, and CYP2E1 were estimated using qRT- polymerase chain reaction. **Results:** *In vitro* toxicity of 70% aqueous ethanol extract of CP 50 µg/mL showed 62.18% cell viability. Oral administration of CP (50, 500 mg/kg) did not cause any clinical, hematological, serum biochemical, and histopathological changes in rats, whereas CP (1000 mg/kg) showed infiltrative changes in the kidney and lungs. Imbalance in oxidative stress and antioxidant defense was reflected as elevated MDA levels in rat liver. LC-Q-TOF-MS studies could detect tissue distribution of bioactive alkaloids tetrandrine, fangchinoline and their decomposed masses in the liver and kidney, whereas tetrandrine and its decomposed molecule (580.3) ([M + H]-43) + could cross blood–brain barrier and were detected in the brain. Evaluation of mRNA expression revealed dose-dependent increase in expression of CYP2D and CYP1A2. **Conclusion:** Oral administration of CP 500 mg/kg for 28 days is safer in rats due to balanced antioxidant defense. Imbalance in antioxidant defense enzymes and toxic metabolites formed through the escalation of CYP2D, CYP1A2 and metabolism of bioactive alkaloids of CP may be the reason for infiltrative changes in kidney, lungs observed after 28 days CP 1000 mg/kg administration.

Key words: Apoptosis, *Cyclea peltata*, cytochrome P450, metabolite, tetrandrine

SUMMARY

- The present study showed CP 500 mg/kg was safer in Wistar rats for 28 days
- Converted to human equivalent dose (HED) of 60 kg adult human as 61 mg/kg extract per day or 25 g *C. peltata* root powder per day for 28 days
- Liquid chromatography quadrupole time-of-flight mass spectrometry studies showed tissue distribution of bioactive alkaloids tetrandrine, fangchinoline in the liver, kidney, and tetrandrine could cross blood–brain barrier
- Imbalance in antioxidant enzymes and metabolites formed through the escalation of CYP2D, CYP1A2 may be the reason for infiltrative changes in the kidney and lungs after 28 days CP1000 mg/kg administration.



Abbreviations used: CP: 70% aqueous ethanol extract of *Cyclea peltata;* HED: Human equivalent doses; NOAEL: No observed adverse effect levels.

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INTRODUCTION

The use of medicinal plants and their products is increasing worldwide because of their therapeutic value and lesser side effects. Hence, a large number of people are using herbal medicine alone, or a few are using along with modern/other complementary medicine systems. Many people consume these herbs for long term or as a lifelong treatment. In this circumstance, detailed toxicity study, herb-drug interaction, and metabolism studies are very essential prerequisites in the use of herbal medicine. This could facilitate the medical practitioner to choose the correct combination, effective dose, and duration of administration of herbal medicine.

Cyclea peltata (CP) (Lam.) Hook. f. and Thoms. (Menispermaceae), is locally called "Padakkilangu" in Kerala, India. CP roots are used against jaundice and digestive disorders.[1] In "Astangahridaya," CP root is one of the ingredients of Hinguvachadi Choornam, administered in hot

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Table 1: Primer sequence used in the present study

Target gene	Forward sequence	Reverse sequence
CYP1A2	5'-TGTCACCTCAGGGAATGCT-3'	5'-GACCACCGTTGTCTTTGTAG-3'
CYP2D	5'-TGGACCTCAGTAACATGCCA-3'	5'-GATGCAAGGATCACACCTTG-3'
CYP2C6	5'-CGGGAAGTCATACGACATTAGC-3'	5'-GCAGAGAGGCAAATCCATTG-3'
CYP2E1	5'-GCTGTCAAGGAGGTGCTAC-3'	5'-GCCTCATTACCCTGTTTCC-3'
β-Actin	5' -GTCAGGTCATCACTATCGGCAAT-3'	5'-AGAGGTCTTTACGGATGTCAACGT-3'

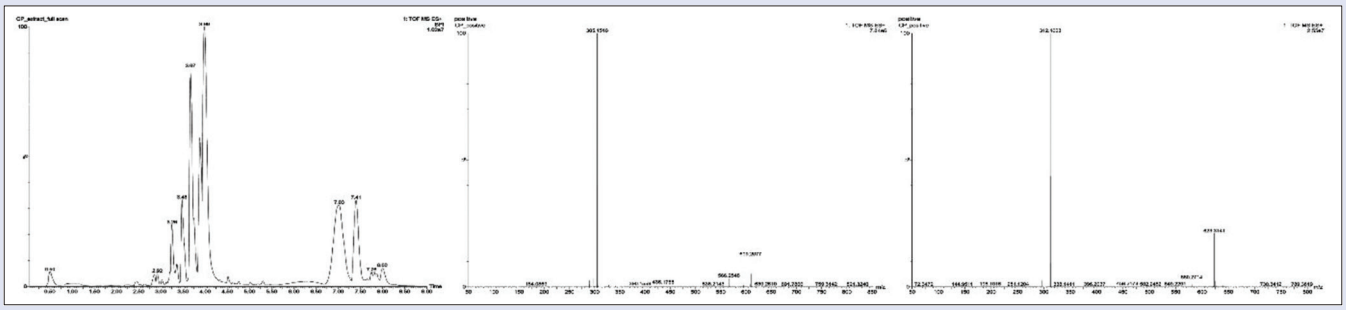

Figure 1: LC-Q-TOF analysis of CP extract; tetrandrine and fangchinoline were detected with M + H value 623.3141 and 609.2977, respectively. CP: *Cyclea peltata*; Liquid chromatography quadrupole time-of-flight

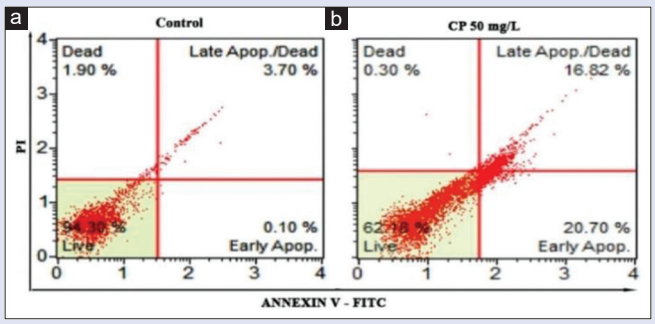

Figure 2: (a and b) *In vitro* cytotoxicity assessment of CP (50 µg/mL) using Annexin5/PI dual staining in L-929 cells. (a) Control cells without drug treatment. (b) Cells treated with CP (50 µg/mL). X-axis represents FITC-labeled Annexin5 positive cells and Y-axis represents the PI-labeled cells. CP: *Cyclea peltata*; PI: Propidium iodide; FITC: Fluorescein isothiocyanate

water (4 g daily) to treat stomach ulcer. In "Patoladigana" decoction used to treat liver diseases, CP root is an ingredient (8 g/day) among other six ingredients.[2-4] CP roots are used as nerve stimulant, antidote against snake bite, remedy against smallpox, to treat stomach pain, diarrhea, wounds, certain skin disorders, for toothache, and also the leaves are used to stop bleeding, against bronchitis, cough, and dandruff.[5-9]

Alkaloids fangchinoline, tetrandrine, d-isochondrodendrine, cycleapeltine, cycleadrine, cycleacurine, cycleanorine were reported from CP roots.[10,11] Tetrandrine was found to be the major alkaloid present in the Indian sample[12] and the amount of tetrandrine in the ethanol extract of CP was found to be 9.8 mg/g.[3] Bioassay-guided fractionation of CP showed tetrandrine as major bioactive molecule present.[13]

Tetrandrine (TET) is a bisbenzylisoquinoline alkaloid validated with promising pharmacologic activities such as antidiabetic,[14] anti-inflammatory, immunosuppressive,[15] free radical scavenging,[16] antifibrotic, and anticancer properties. Moreover, TET has a great potential for clinical use to treat hypertension and silicosis.[17,18] Continuous administration of TET in dogs caused liver damage[19] and TET could induce apoptosis and mitochondrial dysfunction in rat liver.[20]

It was previously reported that the formation of the reactive intermediate quinone methide-derived metabolite is proposed to be responsible for the pulmonary toxicity by tetrandrine.[21,22] Previous studies also reported that inhibitors of cytochrome P450 (CYP 450) subfamilies CYP2D, CYP2C, and CYP2E1 diminished the adenosine triphosphate reduction after TET administration, although the inhibitors of CYP2D and CYP2E1 reduced the reactive oxygen species generated through TET administration.[23] As TET is the major alkaloid of CP, the current research, evaluated the tissue distribution of TET in the brain, liver, and kidney using liquid chromatography-mass spectrometry. Further messenger ribonucleic acid (RNA) (mRNA) expression of tetrandrine metabolizing CYP 450 such as CYP1A2, CYP2D, CYP2C6, and CYP2E1 in Wistar rats were estimated after short term, 28 days repeated oral administration of CP. Our previous study on the acute toxicity of CP showed nontoxic nature up to 2500 mg/kg.[3]

The objectives of the present study were focused on the effect of 28 days of repeated oral administration of CP on different parameters such as serum and tissue biochemical parameters, hematological parameters, clinical observations, histopathological observations, tissue distribution of major bioactive alkaloid tetrandrine in the liver, kidney, brain, and evaluation of mRNA expression of tetrandrine metabolizing CYP 450 enzymes such as CYP1A2, CYP2D, CYP2C6, and CYP2E1.

MATERIALS AND METHODS

Chemicals

Trizol, Qubit 3.0, and cDNA preparation kits were purchased from Thermo Scientific, USA. SYBR Green was purchased from Bioline, London, UK. Bioanalyzer Kits were purchased from Crest Biosystems India. 5,5 Dithiobis (2-Nitrobenzoic acid), 1-Chlororo 2,4 dinitrobenzene, nitroblue tetrazolium chloride, hydrogen peroxide (H_2O_2), sodium pyrophosphate, L-glutamine, fetal bovine serum, trypsin, hematoxylin and eosin, phenazine methosulfate (PMS), were purchase from Hi-media, Mumbai, India. Annexin 5 and propidium iodide (PI) kit were purchased from Life Technologies Corporation Eugene, Oregon, USA.

Plant material

CP roots were collected from Kerala, India, and the plant material was authenticated by taxonomist of the institute and deposited at the institute herbarium (TBGT 13814).

Extract preparation

CP tuberous roots were washed, dried, and pulverized. Plant powder (100 g) was extracted with 70% hydro-alcohol (1 L) 24 h at 37°C; the procedure was repeated twice and the filtrate was concentrated using a rotary evaporator (Buchi R-210, Flawil, Switzerland), dried the crude extract (yield 15% w/w), and referred to as CP.

Liquid chromatography quadrupole time-of-flight mass spectrometry and Inductively Coupled Plasma-Mass-Spectrometry analysis

Liquid chromatography quadrupole time-of-flight mass spectrometry (LC-Q-TOF-MS) analysis of CP was carried out on Xevo G2-+ (Waters) Q-TOF fitted with UPLC BEH C_{18} column.[24]

Trace metal analysis of CP was carried out using inductively coupled PMS (ICPMS) system (Thermo Scientific ICAP Qc). The external calibration solutions were prepared from standard certified multielement solution (MERCK). Thermo scientific BRANSTEAD Smart2pure water containing 1% suprapur grade nitric acid (MERCK) was used to get a range of concentrations 25 ppb, 50 ppb, and 100 ppb for all elements. Samples containing higher concentration of elements than this calibration range were diluted and analyzed by applying dilution factors. The ion optics was tuned using Thermo scientific Tune-B ICAP-Q solution in standard mode and KED mode. Mass and detector calibration was conducted using Thermo Scientific Setup solution ICAPQ.

In vitro cytotoxicity assessment of *Cyclea peltata* using Annexin-5/PI dual staining

A quantitative assessment of apoptosis was performed using fluorescein isothiocyanate (FITC), annexin5/PI dead cell apoptosis detection kit I (Life Technologies Corporation, Oregon, USA). Briefly, mouse fibroblast L-929 (National Centre for Cell Science, Pune, India) cells were cultured in eagle's minimum essential medium with L-glutamine and supplemented with 10% fetal bovine serum in a standard condition at 37°C in 5% carbon dioxide (CO_2). Cells were then treated with CP 50 µg/mL (half maximal inhibitory concentration (IC-50) value based on MTT assay) and incubated at 37°C for 24 h in 5% CO_2. The L-929

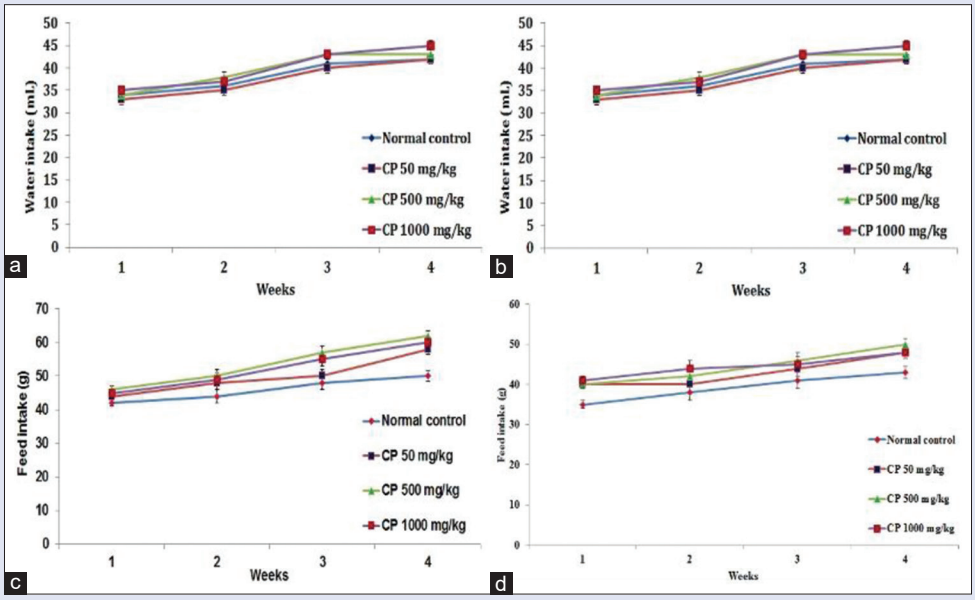

Figure 3: (a-d) Effect of 28 days oral administration CP (50, 500, and 1000 mg/kg) on water and feed intake in male and female rats. (a) Water intake of male rats. (b) Water intake of female rats. (c) Feed intake of male rats. (d) Feed intake of female rats. Values are presented as mean ± SEM (*n* = 8). SEM: Standard error of mean; CP: *Cyclea peltata*

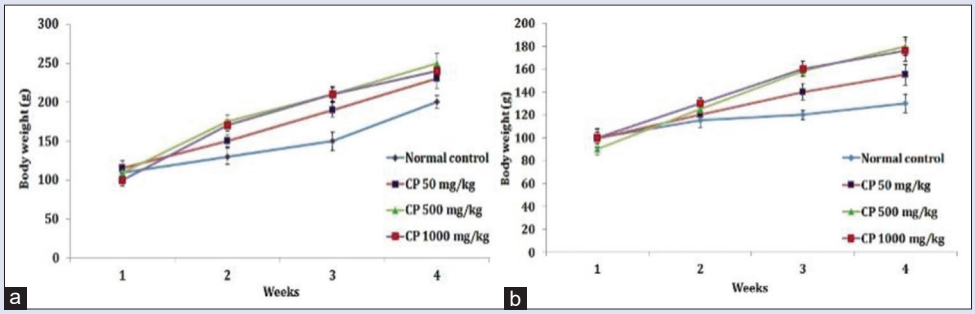

Figure 4: (a and b) Effect of 28 days oral administration CP (50, 500, and 1000 mg/kg) on body weight of male and female rats. (a) Body weight of male rats. (b) Body weight of female rats. Values are presented as mean ± SEM (*n* = 8). SEM: Standard error of mean; CP: *Cyclea peltata*

cells were incubated without plant extract taken as control. Trypsinized cells were transferred to tubes and added 0.0001 L of Annexin 5 and PI to each tube, incubated at room temperature for 20 min in the dark.[25] The cells were analyzed in Muse cell analyzer (Merck Millipore, Darmstadt, Germany) and Muse FCS 3.0 software used for analysis (Merck Millipore, Darmstadt, Germany).

Experimental animals

Wistar albino rats (males and female; 150–200 g) were maintained under standard conditions in polyacrylic cages at 24°C–28°C, humidity 60% with a light–dark cycle (12 h and each), fed commercial rat feed, and boiled water. The experiment was conducted according to NIH guidelines, with approval from Institute's Animal Ethics Committee (CPCSEA/IAEC/TBGRI/B1/04/2016/EMandEP-01).

Short-term toxicity study of *Cyclea peltata*

The short-term toxic effect of CP was evaluated as per Organization for Economic Cooperation and Development guidelines.[26] Male and female healthy Wistar rats (100–120 g body weight) were maintained underregulated standard environmental conditions. Group 1 concurrent control administered 2 mL 1% tween-80 for 28 days. Groups 2, 3, 4 served as drug administered groups; CP 50 mg/kg, 500 mg/kg and 1000 mg/kg was administered orally for respective groups. Drug/vehicle was administered p. o. daily once for 28 days consecutively. Each group contained 8 rats (4 males and 4 females; 2 animals per cage). One animal each (per sex) in the control (group 1) and in the highest dose group (group 4) were designated as satellite groups for observation of 14 days post-treatment period for any toxic effect persistence or reversibility. Animals were weighed and visual observations for mortality, behavioral pattern, and changes in

Table 2: Relative organ to body weight ratio of Wistar rats administered *Cyclea peltata* (50, 500, 100 mg/kg) intragastrically for 28 days and the satellite groups *Cyclea peltata* (50 and 100 mg/kg) for 42 days

Parameters	Normal control	CP (50 mg/kg)		CP (500 mg/kg)		CP (1000 mg/kg)	
	28 days	28 days	42 days	28 days	42 days	28 days	42 days
Males							
Initial BW	110±2.47	115±1.94	110±1.21	110±2.17	-	100±3.10	110±2.50
Final BW	220±1.98	230±3.07	250±4.12	250±1.52	-	240±3.46	280±2.32*
Percentage BW change	101.82	130	140	150	-	140	170
Liver	9.24±0.22	10.14±0.3	10.01±0.21	10.64±0.31	-	10.17±0.41	11.28±0.50
Spleen	0.99±0.12	1.08±0.10	0.98±0.10	1.12±0.17	-	1.03±0.09	1.22±0.10
Kidney	0.85±0.10	0.90±0.13	0.88±0.23	1.06±0.14	-	0.99±0.11	1.07±0.21
Heart	0.70±0.09	0.72±0.10	0.70±0.20	0.91±0.08	-	0.79±0.10	0.91±0.12
Pancreas	1.01±0.22	1.15±0.14	1.05±0.24	1.30±0.10	-	1.26±0.09	1.38±0.11
Brain	1.14±0.30	1.50±0.22	1.60±0.12	1.54±0.23	-	1.52±0.19	1.69±0.20
Lungs	2.05±0.24	2.23±0.16	2.20±0.10	2.41±0.10	-	2.32±0.23	2.50±0.18
Testes	3.16±0.19	3.34±0.14	3.44±0.10	3.35±0.15	-	3.37±0.10	3.45±0.20
Liver: BW	4.20	4.452	4.004	4.258	-	4.24	4.029
Spleen: BW	0.450	0.471	0.392	0.449	-	0.43	0.44
Kidney: BW	0.386	0.39	0.352	0.425	-	0.413	0.382
Heart: BW	0.318	0.311	0.28	0.364	-	0.328	0.325
Pancreas: BW	0.459	0.498	0.42	0.521	-	0.524	0.493
Brain: BW	0.518	0.65	0.64	0.616	-	0.634	0.604
Lungs: BW	0.932	0.97	0.88	0.964	-	0.966	0.893
Testis: BW	1.436	1.452	1.376	1.34	-	1.40	1.23
Females					-		
Initial BW	100±2.12	99±3.23	100±2.44	90±3.54	-	100±2.64	100±2.64
Final BW	152±3.42	155±4.15	180±3.20	180±5.80	-	176±5.21	200±5.21
Percentage BW change	52	55	80	80	-	76	100
Liver	7.87±0.41	8.10±0.32	8.40±0.22	8.89±0.21	-	8.423±0.20	8.93±0.10
Spleen	0.82±0.20	0.85±0.19	0.88±0.20	0.90±0.25	-	0.856±0.31	0.96±0.20
Kidney	0.80±0.18	0.81±0.11	0.85±0.21	0.84±0.15	-	0.841±0.17	0.91±0.27
Heart	0.52±0.20	0.60±0.15	0.60±0.18	0.61±0.09	-	0.595±0.08	0.615±0.08
Pancreas	0.88±0.17	0.91±0.09	0.98±0.10	0.97±0.12	-	0.948±0.14	1.08±0.20
Brain	0.98±0.20	1.02±0.21	1.12±0.21	1.10±0.23	-	1.089±0.19	1.20±0.20
Lungs	1.74±0.18	1.88±0.20	1.99±0.22	1.98±0.15	-	1.901±0.18	2.11±0.14
Ovary	2.75±0.31	2.97±0.34	3.07±0.24	3.02±0.27	-	2.981±0.20	3.08±0.15
Liver: BW	5.177	5.225	4.667	4.77	-	4.786	4.465
Spleen: BW	0.539	0.545	0.489	0.498	-	0.486	0.48
Kidney: BW	0.526	0.524	0.472	0.468	-	0.478	0.455
Heart: BW	0.342	0.373	0.333	0.341	-	0.338	0.308
Pancreas: BW	0.579	0.589	0.544	0.536	-	0.538	0.540
Brain: BW	0.644	0.654	0.622	0.613	-	0.618	0.60
Lungs: BW	1.145	1.21	1.106	1.098	-	1.08	1.06
Ovary: BW	1.81	1.91	1.706	1.675	-	1.69	1.54

*Significantly different compared to normal control at $P \leq 0.05$. Values are presented as mean ± SEM (n = 8). BW: Body weight; SEM: Standard error of mean; CP: *Cyclea peltata*

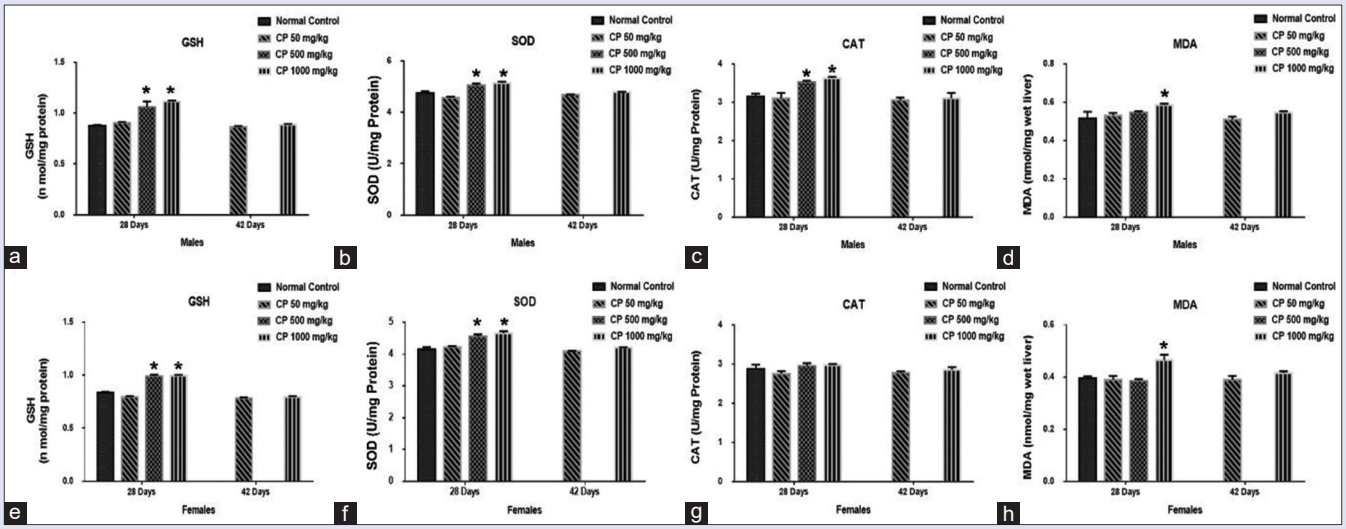

Figure 5: (a-h) Effect of 28 days oral administration CP (50, 500, and 1000 mg/kg) and the satellite groups CP (50 and 1000 mg/kg) for 42 days on liver GSH, SOD, CAT, MDA level in rats. (a) GSH in male rats. (b) SOD in male rats. (c) CAT in male rats. (d) MDA in male rats. (e) GSH in female rats. (f) SOD in female rats. (g) CAT in female rats. (h) MDA in female rats. CP: *Cyclea peltata*; GSH: Reduced glutathione; SOD: Superoxide dismutase, CAT: Catalase; MDA: Malondialdehyde

physical appearance, injury, pain, and signs of illness were monitored. The amounts of feed supplied, remaining feed, and water intake were measured daily and average weekly consumption was calculated. On day 28 (day 42 in satellite groups), rats were housed in metabolic cages to collect urine and volume was calculated. At the end of the experiment (28th and 42nd day), the rats were sacrificed by CO_2 inhalation; blood collected by cardiac puncture and serum parameters were estimated. Rat brain, heart, lungs, liver, spleen, kidney, and testes/ovaries were collected and weighed. One part of all organs was fixed in 10% buffered formalin for histopathological analysis and the other part was frozen in liquid nitrogen and stored at -80°C for biochemical, molecular, and metabolite analysis.

Conversion to human equivalent doses (HED) was calculated based on the following formula;[27] no observed adverse effect levels (NOAEL).

HED (mg/kg) = Animal NOAEL (mg/kg) × (weight $_{animal}$ [kg]/weight $_{human}$[kg])$^{(1-0.67)}$

Estimation of reduced glutathione, superoxide dismutase, catalase, and malondialdehyde

Reduced glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), and malondialdehyde (MDA) levels in the liver tissue homogenates were estimated as per the previous methods.[12]

Tissue distribution of tetrandrine in the liver, brain, and kidney using Liquid chromatography quadrupole time-of-flight mass spectrometry

The tissue was suspended in 0.0002 L volume of chloroform/methanol/water (1:3:1 ratio) at 4°C. Tissue homogenate was sonicated and further vortexed for 1 h at 4°C. Supernatant was collected after centrifugation at 13,000 g for 5 min. The supernatant was collected (0.00018 L) and stored at −80°C until analyzed by liquid chromatography-mass spectrometry.[28]

LC-Q-TOF-MS analysis of the brain, liver, and kidney supernatants was carried out on Xevo G2 (Waters) Q-TOF fitted with UPLC BEH C_{18} column. Gradient elution using 0.1% formic acid in water (Solvent A; 95%) and methanol (Solvent B; 5%), was continued up to 6 min (solvent A; 5% and solvent B; 95%), at flow rate of 0.3 mL/min. Mass spectra were collected in positive and negative ionization mode.[24]

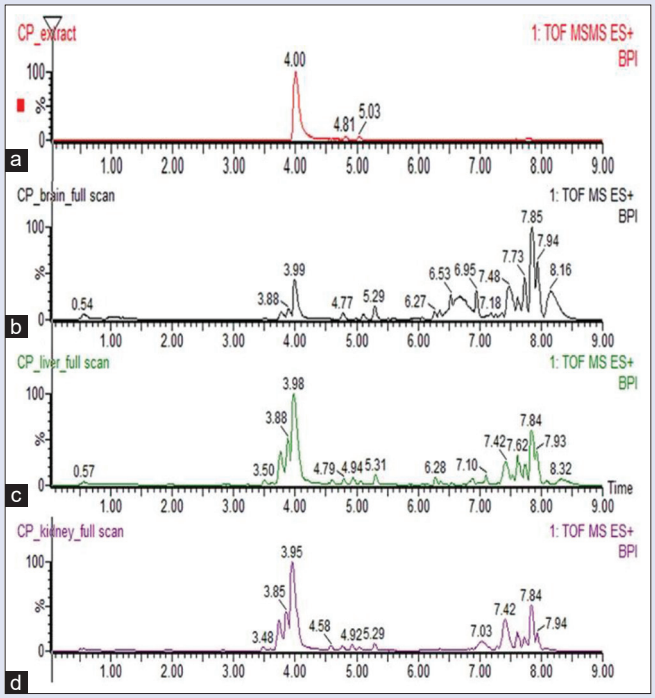

Figure 6: (a-d) LC-Q-TOF-TIC analysis of CP extract, CP (1000 mg/kg) treated rat brain, liver, and kidney. (a) LC-Q-TOF-TIC of plant extract CP. (b) LC-Q-TOF-TIC of CP (1000 mg/kg) treated rat brain. (c) LC-Q-TOF-TIC of CP (1000 mg/kg) treated rat liver. (d) LC-Q-TOF-TIC CP (1000 mg/kg) treated rat kidney. CP: *Cyclea peltata*; LC-Q-TOF-MS: Liquid chromatography quadrupole time-of-flight mass spectrometry

Quantitative real-time polymerase chain reaction measurement of rat liver CYP1A2, CYP2D, CYP2C6, and CYP2E1

Total RNA was extracted from liver tissue (Trizol, Ambion Life Technologies, USA). Total RNA purity and concentration was determined using Qubit 3.0 (Life Technologies, USA). Template complementary DNA was synthesized using High Capacity cDNA synthesis Kit (Thermoscientific, USA). Quantitative real-time polymerase chain reaction analysis was carried out using SYBR Green Master Mix (Applied Biosystems, Life technologies). Data were analyzed (in triplicates) according to $\Delta\Delta C_t$ method using Lightcycler analysis software (Lightcycler 96 SW 1.1).[24] The primer sequences for Cyp1a2, Cyp2d, Cyp2c6, and Cyp2e1 are summarized in Table 1.

Histopathological analysis

Tissue morphology and toxicity of the liver, kidney, brain, heart, spleen, pancreas, lung, and testis were assessed using hematoxylin and eosin (H and E) staining. Formalin (10%) fixed tissues were embedded in paraffin and 5 µm thick sections were stained with (H and E). The histopathological changes (lymphocyte infiltration, apoptosis, etc.) observed under microscope.[24]

Statistical analysis

The data were expressed as mean ± standard deviation. ANOVA, followed by Duncan's multiple range test was carried out to compare and analyze the data. Results were considered significant at $P \leq 0.01$.[29]

RESULTS

Chromatographic analysis and heavy metal content of *Cyclea peltata*

LC-Q-TOF-MS showed tetrandrine (TET., $C_{38}H_{42}O_8N_2$; MW 622.30), fangchinoline ($C_{37}H_{40}N_2O_6$; MW 608.288) detected as (M + H) + value 623.3141 and 609.2977 respectively in CP [Figure 1]. Detection of the heavy metal content of CP using ICPMS analysis revealed that the heavy metal contents were found to be within tolerable levels (Data not shown).

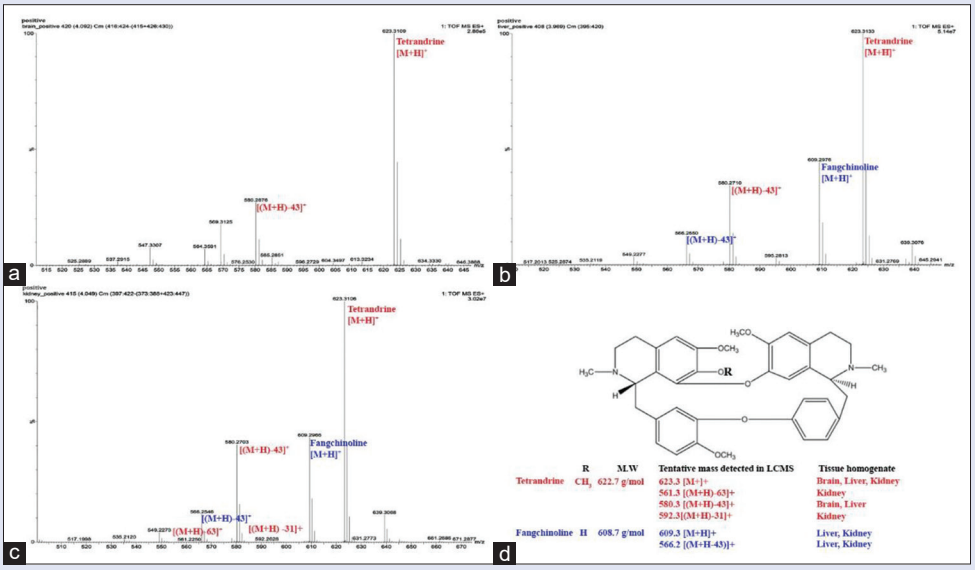

Figure 7: (a-c) LC-Q-TOF-MS analysis of CP (1000 mg/kg) treated rat brain, liver, and kidney at retention time 3.9 min for tetrandrine (TET) (622.762 g/mol) and FAN (608.288). (a) LC-Q-TOF-MS of CP (1000 mg/kg) treated rat brain. (b) LC-Q-TOF-MS of CP (1000 mg/kg) treated rat liver. (c) LC-Q-TOF-MS CP (1000 mg/kg) treated rat kidney. (d) Structures of tetrandrine, fangchinoline, and decomposed molecular masses of both. CP: *Cyclea peltata*; FAN: Fangchinoline; LC-Q-TOF-MS: Liquid chromatography quadrupole time-of-flight mass spectrometry

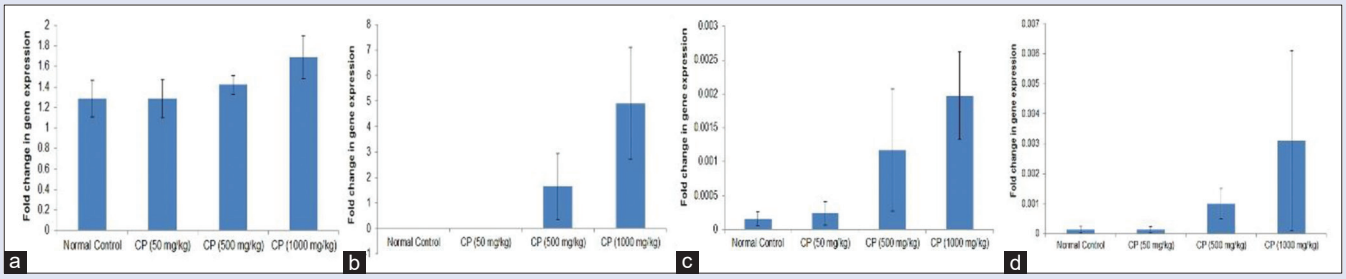

Figure 8: (a-c) Effect of 28 days administration CP (50, 500, and 1000 mg/kg) on mRNA expression of CYP1A2, CYP2D, CYP2C6, and CYP2E1 in Wistar rats. (a) CYP1A2 mRNA levels. (b) CYP2D mRNA levels. (c) CYP2C6 mRNA levels. (d) CYP2E1 mRNA levels. *Significant difference of the normal control between groups. CP: *Cyclea peltata*

In vitro cytotoxicity assessment of *Cyclea peltata* using annexin--V/propidium iodide dual staining

L-929 cells without any plant extract treatment showed 94.5% viable cells, were detected as unstained cells, whereas 24 h treatment of CP 50 µg/mL (IC_{50} value; data not shown) with L-929 cells showed 62.18% viable cells, whereas 20.7% cells were detected as early apoptotic, bound with Annexin 5/FITC, 0.3% cells were detected as dead cells, they stained with PI and 16.82% cells were late apoptotic cells they bound with both Annexin 5-FITC and PI [Figure 2].

Short-term toxicity study of *Cyclea peltata*

Effect Cyclea peltata on clinical observations

Short-term (28 days) treatment with CP up to 1000 mg/kg p. o. did not show significant clinical variations, such as hyperactivity, restlessness, circling, writhing, kicking of rear legs, standing on front legs, or any change in behavior. There was no mortality recorded in rats during the 28 days CP administration period and 14 days post-treatment period.

28 days treatment with CP did not cause any significant changes in mean feed and water intake among both the sexes of experimental animals when compared to normal control**.** The observed results showed that there was a normal increase in feed and water intake as the experimental days progressed [Figure 3a-d].

The mean body weights of the animals treated with CP (50, 500, and 1000 mg/kg) for 28 days and satellite group were compared to the values of normal control of both sexes. Drug treatment groups showed significant weight gain when compared to normal control of both sexes [Figure 4a and b].

The organ weights of the drug-administered animals did not show any statistical differences when compared to the normal control [Table 2].

Table 3: Hematology parameters of rats administered *Cyclea peltata* (50, 500, and 100 mg/kg) intragastrically for 28 days and the satellite groups *Cyclea peltata* (50 and 100 mg/kg) for 42 days

Parameters	Normal control	CP (50 mg/kg)		CP (500 mg/kg)		CP (1000 mg/kg)	
	28 days	28 days	42 days	28 days	42 days	28 days	42 days
Males							
WBC (103/µL)	9.5±1.21	9.8±1.21	9.7±1.56	10.2±1.10	-	11.2±2.20	9.9±1.26
Lymphocyte (103/µL)	6.8±1.30	7.0±1.30	6.9±1.45	7.1±1.20	-	7.4±2.40	7.0±1.21
Monocyte (103/µL)	0.48±0.09	0.45±0.09	0.45±0.10	0.5±0.10	-	0.5±0.15	0.45±0.11
Granulocyte (103/µL)	2.8±1.05	3.0±1.05	2.9±1.24	3.2±1.20	-	3.3±1.05	3.0±1.30
RBC (106/µL)	7.2±1.18	7.6±1.07	7.3±1.15	7.8±1.20	-	8.3±1.19	7.4±1.02
HGB (g/dL)	13.4±1.18	13.9±1.50	13.8±1.26	14.1±1.20	-	15.1±1.40	14.0±1.51
MCV (fL)	59.0±2.12	60.1±1.95	60.0±1.20	58.0±2.80	-	54.6±4.20	58.0±1.22
PLT (103/µL)	942±10.2	940±8.40	945±7.58	950±12.50	-	897±20.60*	944±6.82
Females							
WBC (103/µL)	9.2±0.74	9.3±0.95	9.2±1.05	9.5±1.01	-	8.7±1.20	9.0±2.20
Lymphocyte (103/µL)	5.5±1.12	5.2±1.40	5.2±1.21	5.6±1.10	-	5.1±1.06	5.5±1.06
Monocyte (103/µL)	0.43±0.07	0.40±0.10	0.42±0.15	0.44±0.06	-	0.39±0.05	0.40±0.09
Granulocyte (103/µL)	2.2±1.25	2.5±0.95	2.3±1.10	2.7±1.15	-	2.4±1.04	2.2±1.21
RBC (106/µL)	7.0±0.95	6.9±1.10	6.9±1.24	7.2±1.05	-	6.1±1.10	7.1±1.16
HGB (g/dL)	13.2±1.21	13.0±1.06	12.8±1.12	13.1±1.10	-	12.9±0.90	13.0±1.42
MCV (fL)	54.0±1.15	55.0±1.21	54.0±1.04	57.7±2.20	-	55.1±1.90	54.2±1.10
PLT (103/µL)	935±2.12	927±5.20	930±4.14	918±8.90	-	905±7.20*	934±3.78

*Significantly different compared to normal control at $P \leq 0.05$. Values are presented as mean ± SEM (n = 8).

WBC: White blood cells; RBC: Red blood cells; HGB: Hemoglobin; MCV: Mean corpuscular volume; PLT: Platelet

Table 4: Serum biochemical parameters of rats administered *Cyclea peltata* (50, 500, and 100 mg/kg) intragastrically for 28 days and the satellite groups *Cyclea peltata* (50 and 100 mg/kg) for 42 days.

Parameters	Normal control	CP (50 mg/kg)		CP (500 mg/kg)		CP (1000 mg/kg)	
	28 days	28 days	42 days	28 days	42 days	28 days	42 days
Males							
AST (U/L)	50±2.15	48±1.25	50±2.15	51±2.10	-	58±1.01*	49±1.20
ALT (U/L)	45±3.05	40±4.40	45±2.12	46±2.18	-	52±2.15*	46±2.25
ALP (KA units)	50±3.15	45±2.14	50±1.20	52±1.21	-	55±2.20	51±2.30
Cholesterol (mg/dL)	50±4.12	47±3.50	45±2.15	55±2.20	-	52±3.15	50±2.35
Creatinine (mg/dL)	0.5±0.10	0.46±0.20	0.48±0.1	0.51±0.15	-	0.9±0.15*	0.5±0.10
SB (mg/dL)	0.45±0.15	0.45±0.18	0.50±0.15	0.48±0.20	-	0.47±0.18	0.45±0.1
Females							
AST (U/L)	45±2.18	46±2.14	48±2.42	50±1.98	-	58±2.14	45±2.12
ALT (U/L)	42±2.25	42±2.10	40±2.40	42±1.54	-	65±2.67	42±1.45
ALP (KA units)	46±2.15	44±1.54	45±2.10	48±2.14	-	49±1.80	50±1.54
Cholesterol (mg/dL)	48±3.60	49±1.24	46±2.26	49±1.90	-	50±2.85	48±1.24
Creatinine (mg/dL)	0.4±0.12	0.44±0.10	0.42±0.12	0.44±0.10	-	1.0±0.12*	0.45±0.1
SB (mg/dL)	0.40±0.10	0.41±0.10	0.40±0.12	0.40±0.12	-	0.45±0.14	0.42±0.12

*Significantly different compared to normal control at $P \leq 0.05$. Values are presented as mean ± SEM (n = 8).. SEM: Standard error of mean; CP: *Cyclea peltata*; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; SB: Serum bilirubin

Hematology and serum biochemical parameters

The hematological parameters of CP (50, 500, and 1000 mg/kg) treatments did not show any significant elevation when compared to both sexes of normal control animals. But CP (1000 mg/kg) treatment showed slightly lower level of platelet count when compared to normal control and other treatment groups but within the normal level. This was completely reverted in the satellite group of CP (1000 mg/kg) rats [Table 3].

Serum biochemical parameters of CP (50 and 500 mg/kg) treatment for 28 days showed no statistical difference with normal control groups. CP (1000 mg/kg) treated animals showed slightly higher serum biochemical parameters, especially creatinine and alanine transaminase levels in both sexes. Both creatinine and alanine transaminase levels were reverted to normal level in satellite group of both sexes [Table 4].

Effect of Cyclea peltata treatment on the levels of reduced glutathione, superoxide dismutase, catalase, and malondialdehyde in the liver

CP (50, 500, and 1000 mg/kg) treatment for 28 days showed an increase in the liver antioxidant enzyme system such as GSH, SOD, and CAT, whereas MDA levels were slightly increased in 28 days of administration of CP 1000 mg/kg [Figure 5].

Liquid chromatography quadrupole time-of-flight mass spectrometry-based metabolite detection in the brain, liver, and kidney

Total ion chromatogram (TIC) of 28 days CP (1000 mg/kg) treated rat liver, brain, and kidney showed the presence of CP metabolites at the retention time of 3.9, 4.7, and 5.31 min [Figure 6a-d], which were correlated with the TIC of plant extract CP [Figure 5a]. We have focused our study on the two major bioactive alkaloids tetrandrine (622.762 g/mol) and fangchinoline (608.288 g/mol). Tetrandrine was detected as 623.31 (M + H) + in the brain, liver, and kidney. And also, the decomposed mass of tetrandrine was detected as 580.3 elimination of CH3N = CH2 ([M + H]-43) +, in the brain, liver, kidney, whereas other decomposed mass of tetrandrine was detected as 592.3 with the elimination of CH_3NH_2 ([M + H]-31) + and as 561.3 with the elimination of 2CH3NH2 ([M + H]-63) + were detected in the kidney only [Figure 7a-c].

Fangchinoline was detected as 609.29 (M + H) + and its decomposed mass as 566.2 elimination of CH3N = CH2 ([M + H]-43) + in the liver and kidney. Fangchinoline and its decomposed mass were absent in the brain [Figure 7a-c].

Effect of Cyclea peltata on the mRNA expression of liver CYP1A2, CYP2D, CYP2C6, and CYP2E1

CP (50, 500, and 1000 mg/kg) treatment for 28 days showed increased mRNA expression of CYP1A2 and CYP2D in a dose-dependent manner. CP (1000 mg/kg) treatment showed 4.92 fold increased expression of CYP2D and 0.41 fold increase in CYP1A2 mRNA expression. CP (1000 mg/kg) treatment showed only a slight increase in the mRNA expression of CYP2C6 and CYP2E1; 0.002 fold and 0.003 fold, respectively [Figure 8].

Histopathological study of vital organs

Histopathological study of 28 days treatment of CP (50 and 500 mg/kg) showed no marked changes in the liver, kidney, heart, lung, spleen, pancreas, brain, and testis. CP (1000 mg/kg) treatment showed mild interstitial hemorrhage, a few glomeruli atrophied, cell cast in tubules of the kidney [Figure 9], mild hepatocellular degeneration (cloudy swelling) of the liver, and focal infiltration of mononuclear cells in the lungs [Figure 10]. Other internal organs of all treatments showed normal architecture without any histological changes [Figures 9 and 10].

Treatment of CP up to 500 mg/kg for 28 days was safer in Wistar rats, Conversion of this dose to human equivalent dose showed 60 kg adult man can take CP extract ≈ 61 mg/kg/day safely for 28 days.

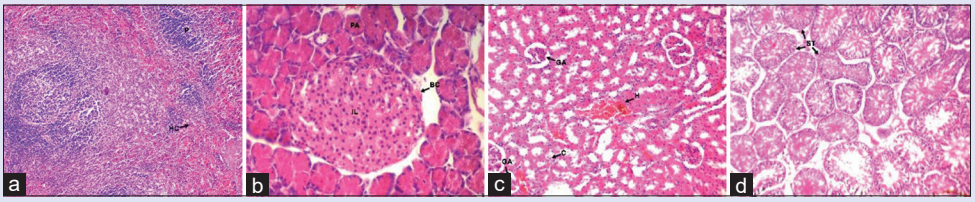

Figure 9: (a-d) Histopathology of the spleen, pancreas, kidney, and testis of rats treated with CP (1000 mg/kg). (a) Spleen (×200) showing islands of HC, per arteriolar lymphoid sheaths (P) with normal architecture. (b) Pancreas (×400) showing IL, BC, normal PA with normal architecture. (c) Kidney (×200) showing mild interstitial hemorrhage (H), a few GA, cell cast in tubules (C). (d) Testis (×200) showing seminiferous tubules (ST) showing normal architecture. HC: Hematopoietic cells; IL: Islet's of Langerhans, BC: Blood capillaries; PA: Pancreatic acini; GA: Glomeruli atrophied; CP: *Cyclea peltata*

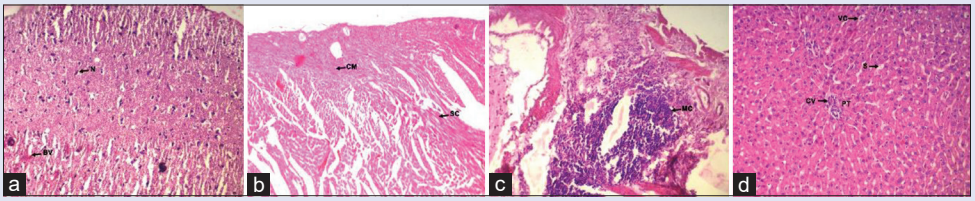

Figure 10: (a-d) Histopathological analysis of the brain, heart, lung, and liver of rats treated with CP (1000 mg/kg). (a) Brain section (×200) showing BV, neurons with nuclei (N) with normal architecture. (b) Heart section (×100) showing SC, CM with normal architecture. (c) Lung section (×200) showing focal infiltration of MC. (d) Liver section (×200) showing PT, CV, sinusoidal space (S), VC, mild hepatocellular degeneration of the liver. BV: Blood vessels; SC: Striated cardiac muscles; CM: Cardiac myocytes; MC: Mononuclear cells; PT: Portal triad; CV: Central vein; VC: Vacuolization of cytoplasm; CP: *Cyclea peltata*

DISCUSSION

Traditional usage of herbal drugs does not always assure safety, since it is difficult for traditional practitioners to detect delayed effects and adverse effects arising from long-term use.[30] Hence, detailed toxicity studies of herbal medicine are indispensable for the safer and effective therapeutic use of the same. Our previous study on the acute toxicity of CP showed that the median lethal dose was >2500 mg/kg and regarded as nontoxic up to 2500 mg/kg single dose.[3]

In the present study, *in vitro* cytotoxicity assay using annexin5/PI apoptosis showed that IC_{50} of CP was higher than that of 50 mg/L. CP 50 and 500 mg/kg oral administration for 28 days did not show any clinical signs of toxicity or mortality in both male and female rats. No significant changes were observed in feed and water intake of CP-treated rats of either sexes. There was a corresponding increase in water and feed intake with respect to the gain in the body weight. Which showed feed and water intake were normal during CP treated rats, this indicates CP treatment did not alter the digestive system of rats. The gain in body weight may also signify the nutritional status of animals. This substantiates the traditional and tribal usage of the plant through oral gavage. There were no significant changes in organ weight to body weight ratio of CP-treated rats, when compared to normal rats of both sexes.

Assessment of blood hematology and serum biochemical parameters of CP administered rats could deliver an insight in to the possible impairment of the internal organs. In the present study, the 28-day administration of CP (500 mg/kg) did not produce any change in hematology and serum biochemical parameters; this gives an insight of the nontoxic nature of plant extract up to 500 mg/kg. Histopathological examinations also revealed the nontoxicity of 28 days oral administration of CP up to 500 mg/kg. However, 28 days of administration of CP (1000 mg/kg) showed slightly elevated levels of alanine aminotransferase, aspartate transaminase, alkaline phosphatase, and creatinine in rats of both sexes. Increased oxidative stress elevated the lipid peroxidation and this can be observed as increased MDA levels; this aldehyde can covalently bind to thiol groups and modify biological macromolecules.[31] The observed elevation of liver MDA levels after 28 days CP 1000 mg/kg oral administration may be due to increased oxidative stress. The imbalance between the oxidative stress and the antioxidant defense system plays a vital role in the cellular damage.[32] This imbalance was observed in CP 1000 mg/kg treatment [Figure 5].

This biochemical observation was further evident in histopathological studies as early degenerative changes of liver and kidney of CP (1000 mg/kg). Hence, from these observations, it was clear that 28 days of administration of CP up to 500 mg/kg are safe but not CP 1000 mg/kg. In this scenario, we have evaluated the LC-MS-based tissue distribution of major bioactive alkaloids in *C. peltate* such as tetrandrine and fangchinoline in the brain, liver, and kidney. LC-MS studies revealed that tetrandrine was detected in the brain, liver, and kidney, whereas fangchinoline was not detected in the brain [Figure 6]. Further LC-MS studies revealed along with tetrandrine, different decomposing fragments of tetrandrine[33] were also detected in brain (580.3) [(M + H)-43] +, liver (580.3) [(M + H)-43] +, kidney (592.3) [(M+H)-31]+,(580.3)[(M+H)-43]+,(561.3)[(M+H)-63]+.Decomposing fragment of fangchinoline (566.2) [(M + H)-43] + was also detected in the liver and kidney.[33] From LC-MS studies [Figure 5], it was clear that liver and kidney were detected with both alkaloids and all metabolites. This may be due to the elimination of compounds after metabolism through the liver and kidney.

Understanding the mechanisms of herbal drug metabolism is crucial because herbal drug utilizes similar drug-metabolizing enzymes, transporters like cytochrome P450 enzymes, glucuronosyltransferases, and permeability glycoprotein, as other modern drugs used.[34] The CYPs are the major enzyme family which catalyse the oxidative biotransformation of most drugs and other lipophilic xenobiotics. Hence, CYPs gain specific importance in clinical and toxicological pharmacology. CYP1A1/2, CYP2A6, CYP2B6, CYP2C8/9/19, CYP2D6, CYP2E1, and CYP3A4/5 are involved in the oxidative metabolism of >90% of prescribed drugs.[34] Previous studies reported that reactive intermediate quinone methide-derived metabolite is responsible for the pulmonary toxicity by tetrandrine.[21,22] The inhibitors of CYP2D, CYP2C, and CYP2E1 decreased the ATP depletion and reduced the ROS after TET exposure.[23] Hence, in the current study, we have quantified the mRNA expression of above mentioned tetrandrine metabolizing CYP 450 such as CYP1A2, CYP2D, CYP2C6, and CYP2E1 in Wistar rats. Among these, CYP2D and CYP1A2 mRNA levels were increased in a dose-dependent manner with CP treatment. The mRNA expression of CYP2C6 and CYP2E1 was not significantly changed with CP treatment. As tetrandrine is the major alkaloid present in CP and the induction of CYP2D after CP administration may also induce the bio-activation of tetrandrine and produce the reactive intermediate. This bio-activation mediated reactive metabolite production may be responsible for the infiltrative changes in the liver, kidney, and lung after CP (1000 mg/kg) treatment, which was supported by LC-MS studies also.

Improvement of liver antioxidant defense without change in MDA level up to CP 500 mg/kg treatment is an indication of less oxidative stress in the liver. An increase in MDA level in CP (1000 mg/kg) indicates the increased lipid peroxidation in the liver. This may be due to the increased reactive metabolite generated through the induction of CYP2D. CP (50 and 500 mg/kg) treatment could optimize the level of antioxidant defense enzyme and reactive intermediate generated through herbal drug metabolism by CYP2D.

Based on the present study, the safer animal dose CP 500 mg/kg was converted to HED of 60 kg adult human as 61 mg extract/kg/day will be safer for 28 days, hence 60 kg adult can take 3.7 g extract per day. The yield of plant root extract was found to be 15%, i.e., 3.7 g plant extract was isolated from 25 g CP root powder. Hence, an adult human can safely intake 25 g CP root powder per day for 28 days.

The clinical therapeutic administration of CP is well within the safer dose, CP root ingredient of "Hinguvachadi Choornam" taken 4 g daily in hot water and "Patoladi gana" decoction in Ashtangahridaya administered 8 g/day CP root among six other ingredients.[2-4] The present study reveals that the current clinical therapeutic dose of CP is well within the safer dose levels.

CONCLUSION

The present study based on biochemical, LC-MS-based metabolite, histopathological, and mRNA expression studies revealed that CP up to 500 mg/kg treatment was safe for short-term 28 days oral administration in Wistar rats. Moreover, the current clinical therapeutic dose of CP administration is also well within the safer dose. The observed histopathological changes in CP (1000 mg/kg) may be due to the oxidative stress induced by the reactive metabolites generated through CYP2D-mediated bio-activation of molecules like tetrandrine.

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Nil.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Valiathan MS. The Legacy of Charak. Chennai: Orient Longman Private Ltd.; 2003. p. 365-7.
2. Lalithamma K. Pharmacopoeia. Thiruvananthapuram, Malayalam ed. Publication Division, Ayurveda College; 1996. p. 138-9.
3. Shine VJ, Latha PG, Shyamal S, Suja SR, Anuja GI, Sini S, *et al.* Gastric antisecretory and antiulcer activities of *Cyclea peltata* (Lam.) Hook. f. & Thoms. in rats. J Ethnopharmacol 2009;125:350-5.
4. Anonymous, The Ayurvedic Formulary of India Part-I, Vol.-II, Second ed. Government of India, Ministry of Health and Family Welfare, Department of Ayurveda, Yoga Naturopathy, Unani, Siddha & Homeopathy, New Delhi, 2003.
5. Sarada S, Nair SG, Reghunath BR. Quantification of medicinally valuable weeds in oil palm plantations of Kerala. J Trop Agric 2002;40:19-26.
6. Ramachandran VS, Nair VJ. Ethnobotanical studies in Cannanore district, Kerala State (India). J Econ Taxon Bot 1981;2:65-72.
7. Prakash JW, Raja RD, Anderson NA, Williams C, Regini GS, Benser K, *et al.* Ethnomedicinal plants used by kani tribes of Agasthiyarmalai biosphere reserve, south Western Ghats. Indian J Tradit Know 2008;7:410-3.
8. Kumar Y, Haridasan K, Rao RR. Ethnobotanical notes on certain medicinal plants among some Garo people around Balphakram Sanctuary in Meghalaya. Bull Bot Surv India 1980;22:161-5.
9. Silja VP, Varma KS, Mohan KV. Ethnomedicinal plant knowledge of Mullu kuruma tribe of Wayanadu district, Kerala. Indian J Tradit Know 2008;7:604-12.
10. Rastogi RP, Mehrotra BN. Cyclea peltata (Menispermaceae). In: Rastogi RP, editor. Compendium of Indian Medicinal Plants. New Delhi: National Institute of Science Communication and Lucknow, India: Central Drug Research Institute; 1999;2:237-40.
11. Reddi TV, Prasanthi S, Ramarao BV. Medicinal and aromatic plants of India. In: Khan IA, Khanum A, editors. Role of Biotechnology in Medicinal and Aromatic Plants. Vol. XII. Hyderabad: Ukaaz Publications; 2005. p. 63-70.
12. Shine VJ, Latha PG, Suja SN, Anuja GI, Raj G, Rajasekharan SN. Ameliorative effect of alkaloid extract of *Cyclea peltata* (Poir.) Hook. f. & Thoms. roots (ACP) on APAP/CCl4 induced liver toxicity in Wistar rats and *in vitro* free radical scavenging property. Asian Pac J Trop Biomed 2014;4:143-51.
13. Shine VJ, Anuja GI, Latha PG, Suja SR, Raj G. Bioassay guided fractionation of Cyclea peltata using *in vitro* RAW 264.7 cell culture, antioxidant assays and isolation/determination of bioactive compound tetrandrine. J Ayurveda Integr Med 2020;11:281-6.
14. Chen WC, Hayakawa S, Yamamoto T, Huang LW, Liu IM, Cheng JT. The plasma glucose lowering action of tetrandrine in streptozotocin-induced diabetic rats. J Pharm Pharmacol 2004;56:643-8.
15. Li SY, Ling LH, Teh BS, Seow WK, Thong YH. Anti-inflammatory and immunosuppressive properties of the bis-benzylisoquinolines: *In vitro* comparisons of tetrandrine and berbamine. Int J Immunopharmacol 1989;11:395-401.
16. Cao ZF. Scavenging effect of tetrandrine of active oxygen radicals. Planta Med 1996;62:413-4.
17. Qian JQ. Cardiovascular pharmacological effects of bisbenzylisoquinoline alkaloid derivatives. Acta Pharmacol Sin 2002;23:1086-92.
18. Xie QM, Tang HF, Chen JQ, Bian RL. Pharmacological actions of tetrandrine in inflammatory pulmonary diseases. Acta Pharmacol Sin 2002;23:1107-13.
19. Tainlin L, Tingyi H, Changqi Z, Peipei Y, Qiong Z. Studies of the chronic toxicity of tetrandrine in dogs: An inhibitor of silicosis. Ecotoxicol Environ Saf 1982;6:528-34.
20. Yan C, Xin-Ming Q, Li-Kun G, Lin-Lin L, Fang-Ping C, Ying X, *et al.* Tetrandrine-induced apoptosis in rat primary hepatocytes is initiated from mitochondria: Caspases and endonuclease G (Endo G) pathway. Toxicology 2006;218:1-2.
21. Jin H, Li L, Zhong D, Liu J, Chen X, Zheng J. Pulmonary toxicity and metabolic activation of tetrandrine in CD-1 mice. Chem Res Toxicol 2011;24:2142-52.
22. Tian Y, Shen S, Jiang Y, Shen Q, Zeng S, Zheng J. CYP3A5 mediates bioactivation and cytotoxicity of tetrandrine. Arch Toxicol 2016;90:1737-48.
23. Qi XM, Miao LL, Cai Y, Gong LK, Ren J. ROS generated by CYP450, especially CYP2E1, mediate mitochondrial dysfunction induced by tetrandrine in rat hepatocytes. Acta Pharmacol Sin 2013;34:1229-36.
24. Anuja GI, Shine VJ, Latha PG, Suja SR. Protective effect of ethyl acetate fraction of Drynaria quercifolia against CCl_4 induced rat liver fibrosis via Nrf2/ARE and NFκB signalling pathway. J Ethnopharmacol 2018;216:79-88.
25. Kwan YP, Saito T, Ibrahim D, Al-Hassan FM, Ein Oon C, Chen Y, *et al.* Evaluation of the cytotoxicity, cell-cycle arrest, and apoptotic induction by *Euphorbia hirta* in MCF-7 breast cancer cells. Pharm Biol 2016;54:1223-36.
26. Organization for Economic Cooperation and Development (OECD). Repeated dose 28-day oral toxicity study in rodents. In: OECD Guideline for the Testing of Chemicals. Sec. 4. Paris, France: Health Effects; 2008. p. 407.
27. Nair AB, Jacob S. A simple practice guide for dose conversion between animals and human. J Basic Clin Pharm 2016;7:27-31.
28. Metabolomics Service Protocol Sample Preparation for Tissues. Glasgow Polyomics; 2013. Available from: http://www.polyomics.gla.ac.uk/assets/downloads/MSMetabolomicsPrepCells-Aug2013.pdf. [Last accessed on 2021 May 14].
29. Raghava RD. Statistical Techniques in Agricultural and Biological Research. New Delhi: Oxford & IBH Publishing Co.; 1987. p. 200.
30. Ernst E. Harmless herbs? A review of the recent literature. Am J Med 1998;104:170-8.
31. Hosseinzadeh CA, Bidmeshkipour A, Gholinezhad CM. Total antioxidant capacity and malondialdehyde levels in seminal plasma among the varicocele-suffering men. Sci J Ilam Univ Med Sci 2009;17:15-23.
32. Zuo L, Zhou T, Pannell BK, Ziegler AC, Best TM. Biological and physiological role of reactive oxygen species – The good, the bad and the ugly. Acta Physiol (Oxf) 2015;214:329-48.
33. Sun YF, Wink M. Tetrandrine and fangchinoline, bisbenzylisoquinoline alkaloids from *Stephania tetrandra* can reverse multidrug resistance by inhibiting P-glycoprotein activity in multidrug resistant human cancer cells. Phytomedicine 2014;21:1110-9.
34. Na DH, Ji HY, Park EJ, Kim MS, Liu KH, Lee HS. Evaluation of metabolism-mediated herb-drug interactions. Arch Pharm Res 2011;34:1829-42.