UGC MAJOR RESEARCH PROJECT FINAL REPORT

Protective efficacy of *Centella asiatica* L. (Apiaceae) on isoniazid induced toxicity in albino rats.

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BY

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Appendix – I

Objectives of the Project

The main objective of the Project is to assess the protective effect of *Centella asiatica* ethanolic leaves extract on isoniazid induced toxicity in albino rats by:

- Estimating the changes in alanine transaminase, aspertate transaminase, alkaline phosphatase lactate dehydrogenase, creatinine and bilirubin in serum.
- Estimating the lipid profiles, like total cholesteral, low density, lipoprotein (LDL), high density lipoprotein (HDL), very low density lipoprotein (VLDL) and triglycerides in serum.
- Estimating the total cholesterol, triglycerides, phospholipids, free fatty acids and protein in liver and kidney tissues.
- Estimating glycogen in liver.
- Analyzing the changes in the activity of lipid peroxidation (TBARS) and antioxidant enzymes such as superoxide dismutase (SOD), Catalase (CAT), Glutathione-S-transferase (GST) and reduced glutathione (GSH) in serum, liver and kidney of rats.
- Analyzing the haemetological parameters such as Red Blood Carpuscles (RBC), White Blood Corpuseles (WBC), Haemoglobin (Hb), Haematocrit (Ht), Mean Cell Haemoglobin (MEH) and Mean Cell Heamoglobin Concentration (MCHC).
- Examining the histological changes in liver and kidney.
- Observing the Transmission electron micrographs (TEM) of liver.
- Analyzing the phytochemical properties of ethanolic extract of *Centella asiatica* L.(Apiaceae) leaves using both qualitative and quantitative screening methods and *in vitro* free radical scavenging and chelating activity.
- To identify and isolate the active principles present in the ethanolic extract of *Centella asiatica* L.(Apiaceae) leaves and elucidate the bioactive molecules using GC-MS, FTIR and NMR (1H and 13C NMR).

Appendix – II

Previous conclusions from Mid-term report

PHASE I

Plant Material collection and identification

Centella asiatica L. (Apiaceae) used in this study was collected freshly from outskirts of Chidambaram, Cuddalore District. The plant was identified at the herbarium of Department of Botany, Annamalai University. The leaves were washed under running tap water to remove dirt and other debris. It was then spread under a clean shade for drying. The dried leaves were milled to coarse power using a mechanical grinder and stored in an air-tight container.

Ethanolic extraction of plant material

Approximately 1 kg of powered *Centella asiatica* L. (Apiaceae) was used for ethanolic extraction using Soxhlet apparatus. The dark green extract obtained was subjected to ultracentrifugation followed by micro-filtration. The final clear dark extract was then concentrated in a rotary evaporator under reduced pressure. The final dried extract was lyophilized and was stored in a glass vials at -20°C for further use. This extract was then subjected to preliminary qualitative and quantitative phytochemical analysis.

Percentage yield of plant extract

The percentage yield of the extract was determined gravimetrically using the dry weight of the crude extract obtained (X) and dry weight of plant powder used for the extraction (Y) by using the following formula:

Percentage yield = X/Y * 100

Qualitative and quantitative analysis

A. Qualitative screening

Phytochemical screening was carried out by using 1 gram of ethanolic extract of *Centella asiatica* L.(Apiaceae) leaves as described below (Harborne, 1973) [1]:

Detection of alkaloids **(Mayer's Test):**

The extracts was dissolved in dilute Hydrochloric acid and filtered. The filtrate was treated with Mayer's reagent (potassium mercuric iodide). Formation of yellow coloured precipitate indicates the presence of alkaloids.

Detection of phenols **(Ferric Chloride Test):**

Extract was treated with 3-4 drops of 10% ferric chloride solution. Formation of green colour indicates the presence of phenols.

***Detection of flavonoids* (Alkaline Reagent Test):**

Extracts were treated with few drops of sodium hydroxide solution. Formation of intense yellow colour, which becomes colourless on addition of dilute acid, indicates the presence of flavonoids.

***Detection of quinones*:**

The extract was treated with few drops of sulphuric acid. Formation of red colour indicates the presence of quinones

***Detection of tannins* (Gelatin Test):**

To the extract, 1% gelatin solution containing sodium chloride was added. Formation of white precipitate indicates the presence of tannins.

***Detection of saponins* (Foam Test):**

0.5 gm of extract was shaken with 2 ml of water. If foam produced persists for ten minutes it indicates the presence of saponins.

***Detection of terpenoids* (Salkowski test):**

The extract was added 2 ml of chloroform. Concentrated H S0 (3 ml) was carefully added to form a layer. A reddish brown colouration of the interface indicates the presence of terpenoids.

B. Quantitative screening

***Determination of Total Flavonoids* (Aluminum chloride colorimetric assay method):**

Total flavonoid contents were measured with the aluminum chloride colorimetric assay [2]. Aqueous and ethanolic extracts that has been adjusted to come under the linearity range i.e. (400µg/ml) and different dilution of standard solution of Quercetin (10-100µg/ml) were added to 10ml volumetric flask containing 4ml of water. To the above mixture, 0.3ml of 5% NaNO2 was added. After 5 minutes, 0.3ml of 10% AlCl3 was added. After 6 min, 2ml of 1 M NaOH was added and the total volume was made up to 10ml with distilled water. Then the solution was mixed well and the absorbance was measured against a freshly prepared reagent blank at 510 nm. Total flavonoid content of the extracts was expressed as percentage of Quercetin equivalent per 100 g dry weight of sample.

***Determination of total phenolic content*:**

The total phenolic content (TPC) assay was performed in accordance to Singleton *et al*., 1999, with modifications [2]. An aliquot of 0.5 ml of each sample was mixed with 1 ml of Folin-Ciocalteu reagent (10% in distilled water) in a universal bottle covered with aluminum foil. After 3 min, 3 ml of 1% sodium bicarbonate was added to each sample bottle, the universal bottles were cap-screwed and vortex. The samples were then incubated for 2 hr at

room temperature in darkness. The absorbance was measured at 760 nm spectrophotometrically (Genesys UV 20, US). A standard curve of gallic acid solutions (ranging from 0 μg ml^{-1} to 250 μg ml^{-1}) was used for calibration. The experiment was done in triplicate. Results were expressed as microgram of gallic acid equivalents (GAE) per milligram of extract (GAE; μg mg^{-1} dry extract).

Quantitative Estimation of Saponins:

Plant extract was dissolved in 80% methanol, 2ml of Vanilin in ethanol was added, mixed well and the 2ml of 72% sulphuric acid solution was added, mixed well and heated on a water bath at 60°c for 10min, absorbance was measured at 544nm against reagent blank [3].

Quantitative estimation of Alkaloids:

5 g of the sample was weighed into a 250 ml beaker and 200 ml of 10% acetic acid in ethanol was added and covered and allowed to stand for 4 h. This was filtered and the extract was concentrated on a water bath to one-quarter of the original volume. Concentrated ammonium hydroxide was added drop wise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitated was collected and washed with dilute ammonium hydroxide and then filtered. The residue is the alkaloid, which was dried and weighed [4].

Gas chromatography-mass spectrometry fingerprinting of crude extract

GC-MS analysis was done at National Chemical Laboratory Pune, Maharashtra, India. GC-MS sample was prepared by dissolving about 1 mg of *Centella asiatica* L. (Apiaceae) extract in 5 mL of methanol. Active extract was dissolved in HPLC grade methanol and subjected to GC and MS JEOL GC mate equipped with secondary electron multiplier. JEOL GCMATE II GC-MS (Agilent Technologies 6890N Network GC system for gas chromatography). The column (HP5) was used with fused silica 50 m x 0.25 mm I.D. Analysis conditions were 20 minutes at 100°C, 3 minutes at 235°C for column temperature, 240°C for injector temperature, helium was the carrier gas and split ratio was 5:4. The sample (1μl) was evaporated in a split less injector at 300°C. Run time was 22 minutes. The components were identified by gas chromatography coupled with mass spectrometry. Interpretation of mass spectra of GC-MS was done using the database of National Institute Standard and Technology (NIST) library search which is having more than 62,000 drug formulation. The mass spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST-08 and Wiley-08 libraries. The name, molecular weight and structure of the components of the test materials were validated.

LC-MS metabolomic fingerprinting of crude extract

In the present work the RP-HPLC (Shimadzu model – LA 3000) with semi preparative C18 HPLC column of Pheomenex (250 x10mm, 4 µm particle size, and 90Å pore size) and analytical column Zorbax C18 (4.6 x250mm, 5 µm particle size, and 80Å pore size) were used for the purification. Solevent system used here is Acetonitrile and Water containing (0.1%) TFA. A three step gradient elution was performed using of 0.1% TFA/water and 0.1% (v/v) TFA in 50% acetonitrile: 0–100% (60 min) held at 100% for 5 min and brought back to 0% (100–0%). Injection volume was 0.5 microlitres. Fractions were collected automatically using the fraction collector (FRC-10-A, Shimadzu model). The absorbance of the fractions was monitored at 280 nm and the peaks were compared with the spectrum of known components in NIST-08, Wiley-08, NAPRALET and CHEMSPIDER databases. The compound names, molecular weight ant the structure of the compound identified in the crude extract were validated.

***In vitro* free radical scavenging activity**

Hydroxyl radical scavenging activity was measured by studying the competition between deoxyribose and the ethanolic leaf extract of *Centella asiatica L. (Apiaceae)* by Fe_3^+–Ascorbate–EDTA–H_2O_2 system (Fenton reaction) according to the method of Elizabeth and Rao [5]. The generation of $OH^{\bullet}$ is detected by its ability to degrade deoxyribose to form products, which on heating with TBA forms a pink colored chromogen. The absorbance of the supernatant was read in a spectrophotometer at 535 nm. The efficiency of *Centella asiatica* L. (Apiaceae) ethanolic leaf extract was compared with dimethyl sulphoxide (DMSO) as standard. Superoxide anion ($O_2^{\bullet-}$) scavenging activity of *Centella asiatica* L. (Apiaceae) ethanolic leaf extract was determined by the method of Liu *et al*., 1997 [6]. Superoxide anion that is derived from dissolved oxygen through the PMS/NADH coupling reaction reduces NBT and absorbance was read in spectrophotometer at 560 nm. The efficiency of *Centella asiatica* L. (Apiaceae) ethanolic leaf extract was compared with ascorbic acid as standard. The effect of *Centella asiatica* L. (Apiaceae) ethanolic leaf extract on $DPPH^{\bullet}$ was assayed using the method of Brand-Williams *et al*., 1995 [7]. $DPPH^{\bullet}$ is a stable free radical and accepts an electron, or hydrogen radical to become a stable diamagnetic molecule. $DPPH^{\bullet}$ reacts with an antioxidant compound that can donate hydrogen and gets reduced. The change in color (from deep violet to light yellow) was measured at 517 nm. The efficiency of *Centella asiatica* L. (Apiaceae) ethanolic leaf extract was compared with BHT as standard. The nitric oxide radical scavenging capacity of the *Centella asiatica* L. (Apiaceae) ethanolic extract was measured by Griess reaction [8]. Various concentrations of *Centella asiatica* L. (Apiaceae) ethanolic leaf extract (12.5, 25, 50, 100 and 200 µg/ml in

95% ethanol) were prepared. Sodium nitroprusside (1.5 mL, 10 mM) in phosphate buffer was added to 0.5 mL different concentrations of the extract. The reaction mixture was incubated at 25°C for 150 min. After incubation, 0.5 mL aliquot was removed and 0.5 mL of Griess reagent (1% (w/v) sulfanilamide, 2% (v/v) H3PO4 and 0.1% (w/v) naphthylethylene diamine hydrochloride) was added. The absorbance was measured at 546 nm. Ascorbic acid was used as reference standard and was treated the same way as that of the extract. Sodium nitroprusside in PBS (2 mL) was used as control. The improved technique for the generation of $ABTS^{\bullet+}$ involves the direct production of the blue/green $ABTS^{\bullet+}$ chromophore through the reaction between $ABTS^{\bullet+}$ and potassium persulphate [9]. The reaction mixture consisted of 0.5 mL of 15 µM H_2O_2, 0.5 mL of 7 mM ABTS and 50 mM sodium phosphate buffer, pH 7.5 and varying concentrations of *Centella asiatica L. (Apiaceae)* ethanolic leaf extract (12.5, 25, 50, 100 and 200 µg/ml). The blank contained water in place of *Centella asiatica L. (Apiaceae)* ethanolic leaf extract. The absorbance was read in spectrophotometer at 734 nm and compared with standard ascorbic acid. Decreased absorbance of the reaction mixture in all the assays indicated increased radical scavenging activity. The % of scavenging or inhibition was calculated according to the following formula:

% of scavenging or inhibition = $[(A_0 - A_1)/A_0] \times 100$

Where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of *Centella asiatica L. (Apiaceae)* ethanolic leaf extract or ascorbic acid/DMSO.

Iron chelating activity

The method of Benzie and strain (1996) was adopted for the assay [10]. The principle is based on the formation of O-Phenanthroline-Fe_2^+ complex and its disruption in the presence of chelating agents. The reaction mixture containing 1 ml of 0.05% O-Phenanthroline in methanol, 2 ml ferric chloride (200µM) and 2 ml of various concentrations ranging from 10 to 500µg was incubated at room temperature for 10 min and the absorbance of the same was measured at 510 nm. EDTA was used as a classical metal chelator. The experiment was performed in triplicates.

% of chelating activity = sample OD/Control OD x 100.

***In vitro* toxicity tests**

Drug preparation

The *Centella asiatica L. (Apiaceae)* extract was dissolved in 0.05% (v/v) of Dimethyl Sulfoxide (DMSO) and it did not affect cell survival.

PBMC proliferation test

Blood samples from healthy volunteers were collected by venepuncture and transferred into

15 ml heparin coated test tubes. It was diluted at 1:1 ratio with PBS, layered onto Ficoll-Histopaque 1077 at a volume ratio of 3:1 and centrifuged at 1,000 x g for 30 min. During the centrifugation the PBMCs moved from the plasma and were suspended in the density gradient, isolating them from erythrocytes and granulocytes. The PBMCs layer was removed and then washed twice with PBS. The supernatant was then removed and the cells were resuspended in complete CDMEM medium supplemented with 1 mM L-glutamine, 100 Units/ml penicillin and 0.1 mg/ml streptomycin, 10% inactivated FCS, and adjusted to pH 7.2 by the addition of 15 mM HEPES. Cell viability was determined by the trypan-blue dye exclusion method. (Results not included in paper)

In vitro Cell viability test

The viability of cells was assessed by MTT assay using primary lymphocyte cells [11]. The PBMC cell density used in the cell viability study was 1 x 10^5 cells/ well of the 96-well tissue culture plate. Dose-response 1- 1000 µg/ml between percentage of cell viability and concentrations of the extracts were constructed.

In vitro hemolytic assay

In vitro hemolytic activity assay was performed according to the method described by Bulmus *et al*., 2003 [12]. Briefly freshly collected human red blood cells were taken and washed three times with 150 mM NaCl by centrifugation method at 2500 rpm for 10 minutes.The serum was removed and the cells were suspended in 100 mM sodium phosphate buffer. Nine different concentrations (0,5,10,30,50,100,200,300, 400µg/ml) of extracts were mixed with 200 µL of RBC solutions and the final reaction mixture volume was made up to 1 ml by adding sodium phosphate buffer. The reaction mixture was then placed in water bath for 1 hour at 37°C. After the incubation time the reaction was collected and the optical density was measured at 541 nm.

Statistical analysis

The data were expressed as mean ± SD (n = 3).

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PHASE II

Animals

Male Wistar rats of body weight 180–200 g were used for this study. The animals were maintained in The Animal house, Annamalai University, Annamalainagar, Chidambaram, India and fed on standard pellet diet and water *ad libitum*. The animals were housed in polycarbonate cages in a room with a 12 h day–night cycle, temperature of 22±20 °C, and humidity of 45–64%. The protocol of this study was approved by the Institutional Ethical Committee of Annamalai University.

Experimental Design

The rats were divided into eight groups each comprising of six rats.

Group 1: Normal control rats (received saline and 0.5% DMSO);

Group 2: INH treated rats (50mg/kg body weight);

Group 3: INH treated rats (50mg/kg body weight) were given *Centella asiatica* ethanolic leaves extract (20 mg/kg body weight) (pre-treatment before 1 hour);

Group 4: INH treated rats (50mg/kg body weight) were given *Centella asiatica* ethanolic leaves extract (40 mg/kg body weight) (pre-treatment before 1 hour);

Group 5: INH treated rats (50mg/kg body weight) were given *Centella asiatica* ethanolic leaves extract (60 mg/kg body weight) (pre-treatment before 1 hour);

Group 6: INH treated rats (50mg/kg body weight) were given *Centella asiatica* ethanolic leaves extract (100 mg/kg body weight);

Group 7: *Centella asiatica* ethanolic leaves extract treated rats (100 mg/kg body weight);

Group 8: INH treated rats (50mg/kg body weight) were given silymarin (50 mg/kg body weight) (pre-treatment before 1 hour).

All the treatments were administered orally using an intragastric tube daily for a period of 30 days [1,2]. The experiment was terminated at the end of 30 days and the animals were fasted overnight, weighed and sacrificed by cervical decapitation. Blood was collected in EDTA tubes for the estimation of haematological parameters. Fresh blood was centrifuged to collect serum for glucose and biochemical parameters.

Estimation of blood glucose

Fresh blood was collected in 10% tricarboxylic acid for glucose estimation by Sasaki *et al.,* 1972 [3].

Evaluation of haematological parameters

The total red blood cells (RBC) and white blood cells (WBC), haemoglobin (Hb), haematocrit (Ht), mean cell haemoglobin (MCH/MEH) and mean cell haemoglobin concentration

(MCHC), were determined and calculated by adopting the method of Daecie and Lewis, 1984 [4].

Evaluation of serum antioxidant and biochemical parameters

In blood serum, the concentration of thiobarbituric acid-reactive substances (TBARS), super oxide dismutase (SOD), catalase (CAT) and reduced glutathione (GSH) were determined by the methods of Hogberg *et al.,* 1974, Kakkar *et al.,* 1973, Sinha 1972 and Moron *et al.,* 1979, respectively [5-8].

The total serum protein (TSP) concentration was determined using the Lowry *et al.,* 1951, method and the albumin concentration by the method of Doumas *et al*., 1971 [9,10]. The globulin concentration was calculated as the difference between albumin concentration and the total protein concentration. Activities of serum alanine transaminase (ALT) and aspartate transaminase (AST) were determined according to the method of Reitman and Frankel, 1957 [11]. Alkaline phosphatase (ALP) serum activity was determined as described by King and Armstrong, 1980 [12]. The level of total bilirubin was measured using the method of Malloy and Evelyn, 1937 [13]. The levels of urea and creatinine were determined using the method of Patton and Crouch, 1977 [14].

Statistical Analysis

All the results were expressed as the mean ±S.D. for six animals in each group. All the grouped data were statistically evaluated with SPSS\10.0 software. Statistical comparisons were performed by one way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT). The results were considered statistically significant if the *p* values were 0.05 or less.

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Results I

Table 1: Percentage yield of plant extract.

Plant	Solvent	Method	Weight of crude extract (g)	% yield
Centella asiatica	Ethanol	Soxhlet extraction	10.89	1.089

Table 2: Qualitative analysis of *Centella asiatica* ethanolic extract.

Secondary metabolites	Test	*Centella asiatica*
Alkaloids	Mayer's Test	+
Phenols	Ferric Chloride Test	+
Flavanoids	Alkaline Reagent Test	+
Quinones	Sulphuric acid test	+
Tannins	Gelatin Test	-
Saponins	Foam Test	+
Terpenoids	Salkowski test	+

+ Presence; - Absence

Table 3: Quantitative analysis of Secondary Metabolites in *Centella asiatica* ethanolic extract.

Plant	Phenols[#]	Flavanoids*	Saponins*	Alkaloids*
Centella asiatica ethanolic extract	12.40	2.70	11.00	3.20

[#]GAE μg mg^{-1} dry extract; * mg/g of crude extract

Table 4: Phytocomponents identified in the ethanolic extract of *Centella asiatica* by GC-MS.

R.T	Compound name	Molecular weight	Peak area %	Molecular formula	Pharmacology
5.183	Methyl pyromeconic acid (maltol)	126	2.24	$C_6H_6O_3$	Catalyst, anti-proliferative, antioxidant, flavour additive
8.617	Methoxy vinyl phenol	150.17	3.06	$C_9H_{10}O_2$	Anti gastritis and blood purifier
11.867	3',5'-Dimethoxyacetophenone	180	1.12	$C_{10}H_{12}O_3$	-
10.675	Beta-D-Ribofuranoside	283.24	12.59	$C_{10}H_{13}N_5O_5$	-
12.825	Cyclohexanecarboxylic Acid	192	28.02	$C_7H_{12}O_6$	Anti-inflammatory
25.733	5-methoxy-2,2,8,8-tetramethyl-acetate	372	1.01	$C_{22}H2_8O_5$	Antioxidant
27.275	Nobiletin	402.39	0.87	$C_{21}H_{22}O_8$	Antioxidant and anti-inflammatory

RT: Retention Time

Figure 1: GC-MS spectrum of *Centella asiatica* ethanolic leaf extract.

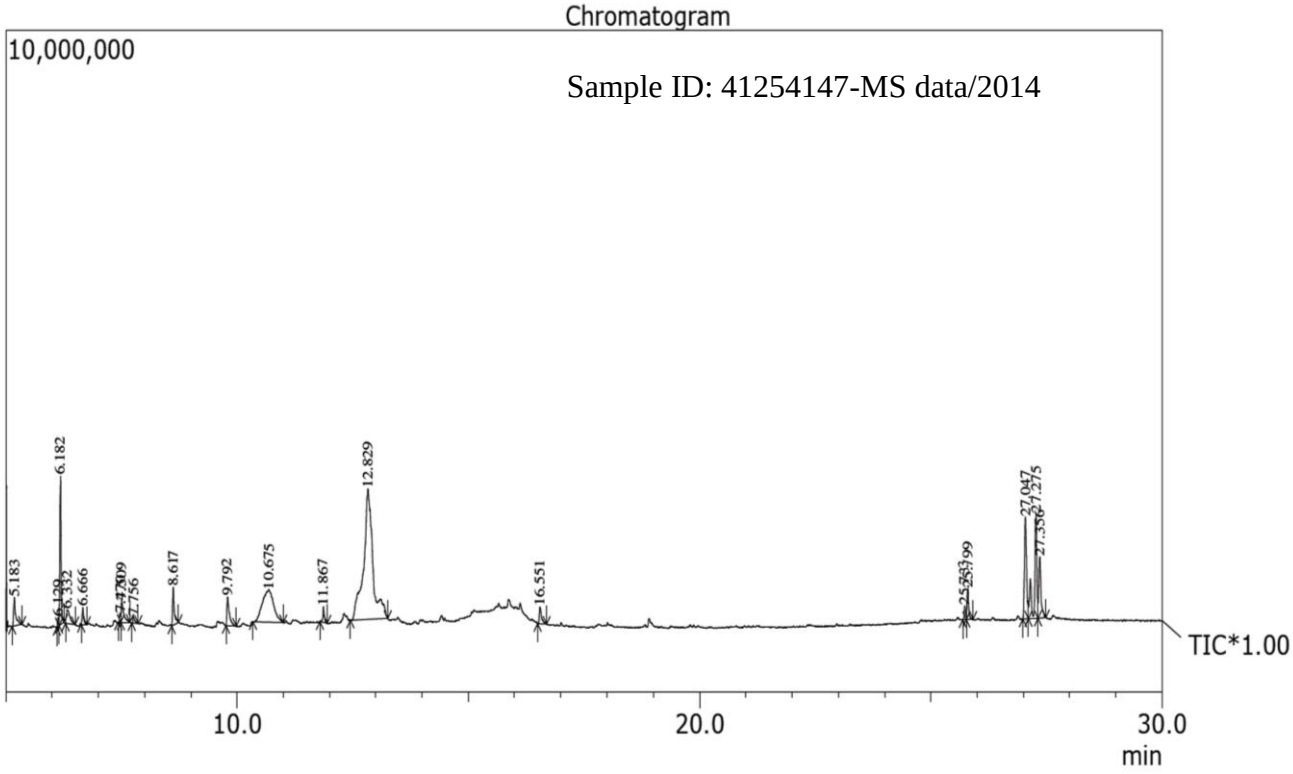


Table 5: LC-MS mass library results for the ethanolic leaf extract of *Centella asiatica*.

S. No.	Compound name	Molecularweight	Pharmacology
1	Maltol	150.17	Anti gastritis and blood purifier
2	3',5'-Dimethoxyacetophenone	180	Antioxidant
3	Papyriogenin A	466.308	Hepatoprotective
4	Asiatic acid	488.70	Neuro protective, anti-bacterial, anti-fungal, antioxidant, wound healing
5	Asiaticoside	959.12	wound healing
6	Madecassoside	975.1	wound healing
7	Madecassic acid	504.17	Anti-inflammatory

Figure 2: Effect of *Centella asiatica* (Ca) extract on (a): hydroxyl (OH•); (b): superoxide anion (O2•−); (c): DPPH•; (d): Nitric oxide and; e: ABTS•+ radical scavenging ability. (f): Chelating activity of *Centella asiatica* extract. The values are given as mean ± SD of three experiments in each group.

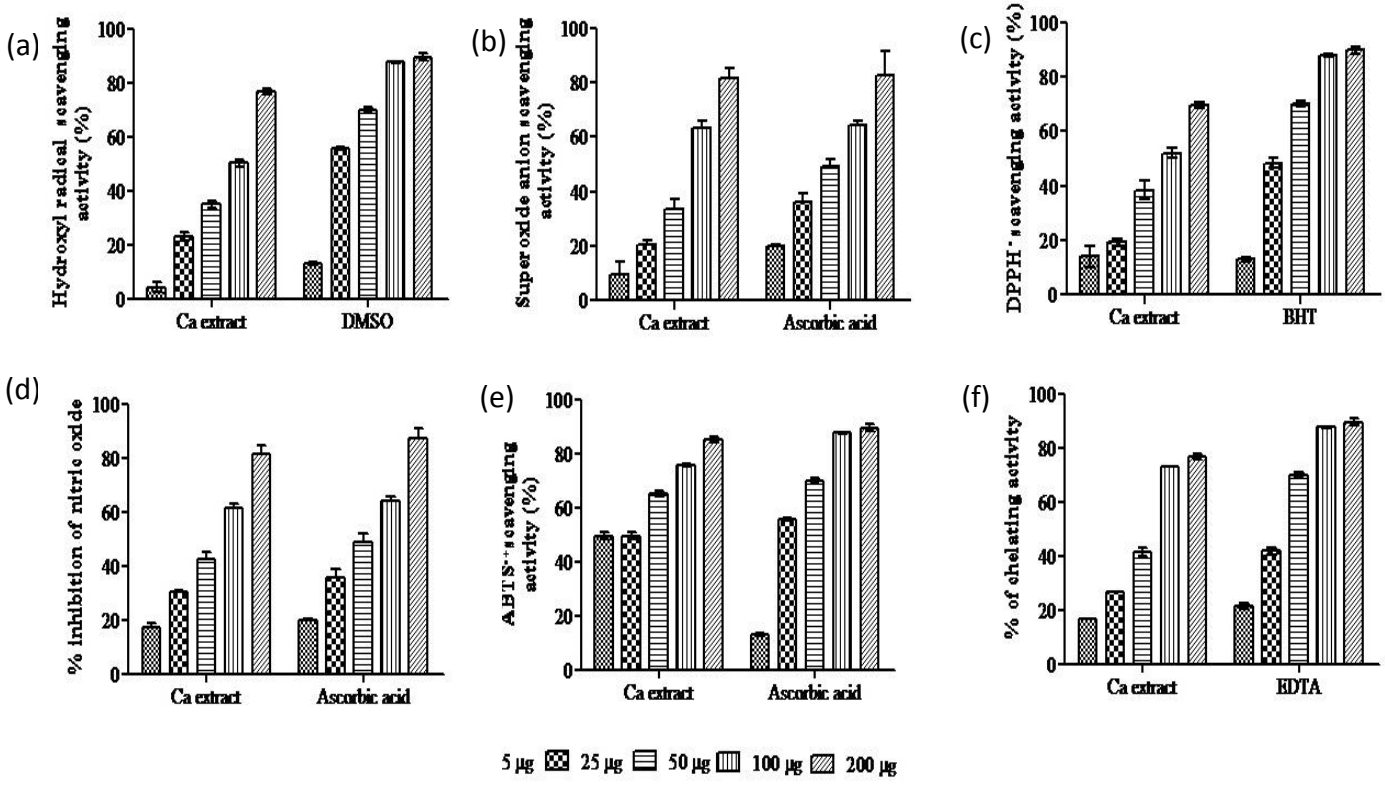
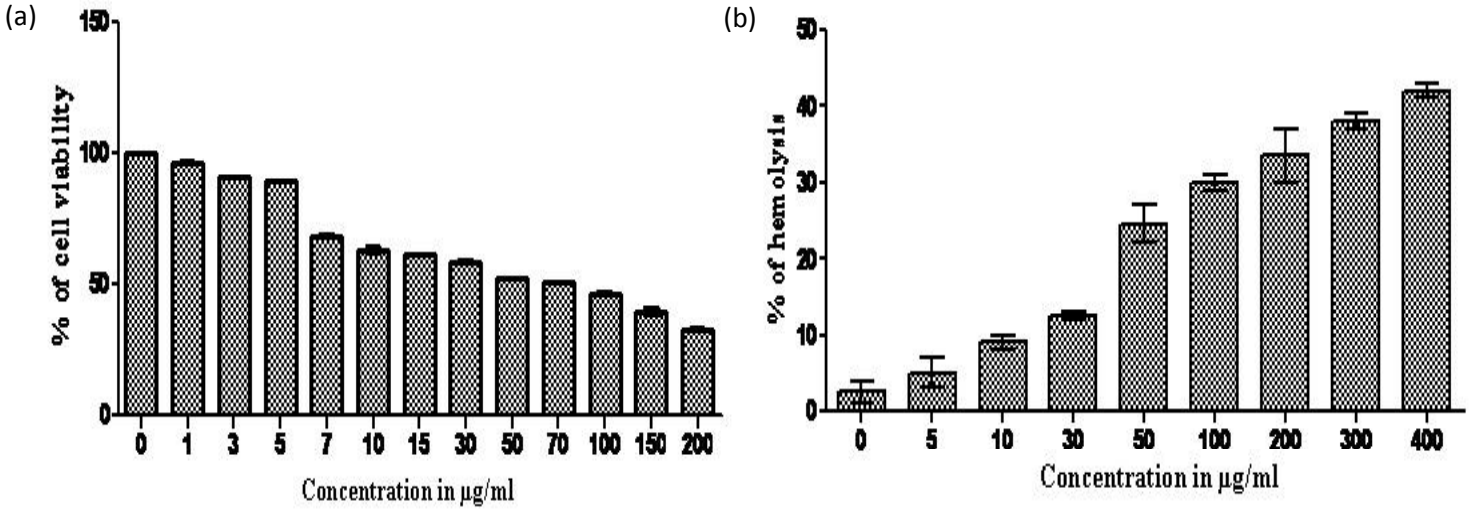


Figure 3: (a) Percentage cell viability of *Centella asiatica* extract in PBMC. Lethal concentration 50 (LC_{50}) value for *Centella asiatica* extract was found to be 69.17 ±3.2 µg/ml. (b) Percentage of hemolysis induced by *Centella asiatica* extract at concentrations ranging from 5-400 µg/ml. Lethal concentration 50 (LC_{50}) value for *Centella asiatica* extract was found to be 476.19 ±5.9 µg/ml. The values are given as mean ± SD of three experiments in each group.



Results II

Body weight

Table 1 shows the body weight of control and experimental animals in each group. The mean body weight was significantly decreased ($p < 0.05$) in isoniazid (INH) treated rats as compared to control animals. The body weight was found to be increased in INH treated rats with 1 hour pre-treatment with the reference drug silymarin (50 mg/kg body weight) and *Centella asiatica* ethanolic leaves extract in a concentration dependent manner. Extract at a concentration of 100 mg/kg body weight was found to be most effective.

Estimation of blood glucose

Table 2 shows the levels of blood glucose in normal and experimental rats. A significant increase ($p < 0.05$) in blood glucose level was observed in INH treated rats and it was found to be decreased after 1 hour pre-treatment with the reference drug silymarin (50 mg/kg body weight) and *Centella asiatica* (Ca) leaves ethanolic extract in a concentration dependent manner. Extract at a concentration of 100 mg/kg body weight was found to be most effective.

Evaluation of haematological parameters

The total RBC, Hb, Ht, MCH/MEH and MCHC, was found to be significantly decreased ($p < 0.05$) in INH treated group. Whereas, a significant increase ($p < 0.05$) in the white blood cells (WBC) level was found in the INH treated group. These levels were found to be restored in the reference drug silymarin (50 mg/kg body weight) and extract pre-treated groups in a concentration dependent manner, with 100 mg/kg body weight to be the most effective concentration (Table 3).

Evaluation of serum antioxidant and biochemical parameters

In blood serum of INH treated group, the concentration of TBARS was found to be significantly increased ($p<0.05$). A significant decrease ($p<0.05$) in the levels of SOD, CAT and GSH were also found in INH treated group. These levels were found to be restored in the reference drug silymarin (50 mg/kg body weight) and the extract pre-treated groups, in a concentration dependent manner, with 100 mg/kg body weight being the most effective concentration (Table 4).

The changes in the level of TSP, albumin, globulin, activities of serum ALT, AST and ALP and total bilirubin(liver function test); urea and creatinine (kidney function test), in INH and Ca extract treated groups are summarized in Table 5 and 6. A significant decrease ($p<0.05$) in the levels of TSP and albumin was observed in INH treated group. A significant increase ($p<0.05$) in the activities of ALT, AST and ALP, globulin, total bilirubin, urea and creatinine was also observed in INH treated group. These levels were found to be restored in the reference drug silymarin (50 mg/kg body weight) and the extract pre-treated groups, in a concentration dependent manner, with 100 mg/kg body weight being the most effective concentration.

Table 1: Body weight of control and experimental animals in each group (1st week and 4th week). Values are expressed as mean ±SD (n=6). Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT).

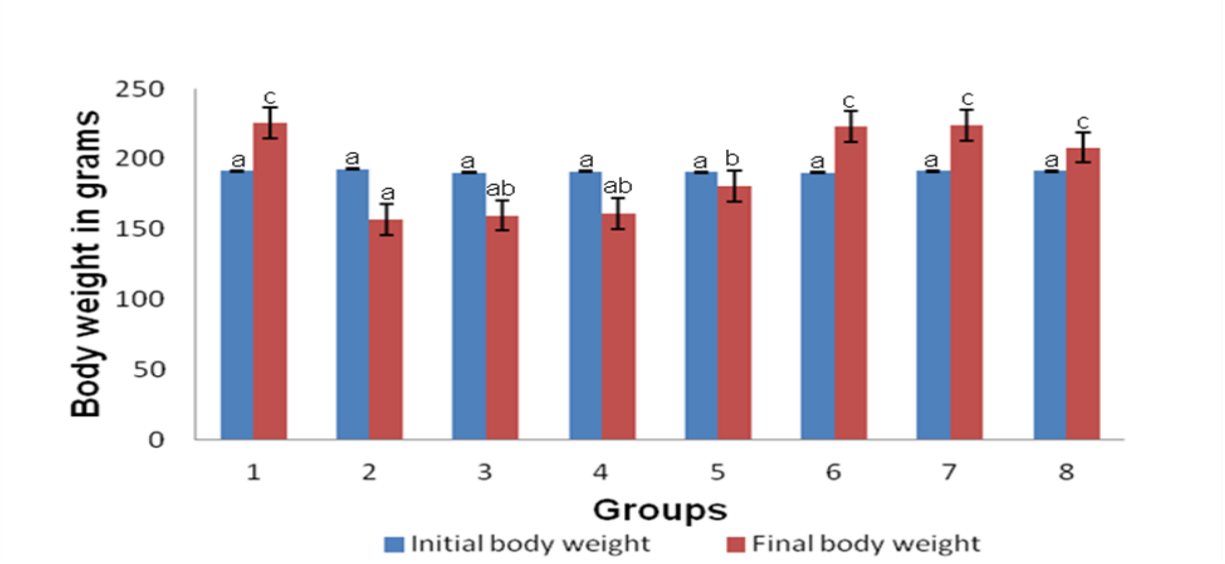


Table 2: Effect of *Centella asiatica* leaf ethanolic extract on blood glucose in control and experimental animals (1st week and 4th week). Values are expressed as mean ± SD (n=6). Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT).

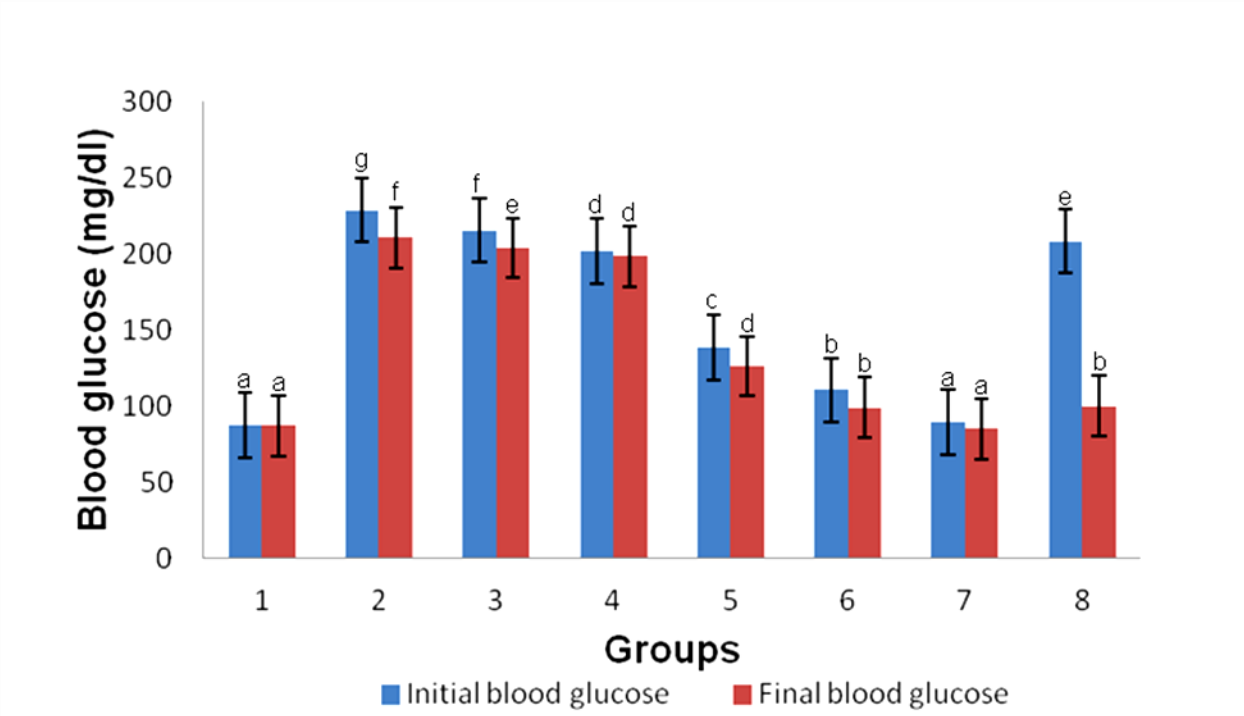
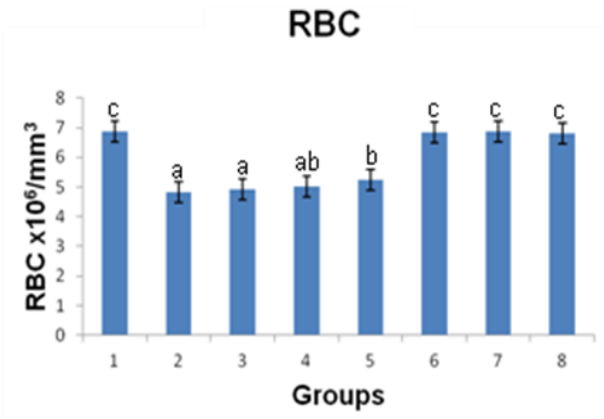
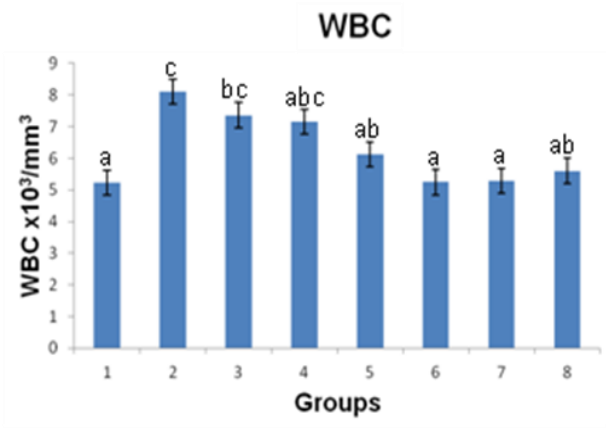
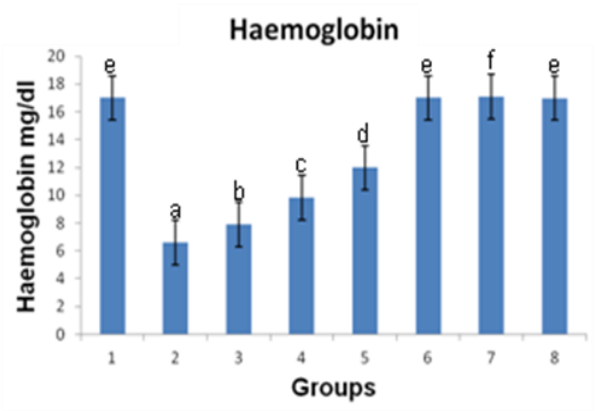
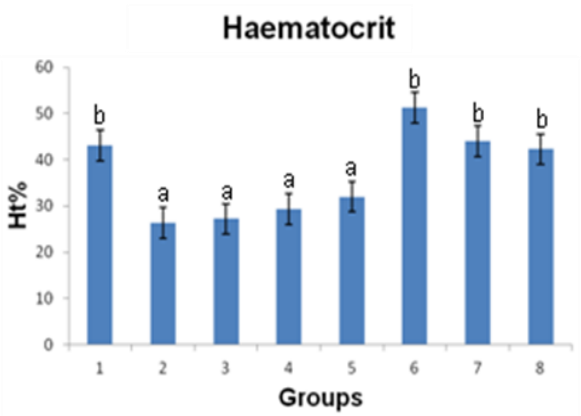


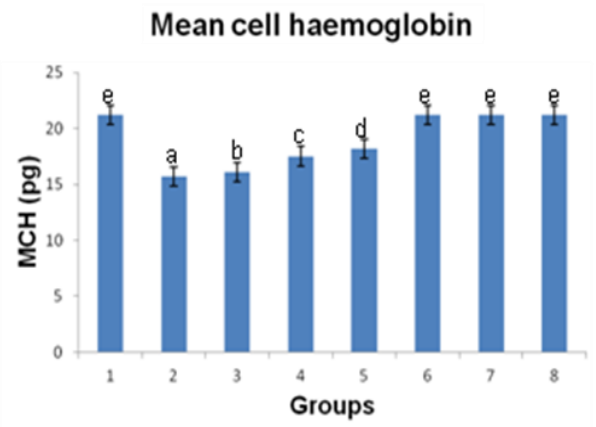
Table 3: Effect of *Centella asiatica* leaf ethanolic extract on haematological parameters in control and experimental animals (4th week). Values are expressed as mean ±SD (n=6). Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT).











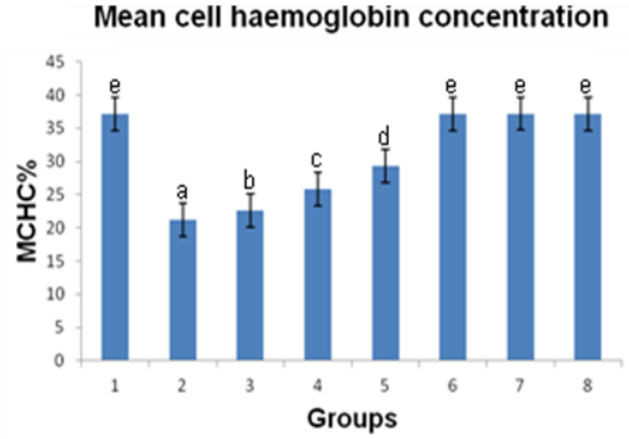
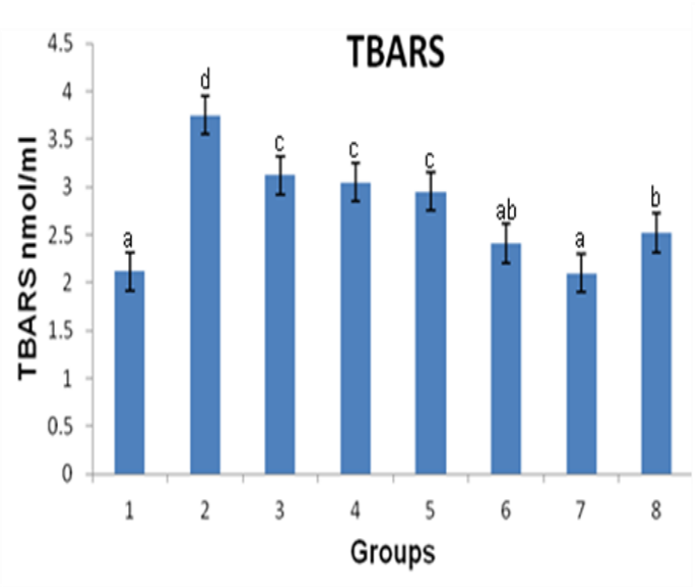
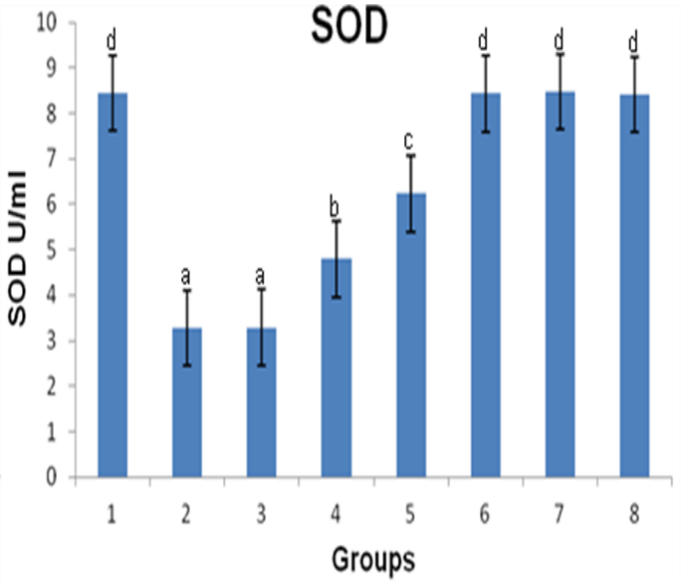
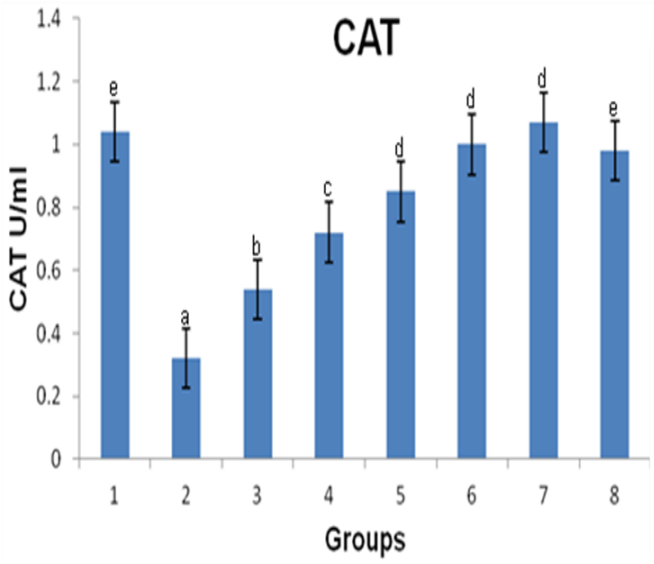


Table 4: Effect of *Centella asiatica* leaf ethanolic extract on TBARS, SOD, CAT and GSH in control and experimental animals (4th week). Values are expressed as mean ±SD (n=6). Values not sharing a common superscript letter differ significantly at $p < 0.05$ (DMRT).







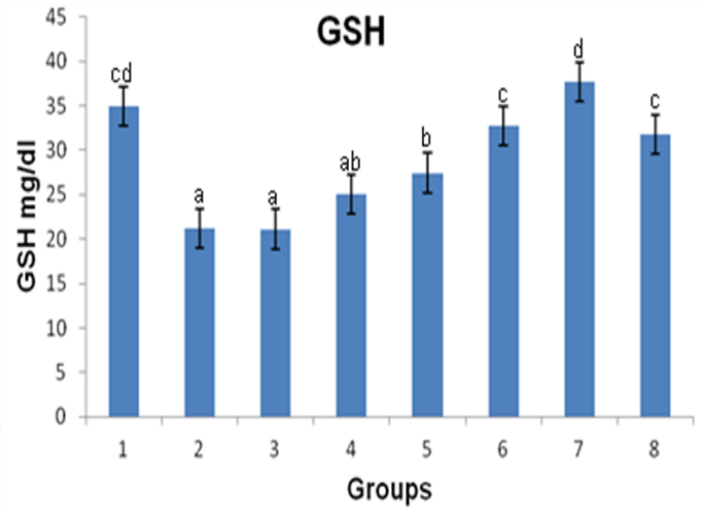
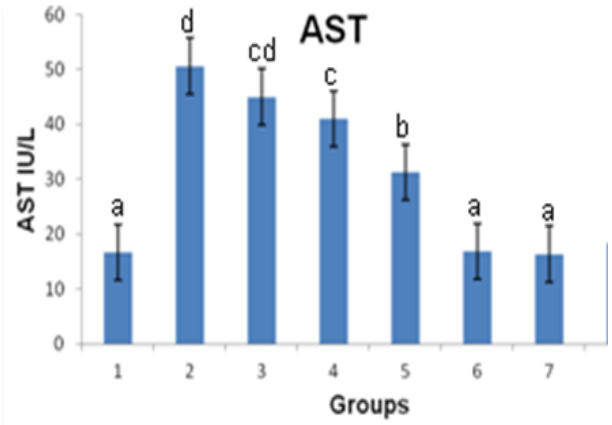
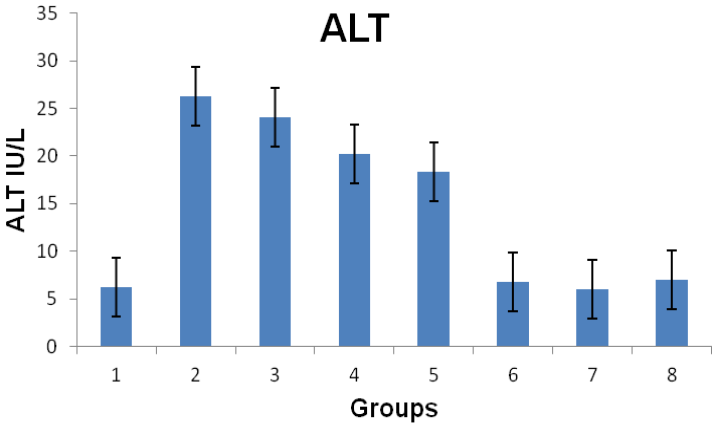
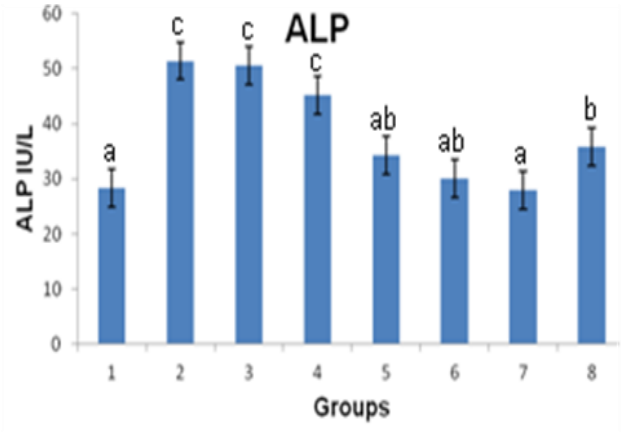
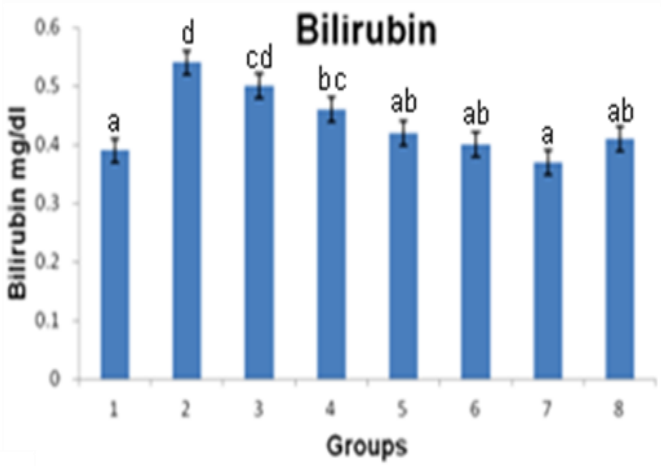


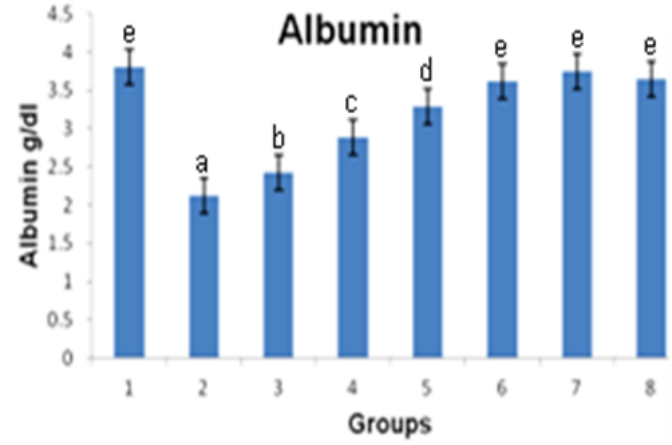
Table 5: Effect of *Centella asiatica* leaf ethanolic extract on liver function tests in control and experimental animals (4th week). Values are expressed as mean ±SD (n=6). Values not sharing a common superscript letter differ significantly at $p < 0.05$ (DMRT).

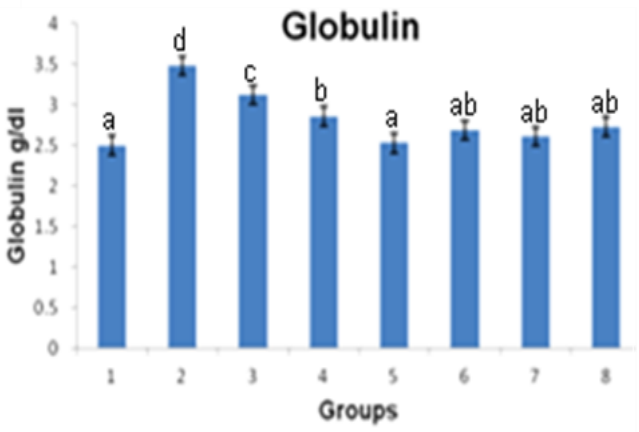












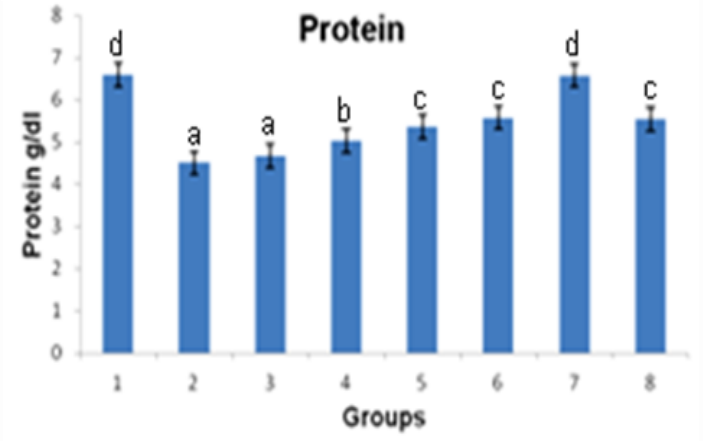
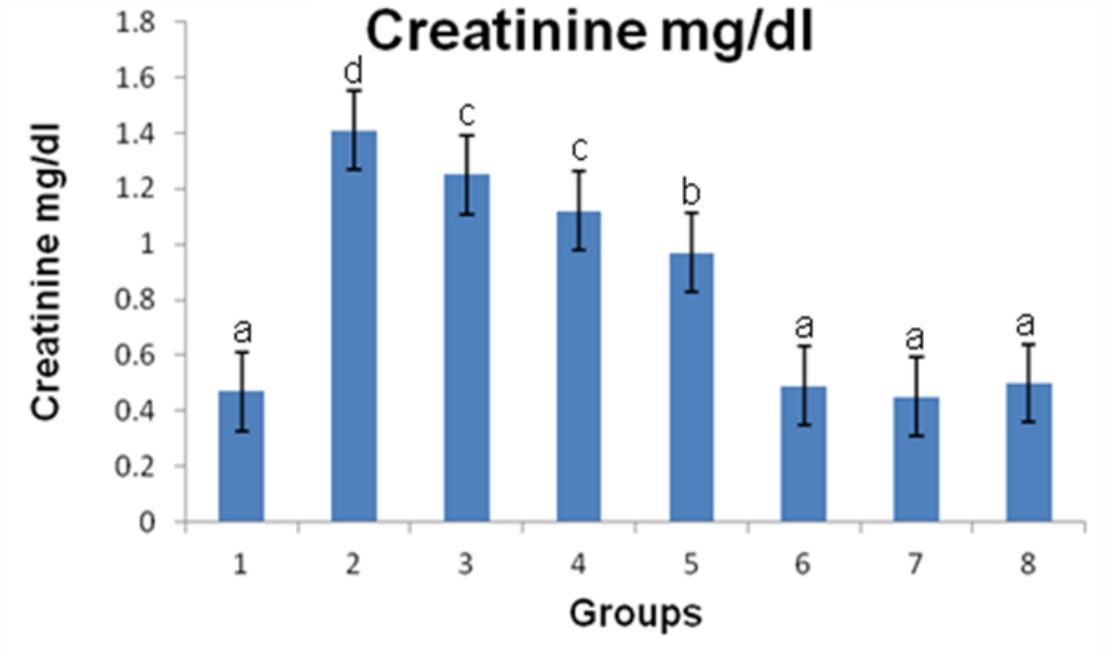
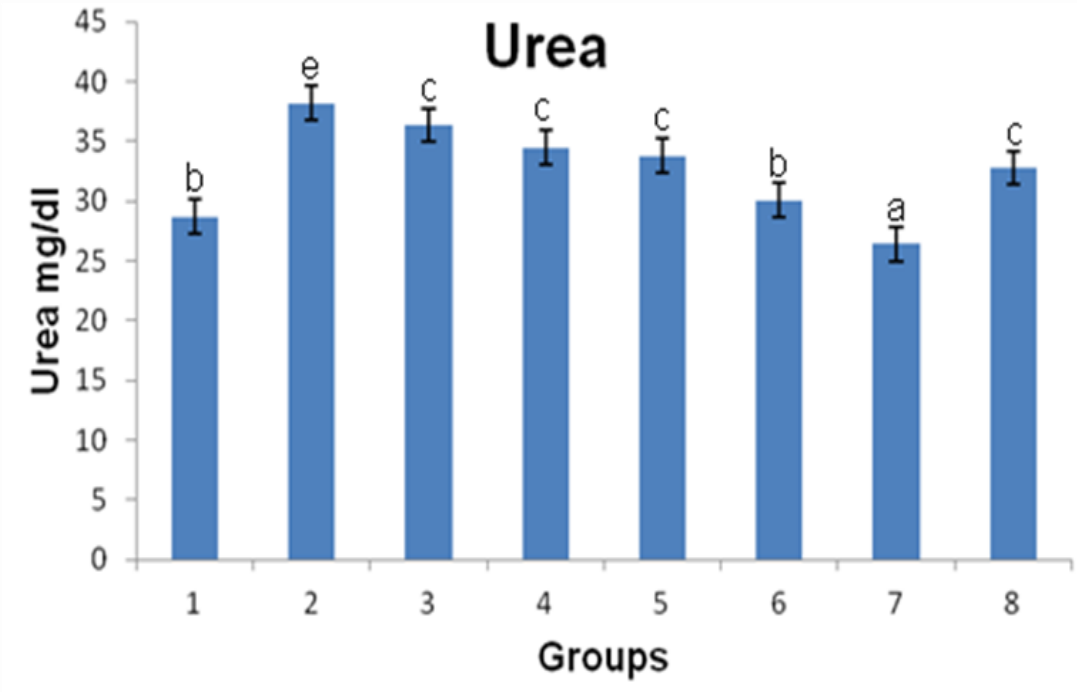


Table 6: Effect of *Centella asiatica* leaf ethanolic extract on kidney function tests in control and experimental animals (4th week). Values are expressed as mean ±SD (n=6). Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT).





Appendix – III

Work carried out in the final phase:

- Estimating the changes in serum alanine lactate dehydrogenase in serum, liver and kidney.
- Estimating the lipid profiles, like total cholesteral, low density, lipoprotein (LDL), high density lipoprotein (HDL), very low density lipoprotein (VLDL) and triglycerides in serum.
- Estimating the total cholesterol, triglycerides, phospholipids, free fatty acids, protein and glycogen in serum, liver and kidney tissues.
- Analyzing the changes in the activity of lipid peroxidation (TBARS) and antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione-s-transferase (GST) and reduced glutathione (GSH) in liver and kidney of rats.
- Levels of AST, ALT and ALP in liver and kidney tissues of rat.
- Examining the histological changes in liver and kidney.
- Observing the Transmission electron micrographs (TEM) of liver.
- The ethanolic extract of *Centella asiatica* L. (Apiaceae) leaves was subjected to FTIR and NMR (1H and 13C NMR) analysis.

MATERIALS AND METHODS

Plant Material collection and identification

Centella asiatica L. (Apiaceae) used in this study was collected freshly from outskirts of Chidambaram, Cuddalore District. The collected plant material was identified by Dr. D. Kumarasamy, Associate Professor, Botany Wing, DDE, Annamalai University, Chidambaram, Tamil Nadu, India. A voucher specimen (Herbarium No. DDE/HER/53) was deposited in the Department Herbarium for future reference. The leaves were washed under running tap water to remove dirt and other debris. It was then spread under a clean shade for drying. The dried leaves were milled to coarse power using a mechanical grinder and stored in an air-tight container.

Ethanolic extraction of plant material

Approximately 1 kg of powered *Centella asiatica* L. (Apiaceae) was used for ethanolic extraction using Soxhlet apparatus. The dark green extract obtained was subjected to ultracentrifugation followed by micro-filtration. The final clear dark extract was then concentrated in a rotary evaporator under reduced pressure. The final dried extract was lyophilized and was stored in a glass vials at -20°C for further use. This extract was then subjected to preliminary qualitative and quantitative phytochemical analysis.

FT-IR analysis of *Centella asiatica* leaves ethanolic extract

The FT-IR spectrum of DHC was recorded using AVATAR – 330 FTIR spectrophotometer (Department of Chemistry, Annamalai University). The sample was grained with KBr and Pellet technique was used to record the spectrum in cm-1.

The analysis of ethanolic extract of *Centella asiatica* leaves extract by RP-HPLC (Reversed Phase – High Performance Liquid Chromatography)

Chromatographic techniques are generally useful in the separation and purification of individual components consistituting the mixture. In RP-HPLC the stationary phase which is used are non-polar hydrocarbons or bonded hydrocarbons (C4, C8, and C18). In the present work the RP-HPLC (Shimadzu model – LA 3000)with semi preparative C18 HPLC column of Pheomenex (250 x10mm, 4 μm particle size, and 90Å pore size) and analytical column Zorbax C18 (4.6 x250mm, 5 μm particle size, and 80Å pore size) were used for the purification.

Solevent system used here is Acetonitrile and Water containing (0.1%) TFA. A three step gradient elution was performed using of 0.1% TFA/water and 0.1% (v/v) TFA in 50% acetonitrile: 0–100% (60 min), held at 100% for 5 min and brought back to 0% (100–0%).Fractions were collected automatically using the fraction collector(FRC-10-A,Shimadzu model) The absorbance of the fractions was monitored at 280 nm.

Molecular mass determination – ESI-MS

The molecular mass of the purified haemolymph samples were determined by electroscopy ionization mass spectrometer with an electrostatic ion spray source. A quadropole is generally used as analyzer in ESI-MS. The quadrapole is essentially a mass filter, which allows ions of a given m/z to pass through to reach the detector. The electron multiplier is a combination of AC and a DC voltage which facilitates the quadrupole to permit ions of a given (m/z) value to pass through at a given time. Calibration of these ions using known ions m/z values enables assignment of peaks unambiguously.

The analysis of ethanolic extract of *Centella asiatica* leaves extract by NMR

The NMR spectrum of *Centella asiatica* leaves extract was recorded on a BRUKER Avance III 400MHz for 1H, 100MHz for 13C NMR spectra (Department of Chemistry, Annamalai University). The two dimensional NMR spectrum was recorded using standard pulse sequences. The chemical shifts are noted in (δ) units PPM relative to the internal standard Tetra methyl silane (TMS) for 1H and 13C spectra. SHIMADZU UV-1650 PC digital spectrophotometer was used for the UV-Visible studies.

Animals

Male Wistar rats of body weight 180–200 g were used for this study. The animals were maintained in The Animal house, Annamalai University, Annamalainagar, Chidambaram, India and fed on a standard pellet diet and water *ad libitum*. The animals were housed in polycarbonate cages in a room with a 12 h day–night cycle, temperature of 22±20 °C, and humidity of 45–64%. The protocol of this study was approved by the Institutional Ethical Committee of Annamalai University (Proposal number: 1021, dated 06.08.2013).

Experimental Design

The rats were divided into eight groups, each comprising of six rats for the preliminary studies.

Group 1: Normal control rats (received saline and 0.5% DMSO);

Group 2: INH treated rats (50mg/kg body weight);

Group 3: INH treated rats (50mg/kg body weight) were given *Centella asiatica* ethanolic leaves extract (20 mg/kg body weight) (pre-treatment before 1 hour);

Group 4: INH treated rats (50mg/kg body weight) were given *Centella asiatica* ethanolic leaves extract (40 mg/kg body weight) (pre-treatment before 1 hour);

Group 5: INH treated rats (50mg/kg body weight) were given *Centella asiatica* ethanolic leaves extract (60 mg/kg body weight) (pre-treatment before 1 hour);

Group 6: INH treated rats (50mg/kg body weight) were given *Centella asiatica* ethanolic leaves extract (100 mg/kg body weight);

Group 7: *Centella asiatica* ethanolic leaves extract treated rats (100 mg/kg body weight);

Group 8: INH treated rats (50mg/kg body weight) were given silymarin (50 mg/kg body weight) (pre-treatment before 1 hour).

All the treatments were administered orally using an intragastric tube daily for a period of 30 days. The experiment was terminated at the end of 30 days and the animals were fasted overnight, weighed and sacrificed by cervical decapitation. Fresh blood was centrifuged to collect serum for biochemical parameters.

The effective groups from preliminary studies for *Centella asiatica* ethanolic extract on INH toxicity in rats were used to further understand the effectiveness of the optimum dose of *Centella asiatica* ethanolic extract (100 mg/kg body weight/day) in 0.5% DMSO (oral pre-treatment before 1 hour). Control and highest dose of *Centella asiatica* ethanolic extract (100 mg/kg body weight) administered groups were also used to understand the effect of *Centella asiatica* ethanolic extract through the different parameters under study. Group administered

with INH was considered as toxic alone group. Silymarin (50 mg/kg body weight) oral pre-treatment before 1 hour on INH toxicity was used as a positive control. All the treatments were orally administered with an intragastric tube daily for 30 days. This study was conducted to understand the effectiveness of *Centella asiatica* ethanolic leaves extract with optimum dose of 100mg/kg body weight/day against INH induced toxicity in albino rats.

For the experimental purposes, the groups were re-designated as follows:

Group 1 : Normal control rats (received saline and 0.5% DMSO)

Group 2 : INH treated rats (50mg/kg body weight)

Group 3 : INH treated rats (50mg/kg body weight) were given *Centella asiatica* ethanolic leaves extract (100 mg/kg body weight)

Group 4 : *Centella asiatica* ethanolic leaves extract treated rats (100 mg/kg body weight)

Group 5 : INH treated rats (50mg/kg body weight) were given silymarin (50 mg/kg body weight) (pre-treatment before 1 hour).

Fresh blood was centrifuged to collect serum for LDH and antioxidant level analysis. A portion of dissected out liver and kidney were washed in ice cold saline. Then these tissues were minced and homogenized (10% *w/v*) in 0.1 M Tris-HCl buffer (pH 7.4). The homogenates were then centrifuged at 3000 x g for 20 minutes at 4°C and the supernatant was utilized for various biochemical assays. After washing, a portion of liver and kidney were processed separately for biochemical analysis. Further, tissues were also fixed in fixatives for histological and/or TEM studies.

The following methodologies were used for carrying out experiments in control and experimental groups of albino rats:

S. No	Parameters & References	Estimation in	Techniques & instruments used
1.	LDH (King, 1965)	Serum, liver & kidney	Spectrophotometry
2.	Glycogen (Morales *et al.,* 1973)	Liver & kidney	Spectrophotometry
3.	Total cholesterol (Zlatkis *et al.,* 1953)	Serum, liver & kidney	Spectrophotometry
4.	Triglycerides (Foster and Dunn, 1973)	Serum, liver & kidney	Spectrophotometry
5.	Phospholipids (Zilversmit & Davis, 1950)	Serum, liver & kidney	Spectrophotometry
6.	VLDL-cholesterol (Friedwald et al., 1972)	Serum	Spectrophotometry
7.	High density lipoprotein (HDL) (Burstein *et al.,* 1970)	Serum	Spectrophotometry
8.	Low density lipoprotein (LDL) (Friedwald et al., 1972)	Serum	Spectrophotometry
9.	Free fatty acids (Falholt *et al.,* 1968)	Serum, liver & kidney	Spectrophotometry
10.	Protein (Lowry *et al.,* 1951)	liver & kidney	Spectrophotometry
11.	Lipid peroxidation (TBARS) (Hogberg *et al.,* 1974)	Serum, liver & kidney	Spectrophotometry
12.	Superoxide dismutase (SOD) (Kakkar *et al*., 1973)	Serum, liver & kidney	Spectrophotometry
13.	Catalase (CAT) (Sinha, 1972)	Serum, liver & kidney	Spectrophotometry
14.	Glutathione – S – transferase (GST) (Habig *et al.,* 1974)	Liver & kidney	Spectrophotometry
15.	Reduced glutathione (GSH) (Ellman,1959)	Serum, liver & kidney	Spectrophotometry
16.	AST (Reitman and Frankel, 1957)	Liver & kidney	Spectrophotometry
17.	ALP (King and Armstrong, 1980)	Liver & kidney	Spectrophotometry
18.	ALT (Reitman and Frankel, 1957)	Liver & kidney	Spectrophotometry
19.	Histological analysis	Liver & kidney	Haematoxylin and eosin (H& E)
20.	Transmission Electron microscopic studies(TEM)	Liver	----------

Histological studies

The tissue samples of liver, kidney and heart from all animal from each group were fixed for 48 hour in 10% formalin-saline and dehydrated by passing successfully in different mixture of ethyl alcohol, water, cleaned in xylene and embedded in paraffin. Sections of the tissues (5-6µm thick) were prepared by using a rotary microtone and stained with haematoxylin and eosin (H& E) dye, which was mounted in a neutral deparaffined xylene medium for microscopical observations.

Transmission electron microscopic (TEM) study

The ultrastructure of the liver was examined by Transmission Electron Microscopy.

Reagents

1. 3% glutaraldehyde
2. 0.1 M phosphate buffer (pH 7.2)
3. 1% osmium tetroxide
4. Ethanol
5. Uranyl acetate
6. Reynold's solution (sodium citrate and lead mitrate)

Procedure

A portion of freshly dissected rat liver was sliced into 1 mm^3 and stored in 3% glutaraldehyde (EM grade) in 0.1 M phosphate buffer (pH 7.2) for fixing (48 hours at 2-4°C). The samples were washed with 0.1 M phosphate buffer (pH 7.2), post fixed in 1% osmium tetroxide prepared in 0.1 M phosphate buffer (pH 7.2), dehydrated in a graded ethanol series, and embedded in epoxy resin. Ultrathin sections with 40–60nm thickness were cut using Leica ultramicrotome with a diamond knife (DiATOME). The ultrathin sections were taken on copper grid and stained with (double metallic) uranyl acetate and Reynold's solution (sodium citrate & lead mitrate). The sections were examined using a Philips Tecnai T12 (120 kV) Electron Microscope (Netherland). The images were acquired using a Gatan Image Filter (GIF; Ultrascan 10000 slow scan) with CCD camera 4Kx4K chip and were processed using Gatan software (T12).

Statistical Analysis

All data were analyzed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT), by using commercially available statistics software package (IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.). Results were presented as mean ± SD of 6 experiments. The value of $p < 0.05$ was regarded as statistically significant.

RESULTS

FT-IR analysis of *Centella asiatica* leaves ethanolic extract

FT-IR spectroscopy of CA ethanolic leaves extract (Figure 1) showed the presence of functional groups of secondary metabolite derivatives such as O–H stretch (3412.14 cm^{-1}), C–H stretch (2999.99 cm^{-1}; 2916.24 cm^{-1}), –C=C– stretch (2854.78 cm^{-1}), –C=C– stretch (1658.63 cm^{-1}), -C-C- stretch (in–ring) (1436.32 cm^{-1}; 1406.63 cm^{-1}), C–O stretch (1312.85 cm^{-1}; 1014.60 cm^{-1}), =C–H bend (951.32 cm^{-1}), O–H bend (931.05 cm^{-1}), C-H(bend) aromatic (894.91 cm^{-1}) and –C≡C–H: C–H bend (700.53 cm^{-1}). The peaks thus revealed the presence of alcohols, phenols, carboxylic acids, alkanes, aromatic rings, esters, ethers, alkenes and alkynes, respectively. These functional groups are the primary domains of compounds like phenolic, terpen, fatty acids, sterols and secondary alkaloids. Further CA extract analysis may reveal the presence of alcohol derivatives, phenolic, carboxylic acid, fatty acid derivatives and phenolic compounds as their bioactive constituents.

The analysis of ethanolic extract of *Centella asiatica* leaves extract by RP-HPLC (Reversed Phase – High Performance Liquid Chromatography)

HPLC-based activity profiling of plant extract, which combines physicochemical data recorded in online databases with biological information in parallel to the time-based HPLC fractionation. Sample of nearly 7 peaks (1 ml/min) was elated. Each fraction (P1-P7) was collected up to 100ml and the eluted fractions were freeze-dried. This also helped in identifying the active compound Asiatic acid, which has a retention time of 23.520. The fraction was collected separately and was then freeze dried for further verification (Figure 2A).

Molecular mass determination – ESI-MS

The ESI-MS (Figure 2B) confirmed the presence of Asiatic acid in the fraction (489.05 m/z).

The analysis of ethanolic extract of *Centella asiatica* leaves NMR (1H and 13C NMR)

Figure 3 A & B shows the 1H & 13C NMR of *Centella asiatica* ethanolic leaves extract fraction containing Asiatic acid (fraction pooled from HPLC fraction collector). The NMR of extract fraction revealed the presence of the compound Asiatic acid, which was confirmed with standard Asiatic acid (Figure 3 C & D).

Evaluation of lipid profile, phospholipids and free fatty acids in serum

The levels of total cholesterol (TC), LDL, VLDL, triglycerides (TG) and phospholipids were found to be significantly increased ($p<0.05$) in INH treated rats. A

significant decrease (p<0.05) in the HDL level was also found in the INH treated rats. The above abnormalities were found to be restored to near normal levels with 1 hour oral pre-treatment of *Centella asiatica* ethanolic leaves extract in a concentration dependent manner. Similar results were observed in silymarin (50 mg/kg body weight/day). Among all the concentrations administered for *Centella asiatica* extract, 100 mg/kg body weight/day was found to be most effective (Table 1).

Changes in tissue protein, and lipid content of liver and kidney

Table 2 shows the levels of tissue protein, free fatty acids (FFA), phospholipids (PL), total cholesterol (TC) and triglycerides (TG) in control and experimental rats in each group. A significant increase (p<0.05) in FFA, TC and TG and significant decrease in protein, PL levels were observed in liver and kidney of INH administered rats. *Centella asiatica* ethanolic leaves extract (100 mg/kg body weight/day) oral pre-treatment in INH rats significantly restored (p<0.05) the protein and lipid levels to near normal when compared to control rats. Similar effects were observed in silymarin (50 mg/kg body weight/day) treated INH intoxicated rats.

Changes in lipid peroxidation (TBARS) and antioxidant defense enzymes

Table 3 and 4 shows the levels of TBARS, SOD, CAT, and GST in control and experimental rats. An increase in tissue (liver and kidney) TBARS was observed in INH treated rats. A significant decrease (p<0.05) in tissue SOD, CAT, and GST were observed in rats administered with INH. The abnormalities seen in all the above parameters were significantly restored (p<0.05) to near normal levels by oral pre-treatment of *Centella asiatica* ethanolic leaves extract (100 mg/kg body weight/day). Similar results were observed in silymarin (50 mg/kg body weight/day) treated INH intoxicated rats.

Effect of *Centella asiatica* leaves ethanolic extract on LDH in serum

A significant decrease (p<0.05) in the levels of total LDH in serum, liver and kidney was observed in INH treated rats when compared to control rats (Figure 4 A, B & C). Administration of *Centella asiatica* ethanolic leaves extract at 100 mg/kg body weight/day or reference drug silymarin (50 mg/kg body weight/day) as a oral pre-treatment before 1 hour significantly restored (p<0.05) the abnormalities in LDH level to near normal levels.

Effect of *Centella asiatica* leaves ethanolic extract on liver glycogen

Figure 5 A & B shows the levels of glycogen in liver and kidney of control and experimental rats in each group. A significant decrease ($p<0.05$) in glycogen level was observed in liver of INH administered rats. *Centella asiatica* ethanolic leaves extract (100 mg/kg body weight/day) oral pre-treatment in INH rats significantly restored ($p<0.05$) the levels to near normal when compared to control rats. Similar effects were observed in silymarin (50 mg/kg body weight/day) treated INH intoxicated rats.

Effect of *Centella asiatica* leaves ethanolic extract on ALT, AST & ALP in liver and kidney

A significant increase ($p<0.05$) in the activities of ALT, AST and ALP, was observed in INH treated group liver and kidney. These levels were found to be restored in the reference drug silymarin (50 mg/kg body weight) and the extract pre-treated group at a concentration of 100 mg/kg body weight restored the levels to near normal (Figure 6).

Effect of *Centella asiatica* leaves ethanolic extract on histology of liver

The histological examination of liver tissue photomicrograph of INH (50 mg/kg body weight/day) treated rats revealed numerous changes such as cellular damage, inflammation, vascularization, hypertrophy, loss of structural integrity of cells as well as loss in characteristic tissue organization. Near normal cellular architecture with normal hepatocyte arrangement, sinusoids, mild central vein congestion were observed in *Centella asiatica* ethanolic extract treated (100 mg/kg body weight/day) INH intoxicated rat liver. Similar effects were observed in silymarin (50 mg/kg body weight/day) treated INH intoxicated rat (Figure 7).

Effect of *Centella asiatica* leaves ethanolic extract on liver ultrastructure by TEM

The analysis of INH treated rat liver ultramicrographs obtained from Transmission Electron Microscopy (TEM) revealed the presence of cell membrane enfolding, damaged nuclear membrane, nuclear chromatin condensation, regression of mitochondrial cisternae and presence of fat droplets with vacuoles. Near normal hepatocyte ultrastructure with euchromatic nucleus, prominent nuclear membrane, prominent nucleolus, many mitochondria, rough endoplastic reticulum, appearance of glycogen granules and less vacuolated cytoplasm were observed in *Centella asiatica* ethanolic extract administered (100

mg/kg body weight/day) INH intoxicated rat hepatocytes. Similar effects were observed in silymarin (50 mg/kg body weight/day) treated INH intoxicated rat livers (Figure 8).

Effect of *Centella asiatica* leaves ethanolic extract on histology of kidney

The histological examination of kidney renal cortex tissue photomicrograph of INH (50 mg/kg body weight/day) treated rats revealed distorted cellular organization accompanied with thickened renal basement, swelled glomerulus filled with blood cells and enclosed in uneven spacing of Bowman's capsule. Near normal renal cortex architecture, glomerulus in Bowman's capsule as well as near normal tubular arrangement (proximal and distal tubules) and decrease in renal basement membrane thickness was observed in *Centella asiatica* ethanolic extract treated (100 mg/kg body weight/day) INH intoxicated rat kidney. Similar effects were observed in silymarin (50 mg/kg body weight/day) treated INH intoxicated rat (Figure 9).

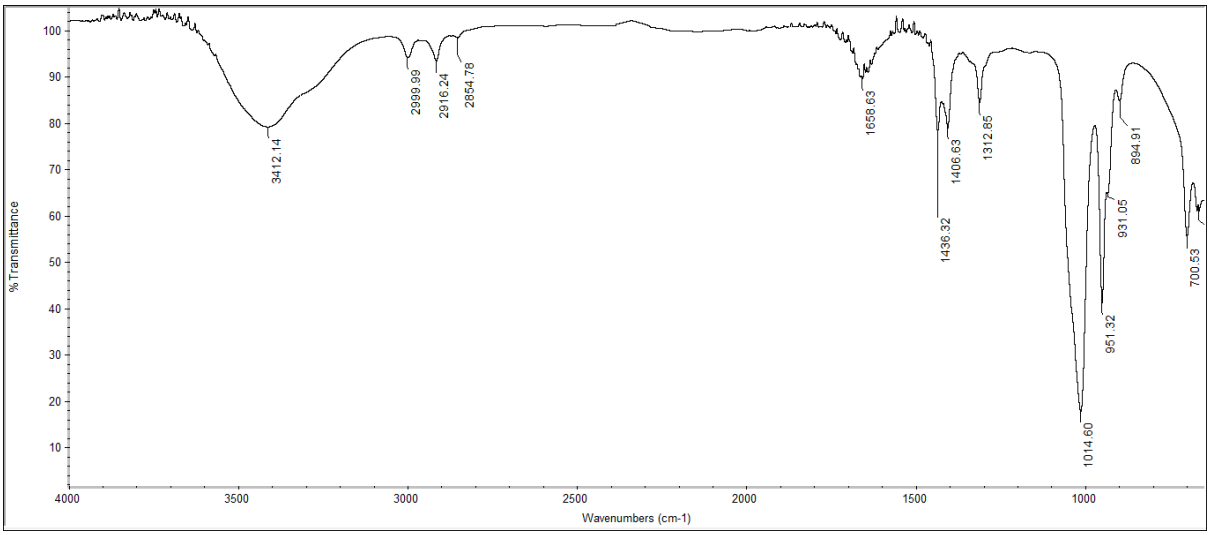


Figure 1. FT-IR spectroscopy of *Centella asiatica* leaf extract

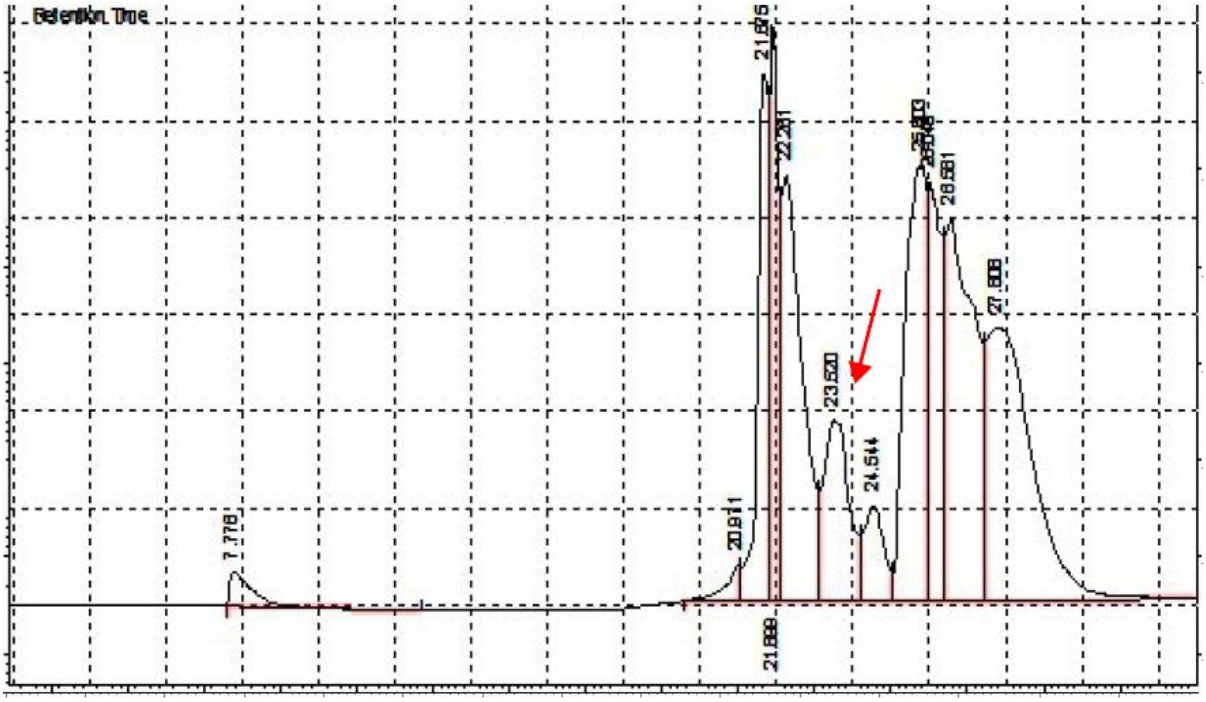


Figure 2A. HPLC of *Centella asiatica* leaf extract

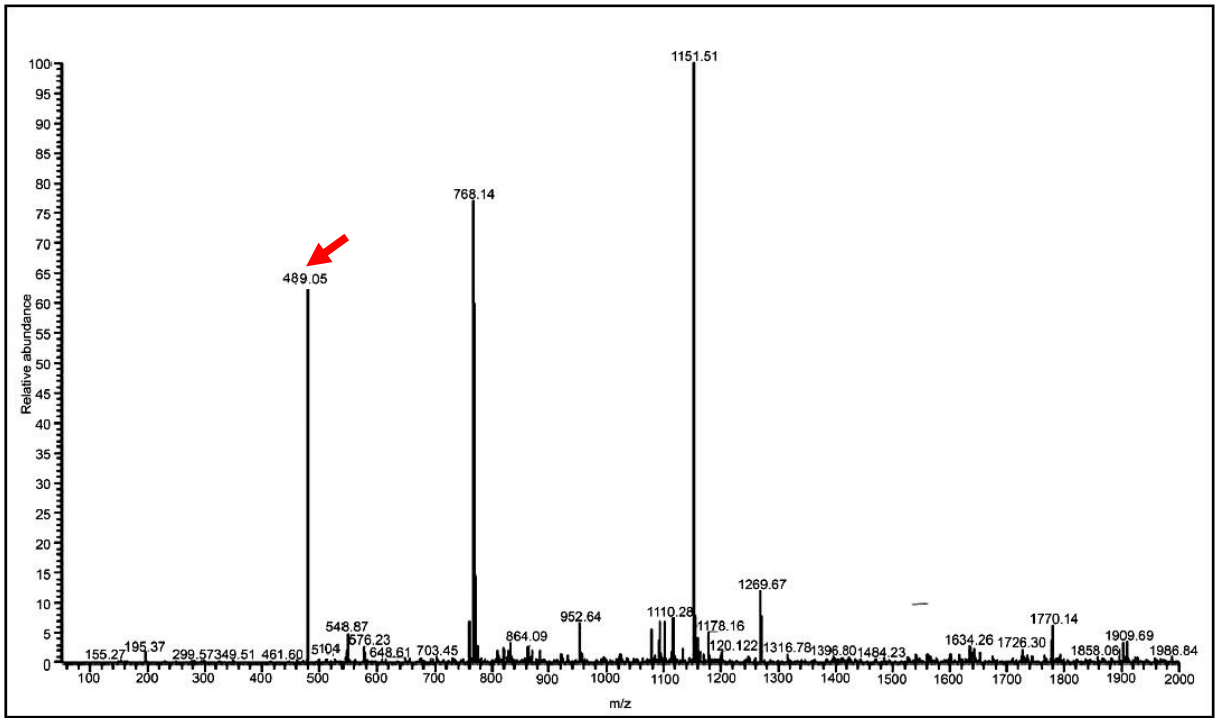
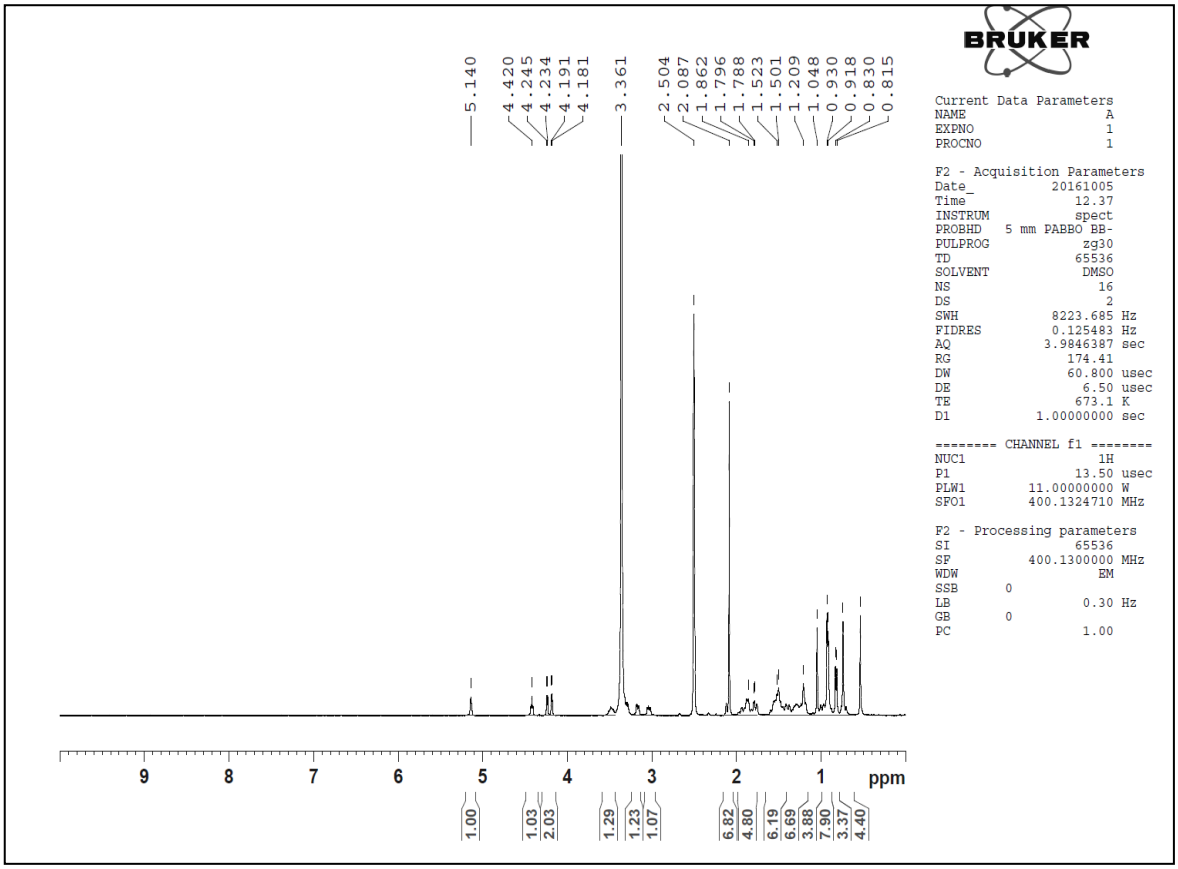


Figure 2B. ESI-MS of *Centella asiatica* leaf extract fraction

A



B

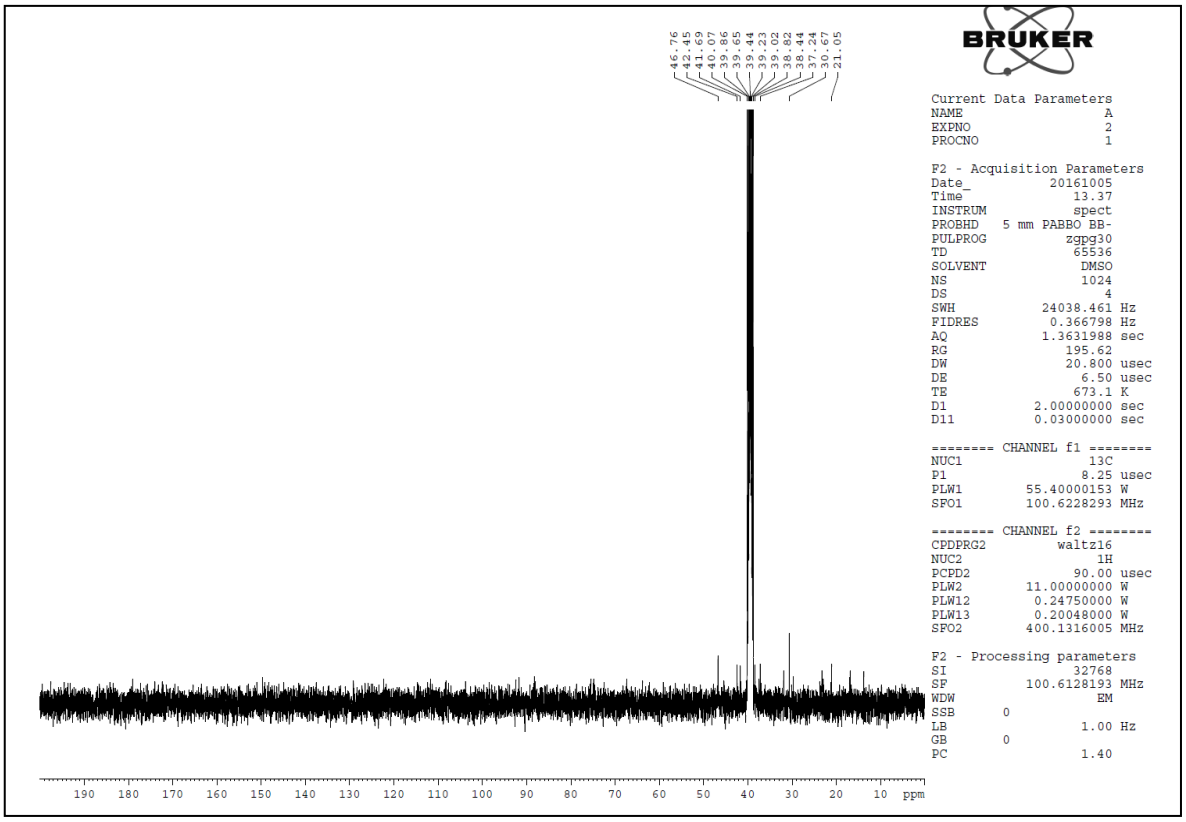
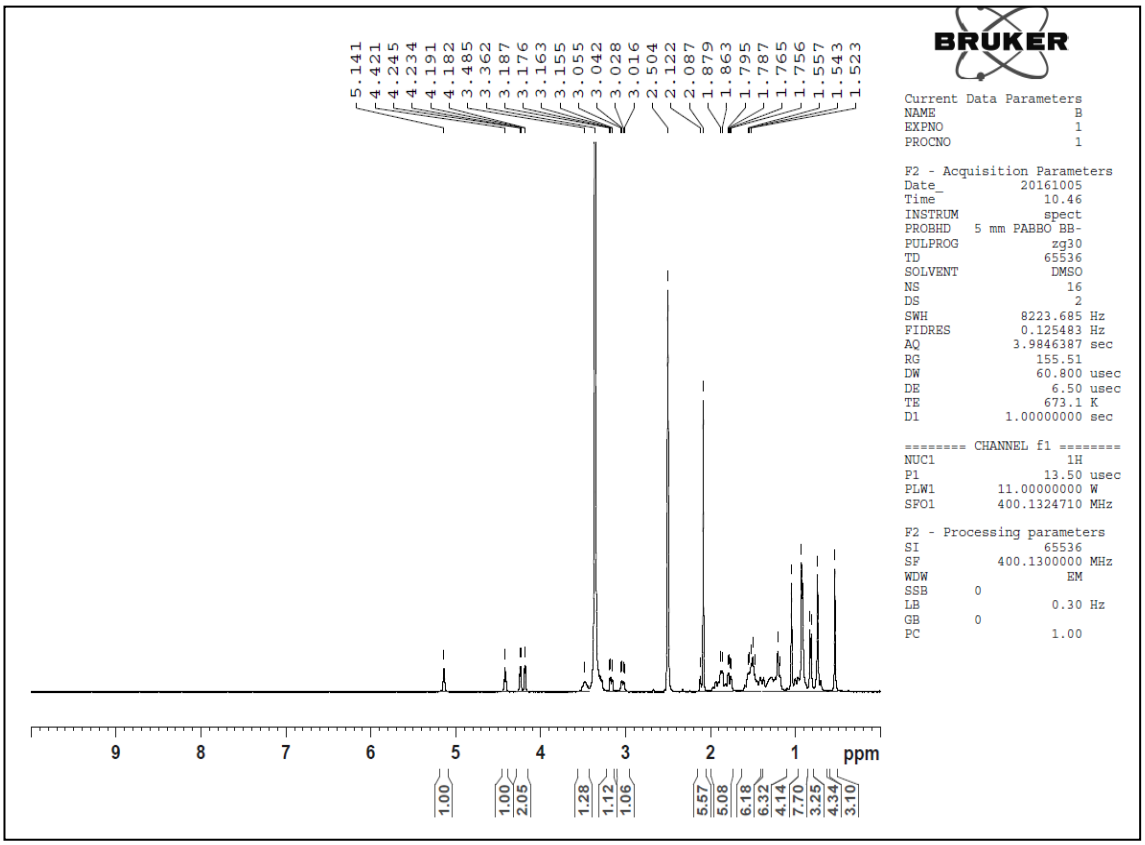


Figure 3 A) & B). *Centella asiatica* leaves ethanolic extract 1H & 13 C NMR, respectively: Fraction containing Asiatic acid

C



D

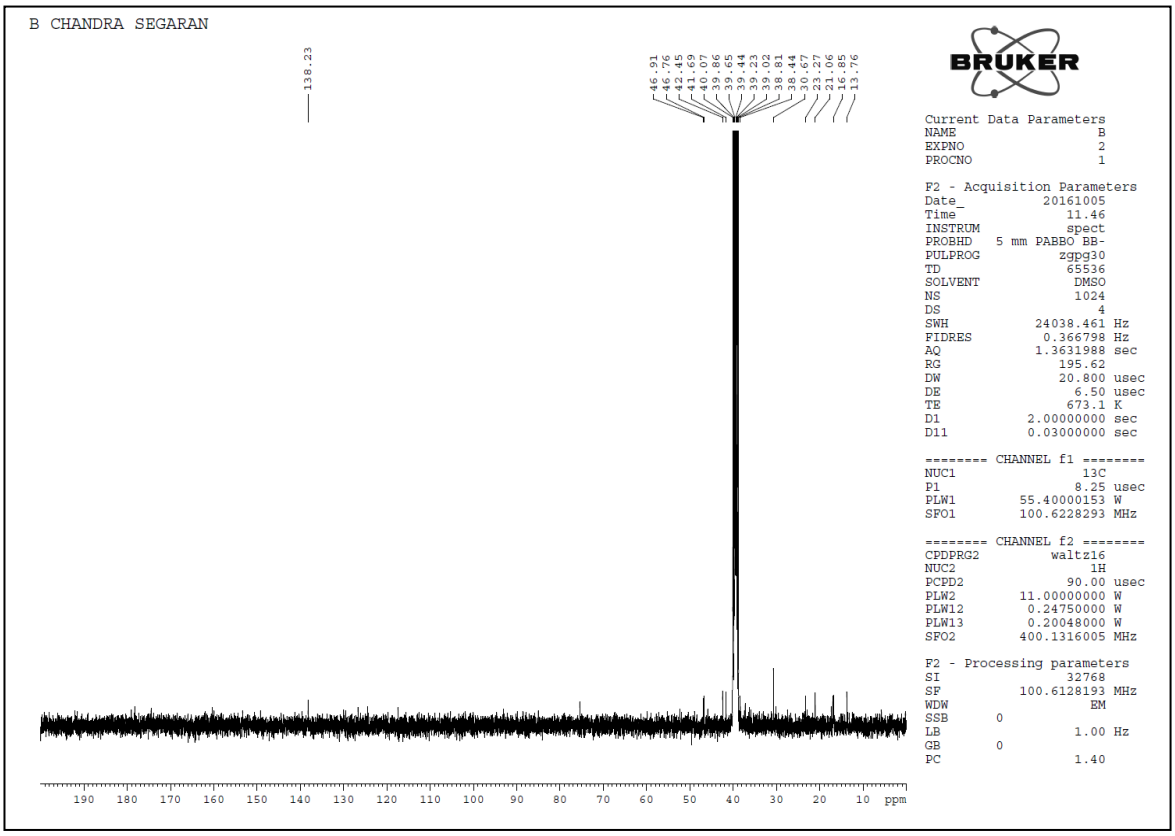


Figure 3 C) & D). Standard Asiatic acid 1H & 13 C NMR, respectively

Tables 1. Effect of *Centella asiatica* leaf ethanolic extract on lipid profile and phospholipids in control and experimental animals. Values are expressed as mean ±SD (n=6). Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT).

Groups	TC (mg/dl)	TG (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	VLDL (mg/dl)	Phospholipids (mg/dl)
Group 1	92.51±5.99^{c}	82.51±6.00^{ab}	45.75±4.66^{cd}	34.46±2.78^{a}	12.25±1.85^{ab}	109.21±0.29^{b}
Group 2	178.20±5.22^{f}	198.20±5.22^{f}	31.17±4.64^{a}	108.72±5.80^{g}	38.12±1.46^{g}	178.07±0.33^{g}
Group 3	166.21±4.58^{e}	166.21±4.58^{e}	32.21±4.11^{a}	89.76±4.78^{f}	31.31±1.90^{f}	175.01±0.57^{f}
Group 4	152.32±4.66^{d}	152.32±4.66^{d}	37.54±3.55^{b}	75.23±4.11^{e}	25.21±1.57^{e}	160.12±0.38^{e}
Group 5	176.17±4.79^{f}	126.17±4.79^{c}	39.89±4.66^{b}	60.12±4.66^{d}	22.32±1.35^{d}	158.07±0.34^{d}
Group 6	85.21±5.66^{ab}	85.21±5.66^{ab}	42.32±3.45^{bcd}	39.72±3.55^{b}	14.01±1.33^{bc}	115.01±0.34^{c}
Group 7	80.31±4.84^{a}	80.31±4.84^{a}	47.11±4.15^{d}	31.29±3.45^{a}	11.21±1.97^{a}	102.20±0.32^{a}
Group 8	88.19±5.14^{bc}	88.19±5.14^{b}	41.52±3.24^{bc}	45.23±4.68^{c}	15.20±1.46^{c}	115.15±0.33^{c}

Table 2. Effect of *Centella asiatica* leaf ethanolic extract on total protein, total cholesterol, triglycerides, free fatty acids and phospholipids in organs, of control and experimental animals. Values are expressed as mean ±SD (n=6). Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT). (INH: Isoniazid; CA: Centella asiatica; S: silymarin).

Groups	**Control**	**INH (50 mg/kg b.wt)**	**INH (50 mg/kg b.wt) +CA (100mg/kg b.wt)**	**CA (100mg/kg b.wt)**	**INH (50 mg/kg b.wt) +S (50 mg/kg b.wt)**
Protein[1]					
Liver	5.78±0.55^{c}	3.43±0.34^{a}	4.89±0.43^{b}	5.93±0.52^{c}	5.47±0.46^{b}
Kidney	5.21±0.51^{c}	3.54±0.32^{a}	4.74±0.11^{b}	5.31±0.52^{c}	5.04±0.12^{b}
Total cholesterol[2]					
Liver	6.90±0.24^{a}	12.87±0.19^{c}	8.01±0.32^{b}	6.81±0.36^{a}	7.94±0.44^{b}
Kidney	4.57±0.14^{a}	12.73±0.29^{b}	5.58±0.30^{a}	4.46±0.45^{a}	5.33±0.37^{a}
Triglycerides[3]					
Liver	319.71±10.28^{ab}	487.75±12.52^{c}	355.40±10.14^{b}	308.78±11.28^{a}	357.35±14.98^{b}
Kidney	416.45±12.30^{ab}	621.54±13.91^{d}	496.10±13.14^{c}	400.89±12.02^{a}	446.79±12.01^{b}
Free fatty acids[4]					
Liver	6.80±0.31^{a}	11.99±1.17^{b}	7.09±0.16^{a}	6.25±1.28^{a}	7.00±0.76^{a}
Kidney	4.90±0.23^{b}	8.23±0.24^{d}	5.42±0.45^{c}	4.01±0.35^{a}	5.10±0.20^{bc}
Phospholipids[5]					
Liver	21.86±1.26^{c}	10.92±1.53^{a}	17.94±1.04^{b}	21.86±1.39^{c}	18.01±1.01^{b}
Kidney	17.20±0.24^{c}	11.03±0.35^{a}	14.89±0.56^{b}	17.35±0.39^{c}	15.09±0.27^{b}

[1]Protein for tissues were expressed as mg protein/g tissue
[2]Total cholesterol for tissues were expressed as mg/g wet tissue
[3]Triglycerides for tissues were expressed as mg/100g wet tissue
[4]Free fatty acids for tissues were expressed as mg/g wet tissue
[5]Phospholipids for tissues were expressed as mg/g wet tissue

Table 3. Effect of *Centella asiatica* leaf ethanolic extract on TBARS in liver and kidney of control and experimental animals. Values are expressed as mean ±SD (n=6). Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT). (INH: Isoniazid; CA: Centella asiatica; S: silymarin).

Groups	**Control**	**INH (50 mg/kg b.wt)**	**INH (50 mg/kg b.wt) + CA (100mg/kg b.wt)**	**CA (100mg/kg b.wt)**	**INH (50 mg/kg b.wt) +S (50 mg/kg b.wt)**
TBARS[1]					
Liver	0.76 ± 0.13^{a}	4.38 ± 0.27^{d}	1.32 ± 0.23^{c}	0.78 ± 0.05^{a}	1.09 ± 0.07^{b}
Kidney	1.31 ± 0.04^{a}	3.72 ± 0.16^{d}	1.72 ± 0.11^{c}	1.28 ± 0.05^{a}	1.53 ± 0.19^{b}

[1]TBARS in tissues were expressed as nmoles/100g wet tissue

Table 4. Effect of *Centella asiatica* leaf ethanolic extract on SOD, CAT and GST in liver and kidney of control and experimental animals. Values are expressed as mean ±SD (n=6). Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT). (INH: Isoniazid; CA: Centella asiatica; S: silymarin)

Groups	Control	INH (50 mg/kg b.wt)	INH (50 mg/kg b.wt) + CA (100mg/kg b.wt)	CA (100mg/kg b.wt)	INH (50 mg/kg b.wt) +S (50 mg/kg b.wt)
SOD[1]					
Liver	7.90 ± 0.25^{c}	3.82 ± 0.30^{a}	6.93 ± 0.60^{b}	8.05 ± 0.41^{c}	6.94 ± 0.47^{b}
Kidney	14.86 ± 1.11^{c}	6.57 ± 1.43^{a}	12.84 ± 1.02^{b}	14.43 ± 0.30^{c}	13.89 ± 1.18^{bc}
CAT[2]					
Liver	78.14 ± 3.62^{c}	43.24 ± 2.80^{a}	56.57 ± 4.64^{b}	80.94 ± 1.46^{c}	59.00 ± 2.65^{b}
Kidney	48.40 ± 2.45^{c}	29.18 ± 3.20^{a}	39.96 ± 2.53^{b}	48.90 ± 3.26^{c}	42.43 ± 2.10^{b}
GST[3]					
Liver	8.81 ± 0.67^{cb}	6.31 ± 0.47^{a}	7.49 ± 0.62^{b}	9.01 ± 0.34^{d}	8.27 ± 0.45^{c}
Kidney	5.83 ± 0.35^{c}	3.32 ± 0.11^{a}	4.63 ± 0.23^{b}	6.21 ± 0.21^{d}	4.92 ± 0.25^{b}

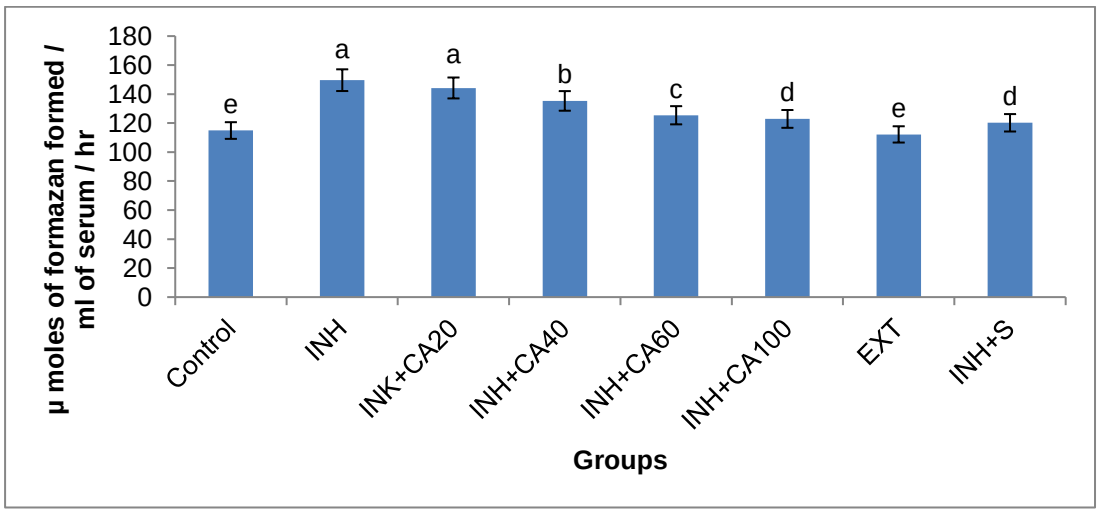
[1]SOD for tissues were expressed as 50% inhibition of nitroblue tetrazolium reduced in 1minute/mg protein

[2]CAT for tissues were expressed as µmoles of H_2O_2 consumed/minute/mg protein.

[3]GST for tissue were expressed as CDNB-GSH conjugate formed/minute/mg protein

Figure 4A, B &C. Effect of *Centella asiatica* leaves ethanolic extract on serum, liver and kidney tissues LDH in control and experimental rats respectively. Values are expressed as mean ±SD (n=6). Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT). (INH: Isoniazid; CA: Centella asiatica 20, 40, 60 & 100 mg/kg b.wt; S: silymarin)

A



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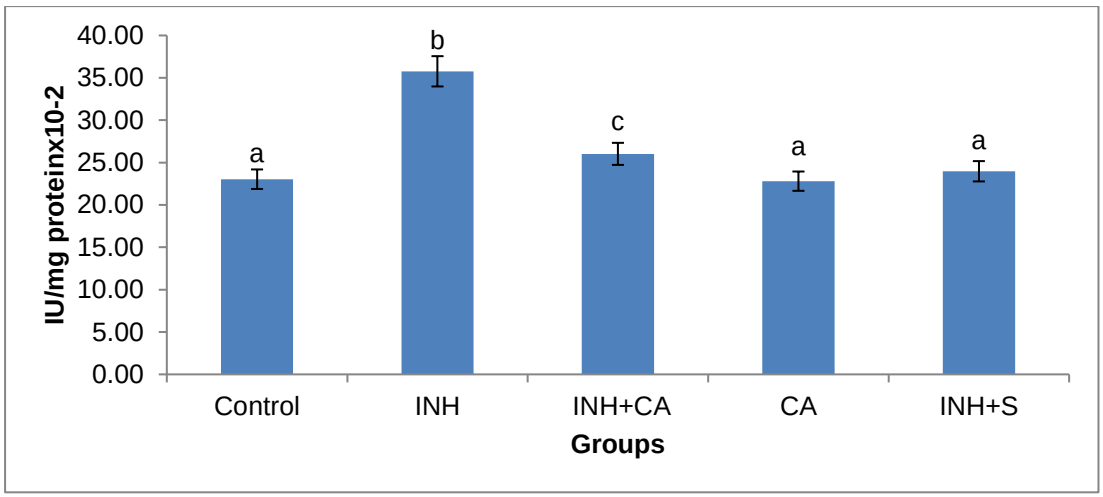

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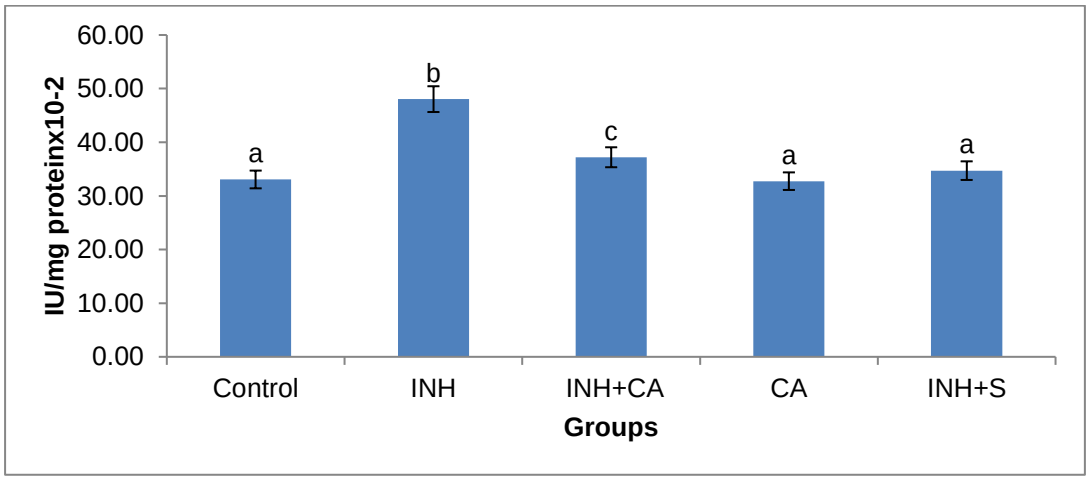
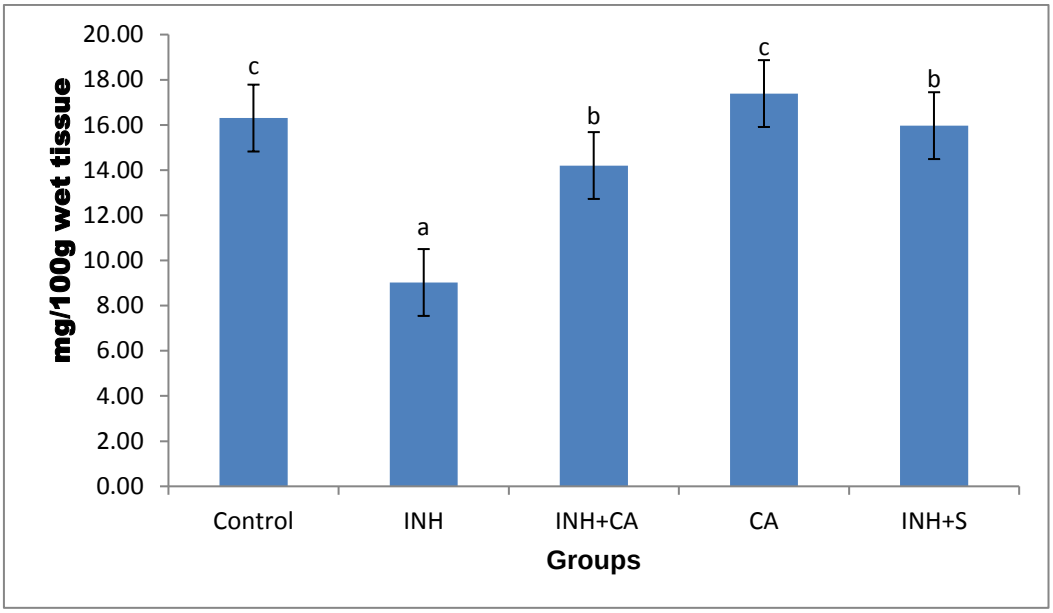


Figure 5 A & B. Effect of *Centella asiatica* leaves ethanolic extract on liver and kidney glycogen in control and experimental rats. Values are expressed as mean ±SD (n=6). Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT). (INH: Isoniazid; CA: Centella asiatica 100 mg/kg b.wt; S: silymarin)

A



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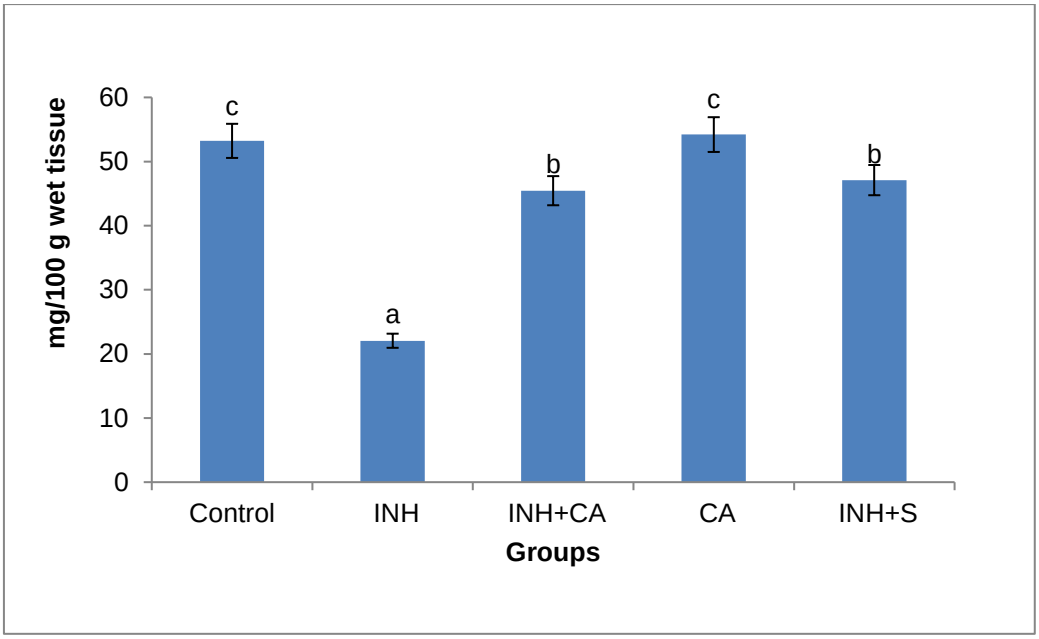
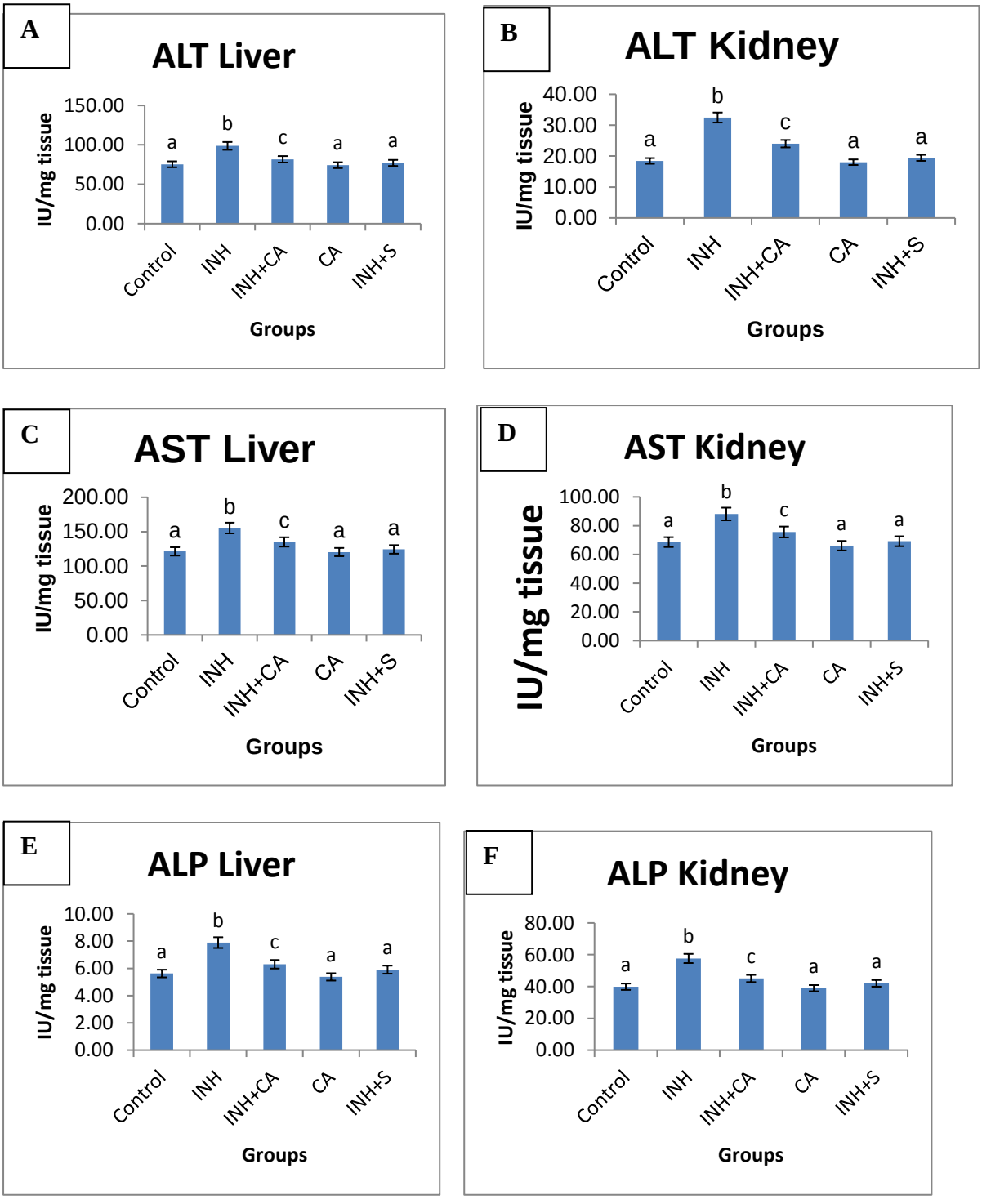


Figure 6 A, B & C. Effect of *Centella asiatica* leaves ethanolic extract on liver and kidney ALT, AST & ALP in control and experimental rats. Values are expressed as mean ±SD (n=6). Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT). (INH: Isoniazid; CA: Centella asiatica 100 mg/kg b.wt; S: silymarin)



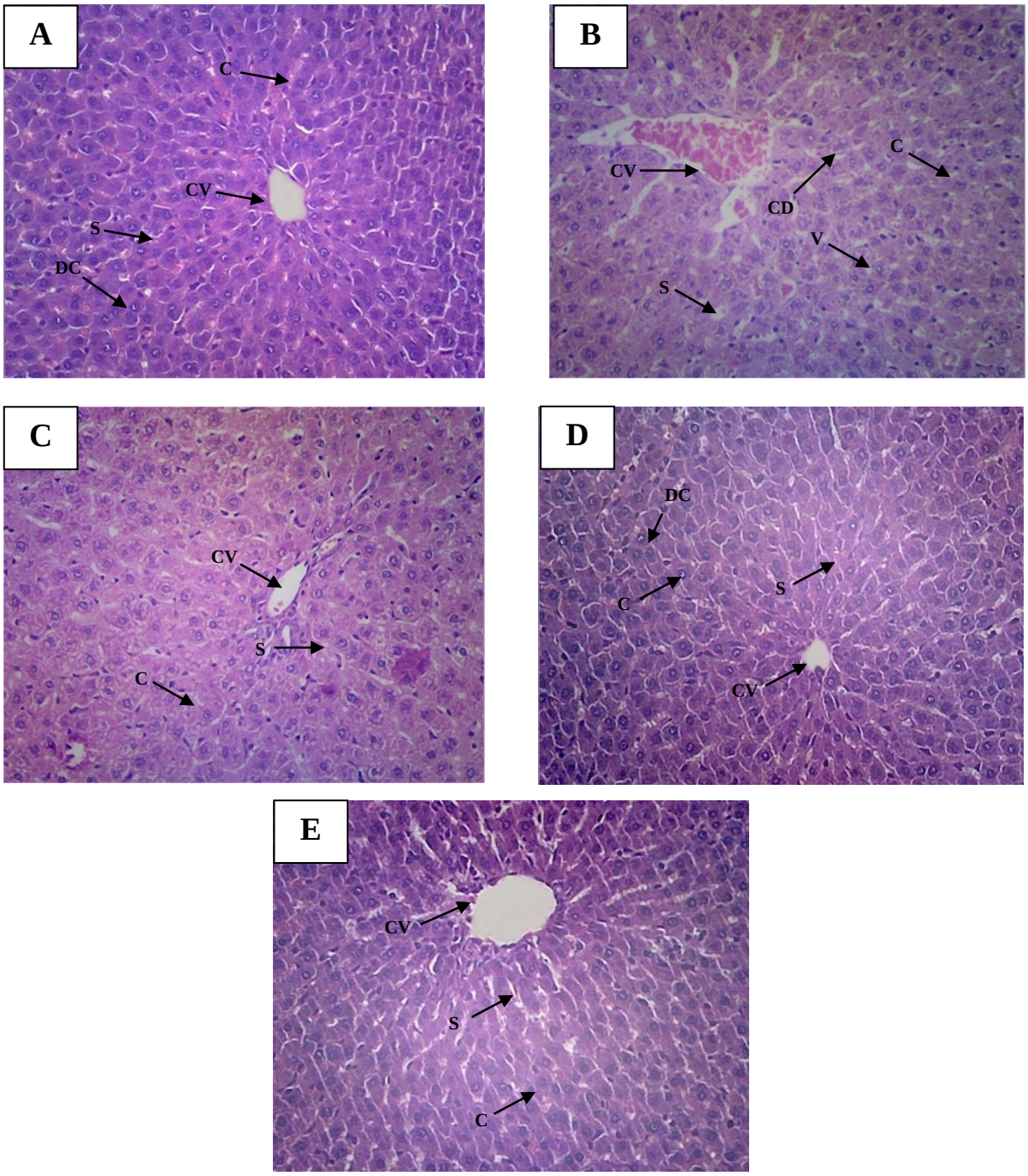


Figure 7. Photomicrographs of histological changes in hematoxylin and eosin (H & E) stained liver sections of control and experimental rats (X 200)

A & D. Control and CA ethanolic treated rats liver showed clear central vein (CV) with hexagonal cells, embedded in connective tissue. All hepatocytes are radiantly arranged as trabecules from the central portal vein, separated by sinusoids (S). The hepatocytes contain clear spheroidal nucleus with distinct nucleolus as well as peripheral chromatin condensation (C). Few cells contain two nucleuses (cells undergoing division) was also observed (DC)

B. INH treated rat liver showed distorted cellular organization accompanied with vacuoles (V), hypertrophic cells, cellular damage (CD), inflammation, widening of inter cellular sinusoids (S) and congestion in central vein (CV). The necleoli is not distinct and the nucleuses appeared to have more condensed chromatin (C)

C & E. CA ethanolic extract or silymarin oral pre-treated INH intoxicated rats liver showed near normal hepatocyte arrangement and sinusoids (S) accompanied with mild central vein congestion (CV). The nucleoli and nucleolus also appeared to be nearly distinct with less condensed chromatin (C)

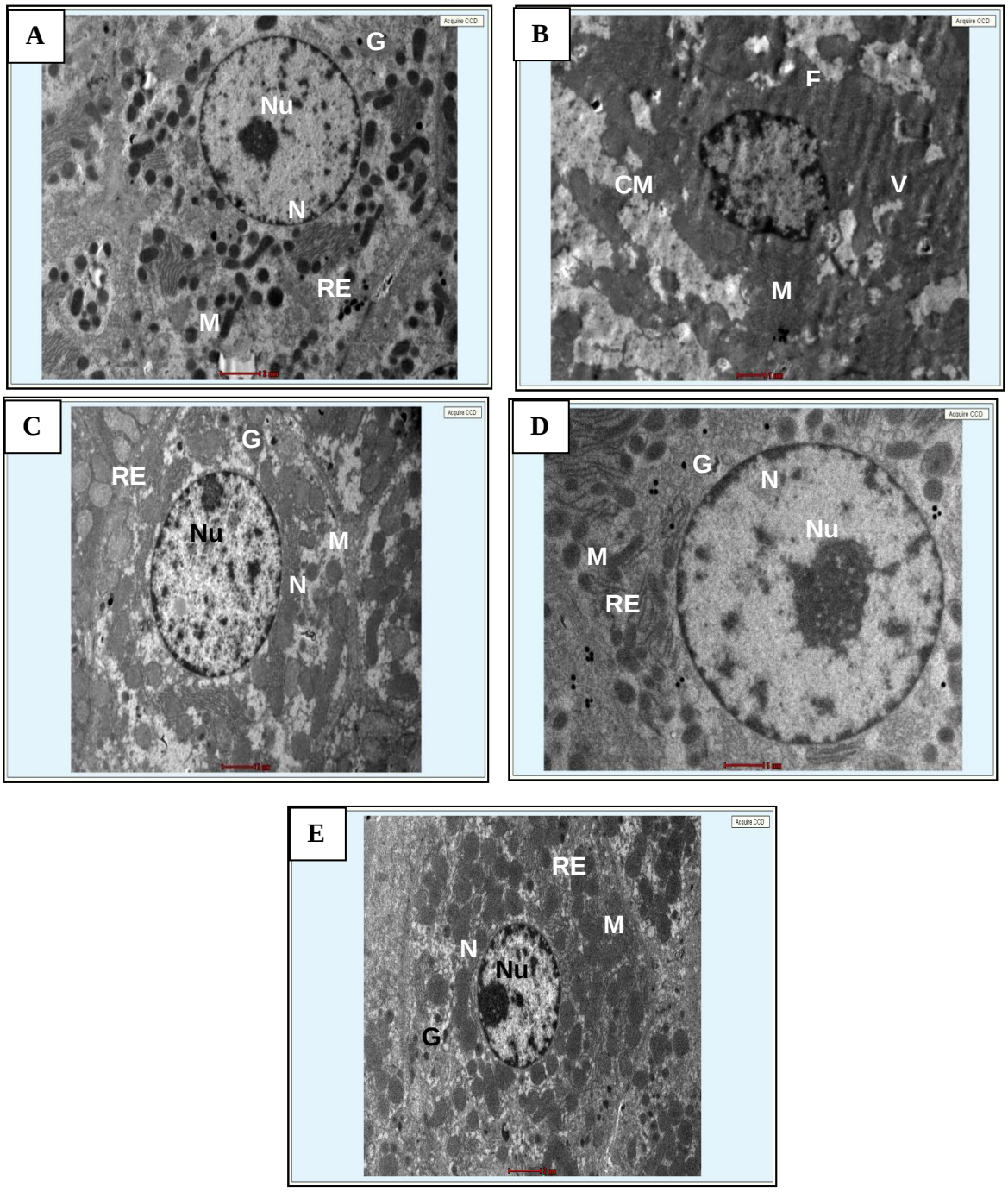


Figure 8. Liver ultramicrographs of control and experimental rats

A & D. Control and CA ethanolic treated rats liver showed a normal hepatocyte with euchromatic nucleus (N) with prominent nuclear membrane, prominent nucleolus (Nu), many mitochondria (M) having normal cristae, rough endoplasmic reticulum (RE) and glycogen granules (G).
TEM mag.for A= 12,000X; TEM mag. For D = 15,000X

B. INH treated rat liver showed hepatocyte with enfolding of cell membrane (CM), damaged nuclear membrane, condensation of nuclear chromatins and pyknotic nucleus (N), regression of mitochondrial cisternae (M), and presence of fat droplets (F) with vacuole (V). TEM mag.= 15,000X

C & E. CA ethanolic extract or silymarin oral pre-treated INH intoxicated rats liver showed hepatocyte with euchromatic nucleus (N) with prominent nuclear membrane, prominent nucleolus (Nu), many mitochondria (M), rough endoplasmic reticulum (RE), glycogen granules (G) with less vacuolated cytoplasm. TEM mag. = 12,000X

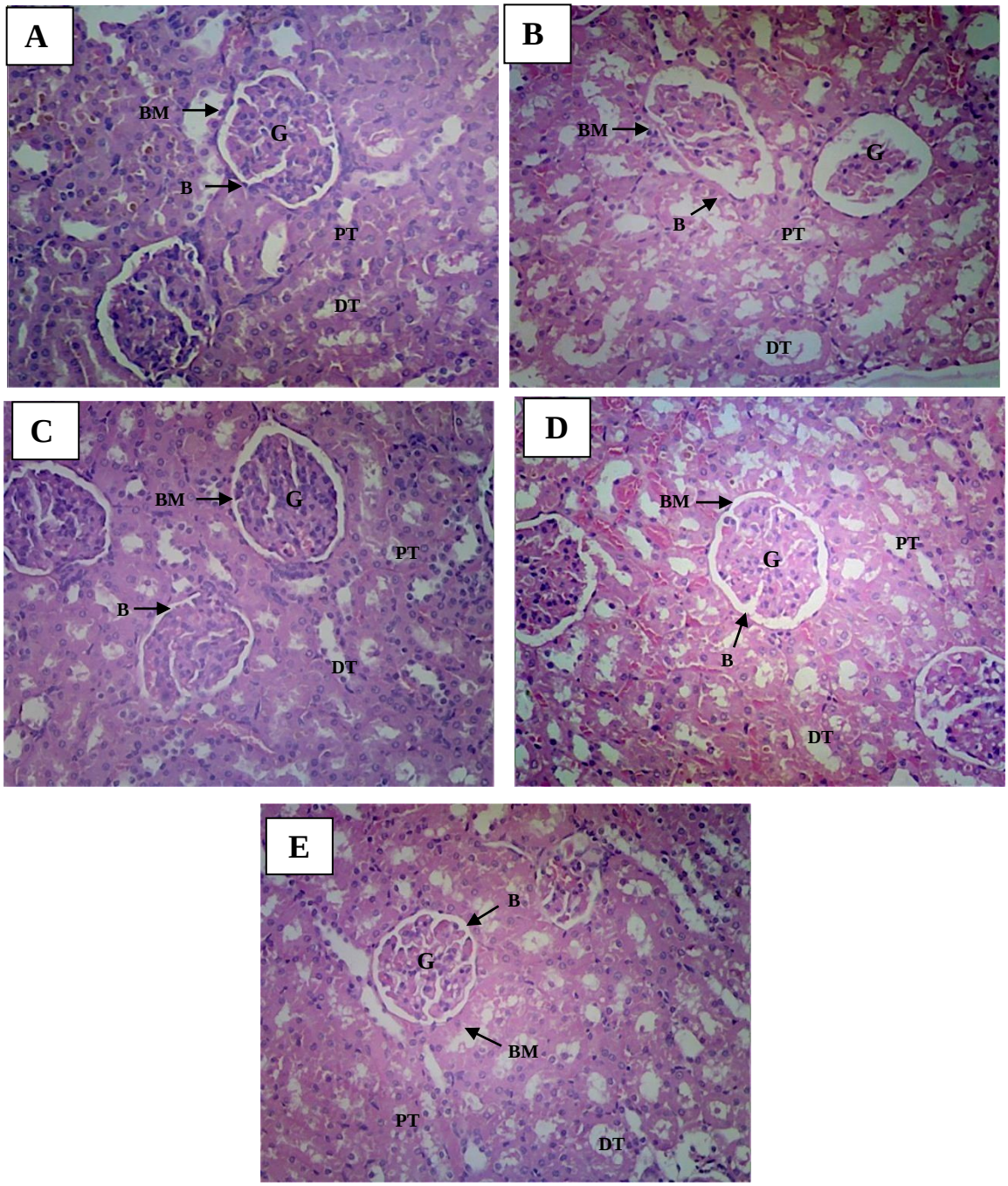


Figure 9. Photomicrographs of histological changes in hematoxylin and eosin (H & E) stained kidney renal cortex sections of control and experimental rats (X 200).

A & D. Control and CA ethanolic treated rat's renal cortex showed clear glomerulus (G) enclosed in a Bowman's capsule (B). Clear proximal (PT) and distal tubules (DT) with regular and distinct lumen were observed around the glomerular capsule. The renal basement membrane appeared normal (BM).

B. INH treated rat's renal cortex showed distorted cellular organization accompanied with thickened renal basement (BM), damaged renal tubules (PT & DT), swelled glomerulus (G) filled with blood cells (BC) and uneven spacing of Bowman's capsule (B).

C & E. CA ethanolic extract or silymarin oral pre-treated INH intoxicated rat's renal cortex showed near normal glomerulus (G) enclosed in Bowman's capsule (B), tubular arrangement of proximal (PT) as well as distal tubules (DT) and decrease in renal basement membrane thickness (BM).

Appendix – IV

Publications:

1. **Ghosh K**, Indra N, Jagadeesan G. "The ameliorating effect of *Centella asiatica* ethanolic extract on liver of isoniazid intoxicated albino rats." International Conference on "Indian Traditional Medicine- A conglomeration of Ancient Knowledge and Modern Science, PHYTOCONGRESS 2016", 21st-22nd July, 2016, organized by Centre for advanced research in Indian system of medicine (CARISM), Sastra University, Thirumalaisamudram, Thanjavur 613 401, Tamil Nadu, India (**Oral presentation**). **Paper under process to be published as conference proceedings.**

2. **Ghosh K**, Indra N, Jagadeesan G. The ameliorating effect of *Centella asiatica* ethanolic extract on albino rats treated with isoniazid. *J Basic Clin Physiol Pharmacol* (De Gruyter) (aop) 2016.

3. **Ghosh K**, Indra N. Phytochemistry, *in vitro* free radical scavenging, chelating and toxicity of *Centela asiatica* L. (Apiaceae) ethanolic leaf extract. *Int J Pharm Sci Rev Res* 2014 29(01):328-334.

Appendix – V

This study was designed to determine the phytocomponents, *in vitro* toxicity, free radical scavenging and chelating activity in the ethanol leaf extract of *Centella asiatica L. (Apiaceae)*. In GC-MS analysis 7 phytochemical compounds were identified in the ethanolic leaf extract of *Centella asiatica* (CA). The identification of the phytochemical compounds is done by comparing the mass spectrum of the unknown component with the spectrum of the known components stored in the NIST-08 and Wiley-08 libraries. Activities of components identified in ethanolic extract were found from Dr.Duke's phytochemical and ethnobotanical databases [Online database]. LC method was used for determining the non-volatile phytochemical contents constituents of *C. asiatica* ethanolic leaf extract. In LC-MS analysis 7 phytochemical compounds were identified in the ethanolic leaf extract of *C. asiatica* (CA). Activities of components identified in ethanolic leaf extract were found from Dr.Duke's phytochemical and ethnobotanical databases [Online database]. The major components which were found in the leaves of the CA leaf ethanolic extract are (Table 5): Maltol, 3', 5'-Dimethoxyacetophenone, Papyriogenin A, Asiatic acid, Asiaticoside, Madecassoside and Madecassic acid. Medicinal plants are rich source of secondary metabolites. The triterpene form the major constituent of *C. asiatica*. Compounds like Asiatic acid, Asiaticoside, Madecassoside and Madecassic acid are already reported to be present in *C. asiatica* (Zheng and Qin, 2007). Papyriogenin A, a triterpenoid, was first time identified in *C. asiatica* extract. It is a potent hepato-protective compound (Valan *et al*., 2010). The presence of various antioxidant and anti-inflammatory compounds may be the reason for the presence of strong antioxidant, free radical scavenging and chelating properties of CA ethanolic leaf extract.

The second phase of the present study was undertaken to understand the efficacy of CA leaves extract on INH induced toxicity in albino rats. Previous finding by Antony *et al*., 2006, showed that CA alcoholic extract had hepatoprotective effect in chemically (CCL_4) induced liver injury (Antony et al., 2006). Based on the acute toxicity works of Abdulla *et al.*, 2010, the ethanolic extract of CA at a dose of 2g and 5g per kg body weight for 14 days did not manifest any significant visible signs of toxicity (Abdulla *et al*., 2010). Silymarin, a flavonolignan from 'milk thistle' (*Silybum marianum*) plant, is a reference drug which is used extensively for hepatoprotection (Pradhan and Girish, 2006).

Oral administration of CA ethanolic leaves extract at a concentration of 100 mg/kg body weight normalized ($p<0.05$) the levels of blood glucose in INH treated rats to near

normal levels. Luntz and Smith, 1953 had reported abnormalities in carbohydrate metabolism and also reported a rise in blood glucose level in diabetic and non-diabetic tuberculosis patients. This may be due to the deleterious effect of INH on pancreas, causing a decrease secretion of insulin, and leading to increased blood glucose level. Drug induced pancreatitis is not a well documented event in patients and is thus rare (Wilmink and Frick, 1996). There had been numerous reports of pancreatitis, either in isoniazid alone or in combination with rifampicin. In the majority of the cases, the effects of isoniazid induced pancreatitis are reversible (Pandey and Surana, 2011).

The INH treated animals showed a significant decrease ($p<0.05$) in body weight, but pre-treatmented with CA ethanolic leaves extract (100 mg/kg body weight) as well as reference drug silymarin (50 mg/kg body weight) significantly improved ($p<0.05$) the body weight of these rats to near normal, as compared to control group ($p<0.05$). We also found elevated levels of total WBC, TBARS, AST, ALT, ALP, LDH total bilirubin, urea, creatinine and globulin, accompanied by decrease in the levels of total haemoglobin, RBC, hematocrit, mean cell haemoglobin concentrations, SOD, CAT, GSH, total serum protein and albumin in INH treated rats when compared with control ($p<0.05$). These changes may be due to increased damage in the RBC, liver and kidney of the INH treated group, accompanied with a significant decreased ($p<0.05$) in antioxidant enzymes like SOD, CAT and GSH, and a significant increase in TBARS level.

WBCs are primarily involved in protecting the body at the event of any infection or injury. T and B lymphocyte cells play a very important role in the immune-defence system of the body. A recent study by Tosif *et al*., 2014, had reported that the anti-TB drug, INH, compromises the immunity in mice by inducing activation-induced cell death in activated CD4+T cells, thus resulting in immune impairment of the host by leukopenia. INH is also used as a preventive medicine for healthy individuals who are in close contact with TB patients (Comstok *et al*., 1979). Thus an impaired immune system may lead to increase susceptibility to various pathogens in the healthy individual. With an increase in infections accompanied with increased oxidative stress in INH treated rats, the level of neutrophils, eosinophils and basophils in the host increases. These events collectively lead to the increased levels of total WBCs in INH treated rats. The antioxidant activity of CA extract (100 mg/kg body weight) and silymarin may help in restoring the oxidant balance and thus prevent the secondary metabolites from compromising the immune system with its toxic effect in the pre-treated INH groups.

Previous study by Yilmaz *et al*., 2008, had reported INH induced oxidative stress in RBCs of rat. The oxidative stress was a result of increased nitric oxide (NO), adenosine deaminase (ADA) and xanthine oxidase (XO) levels in RBCs. This leads to the destruction of RBCs in the blood. A large number of anti-TB drugs, including INH, are known inhibitors of heme biosynthesis (Huang and Benz, 2001). The MCH and MCHC provide the haemoglobin content in RBCs. A decrease in MCH and MCHC indicates INH induced anemia in rats. Hematocrit (Ht) is also known as packed cell volume (PCV) or erythrocyte volume fraction (EVF). It is the volume percentage (%) of RBCs present in the blood. A decrease in Ht reflects that the percentage of total RBCs in the blood is below the normal level (Hutchison *et al*., 2011). A decrease in haemoglobin synthesis, accompanied by an increase in oxidative stress in INH treated rats may be the reason for the decrease in total RBC count and haemoglobin in the blood of INH treated rats. These conditions were found to be restored to near normal in CA leaves extract (100 mg/kg body weight) and silymarin (50 mg/kg body weight) pre-treated INH rats.

Liver is the main organ involved in detoxification of hormones, xenobiotics and drugs. During xenobiotic detoxification, the metabolised produced can damage the liver cells (Singh *et al*., 2011). In liver Isoniazid is metabolized into hydrazine and acetylisoniazid, followed by hydrolysis to acetylhydrazin and oxidization into hepatotoxic intermediaries by CYP 450 (Blair et al., 1985). The enzymes CAT and SOD are inactivated by hydrazine, resulting in significant increase in endogenous H_2O_2 levels in the cells (Hussain and Frazier, 2002). According to various studies, INH causes cellular damage by inducing oxidative stress. This lead to dysfunction of the hepatic antioxidant defence system (Attri *et al*., 2000). The exhaustion of antioxidant defences and increase in free radical production leads to imbalance in the prooxidant–antioxidants and cause oxidative stress-induced cell death in liver. GSH protects against reactive oxygen species (ROS). During this process, GSH is converted into glutathione disulfide (GSSG), its oxidized form (Lei *et al*., 2007). There may be an increase in the utilization of GSH in INH treated rats, leading to the depletion of GSH and increase in lipid peroxidation. Based on previous study, CA ethanolic leaves extract has very good *in vitro* antioxidant and free radical scavenging activity. This may be attributed due to the presence of various antioxidant compounds present in the extract. Increase in ROS and reduction in the levels of GSH, SOD and CAT elevate other parameters, like increase in the levels of serum and tissue AST and ALT in INH treated rats. During drug metabolism, INH produces acetylhydrazine and hydrazine, which can cause immense damage to liver and lead to liver toxicity. Liver cells contain amino transferases. These enzymes are liver specific

and are involved in the catalytic reactions, involving the inter-conversion of amino acid and α-keto acid, by transferring amino groups. Damage to the liver cells due to increased TBARS and decreased SOD, CAT and GSH, lead to cell membrane damage. This is followed by necrosis of liver cells and release of amino transferases enzymes, AST and ALT, into the blood circulation. The healing of liver cells decreases the levels of AST and ALT in CA extract (100 mg/kg body weight) and silymarin (50 mg/kg body weight) treated INH groups to near normal. Hepatotoxicity causes cirrhotic liver condition, leading to defective biliary functioning and increased bilirubin release in blood circulation (Rao, 1973). Pre-treatment with CA ethanolic extract (100 mg/kg body weight) as well as the reference drug silymarin (50 mg/kg body weight), restored the level of bilirubin to near normal levels. This may be due to the stabilization of biliary dysfunction of INH treated rat liver and thus improving the functions of the rat liver to near normal. ALP is also a marker enzyme for evaluating liver damage. Increases in ALP, generally 1-2 times the normal level, occur in liver disorders. Elevated levels of serum ALP are indicative of cellular leakage and loss of functional integrity of liver cell membrane (Santhosh *et al*., 2007). In the present study the damage to the membrane of liver cells by ROS and increase in lipid peroxidation of cell membrane of liver might have caused leakage of cellular enzymes, ALP, into the serum. This was found to be decreased in CA extract (100 mg/kg body weight) and silymarin (50 mg/kg body weight) treated INH groups to near normal.

Albumin is the main protein found in blood and is bio-synthesised in the liver. A decrease in the levels of albumin indicates liver dysfunction (Thapa and Walia, 2007) Various studies have indicated a decrease in albumin levels and increase in serum total protein and serum globulin in TB treated rats (Santhosh *et al*., 2007). The estimation of TSP helps in differentiating amidst normal and damaged liver function. This is because, most of the serum proteins like albumins and globulins are bio-synthesised in the liver (Thapa and Walia, 2007). During hepatocellular injury, total protein may be reduced a little, but there is always a sharp decrease in albumin level and increase in globulin level (Singh *et al*., 2011). In the current study a decrease in the TSP and serum albumin, and an increase in serum globulin were observed in INH treated rats. This may be due to damage and dysfunction of liver by INH administration. The increase in the anti-oxidative status of the liver by CA extract (100 mg/kg body weight) and silymarin (50 mg/kg body weight) treated INH groups may be the reason of restored levels of TSP, serum albumin and globulin to near normal.

After liver, the toxic secondary metabolites produced by phase I and II metabolising enzymes in the liver gets excreted out of the body through the kidneys. The second organ which get

affected by TB drugs toxic metabolites are the kidneys. The nephrotoxicity causes an incomplete filtration of the toxicants like urea and creatinine, leading to increased levels of these toxicants in the blood circulation. Urea and creatinine are thus very important kidney function markers (Daphne *et al*., 2003). In this study, the damage caused by INH treatment in rats lead to an increase in the serum levels of urea and creatinine. Nephrotoxicity may be due to an increase in ROS and decrease in the antioxidant enzyme levels in the system. Our previous study state that CA ethanolic leaves extract GC-MS and LC-MS analysis various pharmacologically active compounds and the extract also has very good *in vitro* antioxidant and free radical scavenging activity, which may be due to the synergic effect of various antioxidant compounds present in the extract. Thus, the increase in the anti-oxidative status of the kidney by CA extract (100 mg/kg body weight) and silymarin (50 mg/kg body weight) treated INH groups might be the reason of restored levels of urea and creatinine to near normal.

The FT-IR results revealed the presence of various functional groups which may belog to phytoconstituents like phenol, alkaloids, flavonoids, etc. The HPLC analysis showed the presence of Asiatic acid which was found to be similar with the results of Borhan *et al*. (2013). The ESI-MS showed the presence of Asiatic acid die to the presence of m/z ~ 489.05, which is similar to Asiatic acid. The NMR of whole plant extract of plants can serve to exploring the complex plant secondary metabolites (Kutyshenko *et al*., 2015; Mahrous and Farag, 2015). The database construction is a crucial part of the NMR library. It is a very constructive process which requires various known standard NMR plot generation and then finally pooling the results for the output of results. Thus, the NMR shows a complex plot which is hard for interpretation due to various phyto-constituents present in a single pool of solution. The generation of database of compounds help in interpreting the presence of a compound in the whole data base. The comparison of whole extract with result showed the presence of Asiatic acid like compound.

Sever distortion in hepatocellular organization was observed in INH (50 mg/kg b.wt for 30 days) treated rats. This observation was in agreement with the results of Jadhav and Mateenuddin (2013) and of Sankar *et al*. (2015). These histological damages were clearly reflected in ultrastructural damages in liver section of INH treated rats. Similarly, distortion and damages in over all histological arrangement were also observed in kidney cortex sections in INH treated rats. These finding were in agreement to that of Hussein *et al*. (2016). These adverse effects on histology and/or ultrastructural changes in INH administered liver

and kidney sections were found to be restored in CA extract (100 mg/kg body weight) and silymarin (50 mg/kg body weight) pre-treatment. This may be due to the presence of various antioxidant and anti-inflammatory compounds present in the extract.

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Appendix – VI

SUMMARY OF THE FINDINGS

Tuberculosis (TB) is a chronic communicable disease caused by the bacterium *Mycobacterium tuberculosis*. It is second in line as the greatest killer after HIV/AIDS. According to the Global Tuberculosis Report 2013 by WHO, in 2012 India had been reported to have the world's one quarter burden of TB patients. Isoniazid is a commonly used drug for the treatment of tuberculosis. The main drawback is its toxic side effects. The present study was designed to examine the protective effect of *Centella asiatica* ethanolic leaves extract on the haematological, lipid profile (TC, TG, HDL, LDL, VLDL & Phospholipids), serum antioxidant, liver function markers (ALT, AST, ALP & LDH)and kidney function markers (urea and creatinine) in serum, and serum biochemical changes, as well as biochemicals (lipid peroxidation marker: TBARS; antioxidant status: SOD, CAT & GST; protein content, glycogen; phospholipids), histological changes and/or ultrastructural changes in liver & kidney induced in the blood of isoniazid treated albino rats. The results of the present study collectively demonstrate that the pre-treatment of INH treated rats with *Centella asiatica* (Ca) leaves ethanolic extract (100mg/kg b.wt/day) can reduce the possible side effects of the TB drug INH. The phytochemical analysis by FT-IR, GC-MS and LC-MS analysis of the extract revealed the presence of pharmacologically active phytochemicals (phytochemicals/phyto-compounds). These provide evidence that the synergetic effect of the phytochemicals may be responsible for reversing the toxic effect of INH treatment in albino rats.

Thus, this study provides further avenues to explore the molecular mechanisms involved in the protective mechanism of the *Centella asiatica* leaves ethanolic extract, and also study the long-term effect of the extract, and explore the effects of the phyto-constituents (phytochemicals/phyto-compounds) identified for their individual as well as their synergic effects on mammalian models.

Appendix – VII

CONTRIBUTION TO THE SOCIETY

Indigenous systems of medicines like "Ayurveda" have been long in use by the natives of India for various ailments. The most popular are the plants which form the basic repository of complex phytochemical mixtures. Various medicinally important plants can possess multifaceted properties in curing many diseases or help prevent a group of shared symptoms involved in many diseases. *Centella asiatica* is well known plant which is consumed as a staple leafy vegetable and has various properties such as memory enhancing, treating ailments like skin diseases, rheumatism, inflammation, syphilis, mental illness, epilepsy, hysteria, dehydration, diarrhoea, wounds and ulcers. Tuberculosis (TB) is a widely spread chronic and communicable disease caused by the bacterium *Mycobacterium tuberculosis*. It is second in line as the greatest killer after HIV/AIDS. Isoniazid also known as isonicotinyl hydrazine (INH) or isotonic acid hydrazide is a broad-spectrum antibiotic. It is the first-line drug, clinically used since 1952, in the treatment and chemoprophylaxis of tuberculosis. It is adversely associated with hepatotoxicity. As the major drugs is also affecting the major organ of the patients, the life saviour drugs start acting as a double edged sword on the patient's overall well being. The use of additive compounds which are naturally derived can curb much of the adverse reactions from these potent life saving medicines and can also help in speedy recovery of the patients. Thus, this study helps in understanding the modulatory effect of *Centella asiatica* in INH induced toxicity in a mammalian model (albino rat). This study opens up new avenues in using naturally available plants for improving allopathic medicine's overall effect on the patients and can also prove to help in restoring their overall health.

Research Article

Phytochemistry, *in vitro* Free Radical Scavenging, Chelating and Toxicity of *Centela asiatica* L. (Apiaceae) Ethanolic Leaf Extract

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ABSTRACT

Centella asiatica L. (Apiaceae) has various pharmaceutical properties and has been widely used for treating various ailments in Ayurvead. The present study was carried out to identify the phytochemicals present in the ethanolic extract of *Centella asiatica L. (Apiaceae)* leaves using both qualitative and quantitative screening methods, GC-MS and LC-MS analysis. The ethanolic extract was also subjected to *in vitro* free radical scavenging and chelating activity. The phytochemical screening showed the presence of triterpenoids, saponins, glycosides, sterols and alkaloids. The major components identified by gas chromatography-mass spectrometry (GC-MS) analysis were Methyl pyromeconic acid (RT: 5.183), Methoxy vinyl phenol (RT: 8.617), 3',5'-Dimethoxyacetophenone (RT: 11.867), Beta-D-Ribofuranoside (RT: 10.675), Cyclohexanecarboxylic Acid (RT: 12.825), 5-methoxy-2,2,8,8-tetramethyl-acetate (RT: 25.733) and Nobiletin (RT: 27.275). The major components identified by liquid chromatography-mass spectrometry (LC-MS) analysis were Maltol, 3', 5'-Dimethoxyacetophenone, Papyriogenin A, Asiatic acid, Asiaticoside, Madecassoside and Madecassic acid. *Centella asiatica* ethanolic extract effectively scavenges hydroxyl (OH•), superoxide anion (O2•–), DPPH•, nitric oxide, ABTS•+ and chelates in a concentration-dependent manner (12.5, 25, 50, 100 and 200 µg/ml). The LC_{50} values for *in vitro* toxicity and haemolytic activity was found to 69.17 ±3.2 and 476.19 ±5.9 µg/ml, respectively. The present study indicates the presence of strong antioxidant, free radical scavenging and chelating properties in the ethanolic extract of *Centella asiatica L. (Apiaceae)* leaves.

Keywords: Triterpenoids, Phytochemical, Toxicity, Haemolytic.

INTRODUCTION

Centella asiatica L. (Apiaceae) is also commonly known as Asiatic pennywort, Indian pennywort, Mandukparni, Spadeleaf or Gutu kola. It belongs to the family Apiaceae, formerly known as Umbelliferae. It is a slender, prostrate, glabrous, perennial creeping herb rooting at the nodes, with simple petiolate, palmately lobed leaves.

It has been widely cultivated in Southeast Asia, India, China, Sri Lanka, Africa, etc., as vegetable or spice. *Centella asiatica L.* (Apiaceae) has various activities like memory enhancing, wound healing, anti-inflammatory, antioxidant, immun- stimulant, anti-anxiety (anti-hypertensive), anti-stress and anti-epilepsy.

The various health benefits of *Centella asiatica L. (Apiaceae)* has lead to the increased usage of this plant in food and beverages. It has been widely used for the treatment of skin diseases, rheumatism, inflammation, syphilis, mental illness, epilepsy, hysteria, dehydration, diarrhea, wounds and ulcers.[1-5]

The present study was conducted to identify the phytochemicals present in the ethanolic extract of *Centella asiatica L.* (Apiaceae) leaves using both qualitative and quantitative screening methods, GC-MS and LC-MS analysis. The ethanolic extract was also used to evaluate the in vitro toxicity, free radical scavenging and chelating activity.

MATERIALS AND METHODS

Chemicals

Sodium carbonate, $KMnO_4$, $FeCl_3$, H_2O_2 and 2, 6-dichlorophenolindophenol was purchased from E. Merck. BHT, trichloroacetic acid, potassium ferricyanide, 1, 1-diphenyl-2-picryl- hydrazyl (DPPH), and ascorbic acidwere purchased from Sigma Chemical Co. Ltd, USA. All other chemicals and solvents used were of analytical grade.

Plant Material collection and identification

Centella asiatica L. (Apiaceae) used in this study was collected freshly from outskirts of Chidambaram, Cuddalore District. The plant was identified at the herbarium of Department of Botany, Annamalai University. The leaves were washed under running tap water to remove dirt and other debris. It was then spread under a clean shade for drying. The dried leaves were milled to coarse power using a mechanical grinder and stored in an air-tight container.

Ethanolic extraction of plant material

Approximately 1 kg of powered *Centella asiatica L.* (Apiaceae) was used for ethanolic extraction using Soxhlet apparatus. The dark green extract obtained was subjected to ultracentrifugation followed by micro-filtration. The final clear dark extract was then concentrated in a rotary evaporator under reduced pressure. The final dried extract was lyophilized and was stored in a glass vials at -20°C for further use. This extract

was then subjected to preliminary qualitative and quantitative phytochemical analysis.

Percentage yield of plant extract

The percentage yield of the extract was determined gravimetrically using the dry weight of the crude extract obtained (X) and dry weight of plant powder used for the extraction (Y) by using the following formula:

Percentage yield = X/Y * 100

Qualitative and quantitative analysis

A. Qualitative screening

Phytochemical screening was carried out by using 1 gram of the dried ethanolic extract which was subjected to phytochemical test as described below (Harborne, 1973).[6]

***Detection of alkaloids* (Mayer's Test)**

The extracts was dissolved in dilute Hydrochloric acid and filtered. The filtrate was treated with Mayer's reagent (potassium mercuric iodide). Formation of yellow coloured precipitate indicates the presence of alkaloids.

***Detection of phenols* (Ferric Chloride Test)**

Extract was treated with 3-4 drops of 10% ferric chloride solution. Formation of green colour indicates the presence of phenols.

***Detection of flavonoids* (Alkaline Reagent Test)**

Extracts were treated with few drops of sodium hydroxide solution. Formation of intense yellow colour, which becomes colourless on addition of dilute acid, indicates the presence of flavonoids.

Detection of quinones

The extract was treated with few drops of sulphuric acid. Formation of red colour indicates the presence of quinines.

***Detection of tannins* (Gelatin Test)**

To the extract, 1% gelatin solution containing sodium chloride was added. Formation of white precipitate indicates the presence of tannins.

***Detection of saponins* (Foam Test)**

0.5 gm of extract was shaken with 2 ml of water. If foam produced persists for ten minutes it indicates the presence of saponins.

***Detection of terpenoids* (Salkowski test)**

The extract was added 2 ml of chloroform. Concentrated H S0 (3 ml) was carefully added to form a layer. A reddish brown colouration of the interface indicates the presence of terpenoids.

B. Quantitative screening

***Determination of Total Flavonoids* (Aluminium chloride colorimetric assay method)**

Total flavonoid contents were measured with the aluminum chloride colorimetric assay.[8] Aqueous and ethanolic extracts that has been adjusted to come under the linearity range i.e. (400µg/ml) and different dilution of standard solution of Quercetin (10-100µg/ml) were added to 10ml volumetric flask containing 4ml of water. To the above mixture, 0.3ml of 5% NaNO2 was added. After 5 minutes, 0.3ml of 10% AlCl3 was added. After 6 min, 2ml of 1 M NaOH was added and the total volume was made up to 10ml with distilled water. Then the solution was mixed well and the absorbance was measured against a freshly prepared reagent blank at 510 nm. Results are provided in (Table 2 and Figure 2). Total flavonoid content of the extracts was expressed as percentage of Quercetin equivalent per 100 g dry weight of sample.

Determination of total phenolic content

The total phenolic content (TPC) assay was performed in accordance to Singleton *et al*., 1999, with modifications.[8] An aliquot of 0.5 ml of each sample was mixed with 1 ml of Folin-Ciocalteu reagent (10% in distilled water) in a universal bottle covered with aluminum foil. After 3 min, 3 ml of 1% sodium bicarbonate was added to each sample bottle, the universal bottles were cap-screwed and vortex. The samples were then incubated for 2 hr at room temperature in darkness. The absorbance was measured at 760 nm spectrophotometrically (Genesys UV 20, US). A standard curve of gallic acid solutions (ranging from 0 µg ml^{-1} to 250 µg ml^{-1}) was used for calibration. The experiment was done in triplicate. Results were expressed as microgram of gallic acid equivalents (GAE) per milligram of extract (GAE; µg mg^{-1} dry extract).

Quantitative Estimation of Saponins

Plant extract was dissolved in 80% methanol, 2ml of Vanilin in ethanol was added, mixed well and the 2ml of 72% sulphuric acid solution was added, mixed well and heated on a water bath at 60°c for 10min, absorbance was measured at 544nm against reagent blank.[9]

Quantitative estimation of Alkaloids

5 g of the sample was weighed into a 250 ml beaker and 200 ml of 10% acetic acid in ethanol was added and covered and allowed to stand for 4 h. This was filtered and the extract was concentrated on a water bath to one-quarter of the original volume. Concentrated ammonium hydroxide was added drop wise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitated was collected and washed with dilute ammonium hydroxide and then filtered. The residue is the alkaloid, which was dried and weighed.[10]

Gas chromatography-mass spectrometry fingerprinting of crude extract

GC-MS analysis was done at National Chemical Laboratory Pune, Maharashtra, India. GC-MS sample was prepared by dissolving about 1 mg of *Centella asiatica L. (Apiaceae)* extract in 5 mL of methanol. Active extract was dissolved in HPLC grade methanol and subjected to GC and MS JEOL GC mate equipped with secondary electron multiplier. JEOL GCMATE II GC-MS (Agilent Technologies 6890N Network GC system for gas chromatography). The column (HP5) was used with fused silica 50 m x 0.25 mm I.D. Analysis conditions were 20 minutes at 100°C, 3 minutes at 235°C for column temperature, 240°C for injector temperature, helium was the carrier gas and split ratio was 5:4. The sample (1µl) was evaporated in a split less injector at 300°C. Run time was 22 minutes. The components were identified by gas chromatography coupled with mass spectrometry. Interpretation of mass spectra of GC-MS was done using the database of National Institute Standard and Technology (NIST) library search which is having more than 62,000 drug formulation. The mass spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST-08 and Wiley-08 libraries. The name, molecular weight and structure of the components of the test materials were validated.

LC-MS metabolomic fingerprinting of crude extract

In the present work the RP-HPLC (Shimadzu model – LA 3000) with semi preparative C18 HPLC column of Pheomenex (250 x10mm, 4 µm particle size, and 90Å pore size) and analytical column Zorbax C18 (4.6 x250mm, 5 µm particle size, and 80Å pore size) were used for the purification. Solevent system used here is Acetonitrile and Water containing (0.1%) TFA. A three step gradient elution was performed using of 0.1% TFA/water and 0.1% (v/v) TFA in 50% acetonitrile: 0–100% (60 min) held at 100% for 5 min and brought back to 0% (100–0%). Injection volume was 0.5 microlitres. Fractions were collected automatically using the fraction collector (FRC-10-A, Shimadzu model). The absorbance of the fractions was monitored at 280 nm and the peaks were compared with the spectrum of known components in NIST-08, Wiley-08, NAPRALET and CHEMSPIDER databases. The compound names, molecular weight ant the structure of the compound identified in the crude extract were validated.

***In vitro* free radical scavenging activity**

Hydroxyl radical scavenging activity was measured by studying the competition between deoxyribose and the ethanolic leaf extract of *Centella asiatica L. (Apiaceae)* by Fe_3^+–Ascorbate–EDTA–H_2O_2 system (Fenton reaction) according to the method of Elizabeth and Rao[11]. The generation of $OH^•$ is detected by its ability to degrade deoxyribose to form products, which on heating with TBA forms a pink colored chromogen. The absorbance of the supernatant was read in a spectrophotometer at 535 nm.

The efficiency of *Centella asiatica L. (Apiaceae)* ethanolic leaf extract was compared with dimethyl sulphoxide (DMSO) as standard. Superoxide anion ($O_2^{•-}$) scavenging activity of *Centella asiatica L. (Apiaceae)* ethanolic leaf extract was determined by the method of Liu *et al*., 1997.[12] Superoxide anion that is derived from dissolved oxygen through the PMS/NADH coupling reaction reduces NBT and absorbance was read in spectrophotometer at 560 nm. The efficiency of *Centella asiatica L. (Apiaceae)* ethanolic leaf extract was compared with ascorbic acid as standard. The effect of *Centella asiatica L. (Apiaceae)* ethanolic leaf extract on $DPPH^•$ was assayed using the method of Brand-Williams *et al*., 1995.[13]$DPPH^•$ is a stable free radical and accepts an electron, or hydrogen radical to become a stable diamagnetic molecule. $DPPH^•$ reacts with an antioxidant compound that can donate hydrogen and gets reduced. The change in color (from deep violet to light yellow) was measured at 517 nm. The efficiency of *Centella asiatica L. (Apiaceae)* ethanolic leaf extract was compared with BHT as standard. The nitric oxide radical scavenging capacity of the *Centella asiatica L. (Apiaceae)* ethanolic extract was measured by Griess reaction.[14]

Various concentrations of *Centella asiatica L. (Apiaceae)* ethanolic leaf extract (12.5, 25, 50, 100 and 200 µg/ml in 95% ethanol) were prepared. Sodium nitroprusside (1.5 mL, 10 mM) in phosphate buffer was added to 0.5 mL different concentrations of the extract. The reaction mixture was incubated at 25°C for 150 min. After incubation, 0.5 mL aliquot was removed and 0.5 mL of Griess reagent (1% (w/v) sulfanilamide, 2% (v/v) H3PO4 and 0.1% (w/v) naphthylethylene diamine hydrochloride) was added. The absorbance was measured at 546 nm. Ascorbic acid was used as reference standard and was treated the same way as that of the extract. Sodium nitroprusside in PBS (2 mL) was used as control.

The improved technique for the generation of $ABTS^{•+}$ involves the direct production of the blue/green $ABTS^{•+}$ chromophore through the reaction between $ABTS^{•+}$ and potassium persulphate.[15] The reaction mixture consisted of 0.5 mL of 15 µM H_2O_2, 0.5 mL of 7 mM ABTS and 50 mM sodium phosphate buffer, pH 7.5 and varying concentrations of *Centella asiatica L. (Apiaceae)* ethanolic leaf extract (12.5, 25, 50, 100 and 200 µg/ml). The blank contained water in place of *Centella asiatica L. (Apiaceae)* ethanolic leaf extract. The absorbance was read in spectrophotometer at 734 nm and compared with standard ascorbic acid. Decreased absorbance of the reaction mixture in all the assays indicated increased radical scavenging activity. The % of scavenging or inhibition was calculated according to the following formula:

$$\% \text{ of scavenging or inhibition} = [(A_0 - A_1)/A_0] \times 100$$

Where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of *Centella asiatica L. (Apiaceae)* ethanolic leaf extract or ascorbic acid/DMSO.

Iron chelating activity

The method of Benzie and strain (1996) was adopted for the assay.[16] The principle is based on the formation of O-Phenanthroline-Fe_2^+ complex and its disruption in the presence of chelating agents. The reaction mixture containing 1 ml of 0.05% O-Phenanthroline in methanol, 2 ml ferric chloride (200µM) and 2 ml of various concentrations ranging from 10 to 500µg was incubated at room temperature for 10 min and the absorbance of the same was measured at 510 nm. EDTA was used as a classical metal chelator. The experiment was performed in triplicates.

% of chelating activity = sample OD/Control OD x 100.

In vitro toxicity tests

Drug preparation

The *Centella asiatica L. (Apiaceae)* extract was dissolved in 0.05% (v/v) of Dimethyl Sulfoxide (DMSO) and it did not affect cell survival.

PBMC proliferation test

Blood samples from healthy volunteers were collected by venepuncture and transferred into 15 ml heparin coated test tubes. It was diluted at 1:1 ratio with PBS, layered onto Ficoll-Histopaque 1077 at a volume ratio of 3:1 and centrifuged at 1,000 x g for 30 min. During the centrifugation the PBMCs moved from the plasma and were suspended in the density gradient, isolating them from erythrocytes and granulocytes. The PBMCs layer was removed and then washed twice with PBS. The supernatant was then removed and the cells were resuspended in complete CDMEM medium supplemented with 1 mM L-glutamine, 100 Units/ml penicillin and 0.1 mg/ml streptomycin, 10% inactivated FCS, and adjusted to pH 7.2 by the addition of 15 mM HEPES. Cell viability was determined by the trypan-blue dye exclusion method. (Results not included in paper)

In vitro Cell viability test

The viability of cells was assessed by MTT assay using primary lymphocyte cells.[17] The PBMC cell density used in the cell viability study was 1 x 10^5 cells/ well of the 96-well tissue culture plate. Dose-response 1- 1000 µg/ml between percentage of cell viability and concentrations of the extracts were constructed.

In vitro hemolytic assay

In vitro haemolytic activity assay was performed according to the method described by Bulmus *et al*., 2003.[18] Briefly freshly collected human red blood cells were taken and washed three times with 150 mM NaCl by centrifugation method at 2500 rpm for 10 minutes.The serum was removed and the cells were suspended in 100 mM sodium phosphate buffer. Nine different concentrations (0,5,10,30,50,100,200,300, 400µg/ml) of extracts were mixed with 200 µL of RBC solutions and the final reaction mixture volume was made up to 1 ml by adding sodium phosphate buffer. The reaction mixture was then placed in water bath for 1 hour at 37°C. After the incubation time the reaction was collected and the optical density was measured at 541 nm.

Statistical analysis

The data were expressed as mean ± SD (n = 3). Statistical analysis of the data was carried out by one-way analysis of variance (Anova) followed by Duncan's Multiple Range Test (DMRT) using a statistical package program (SPSS v11.5 for Windows) $p < 0.05$ were considered as statistically significant.

Table 1: Percentage yield of plant extract

Plant	Solvent	Method	Weight of crude extract (g)	% yield
Centella asiatica	Ethanol	Soxhlet extraction	10.89	1.089

Table 2: Qualitative analysis of *Centella asiatica* ethanolic extract

Secondary metabolites	Test	*Centella asiatica*
Alkaloids	Mayer's Test	+
Phenols	Ferric Chloride Test	+
Flavonoids	Alkaline Reagent Test	+
Quinones	Sulphuric acid test	+
Tannins	Gelatin Test	-
Saponins	Foam Test	+
Terpenoids	Salkowski test	+

+ Presence; - Absence

Table 3: Quantitative analysis of Secondary Metabolites in *Centella asiatica* ethanolic extract.

Plant	Phenols[#]	Flavonoids*	Saponins*	Alkaloids*
Centella asiatica ethanolic extract	12.40	2.70	11.00	3.20

[#]GAE µg mg^{-1} dry extract; * mg/g of crude extract

Table 4: Phytocomponents identified in the ethanolic extract of *Centella asiatica* by GC-MS.

R.T	Compound name	Molecular weight	Peak area %	Molecular formula	Pharmacology
5.183	Methyl pyromeconic acid (maltol)	126	2.24	$C_6H_6O_3$	Catalyst, anti-proliferative, antioxidant, flavor additive
8.617	Methoxy vinyl phenol	150.17	3.06	$C_9H_{10}O_2$	Anti gastritis and blood purifier
11.867	3',5'-Dimethoxyacetophenone	180	1.12	$C_{10}H_{12}O_3$	-
10.675	Beta-D-Ribofuranoside	283.24	12.59	$C_{10}H_{13}N_5O_5$	-
12.825	Cyclohexanecarboxylic Acid	192	28.02	$C_7H_{12}O_6$	Anti-inflammatory
25.733	5-methoxy-2,2,8,8-tetramethyl-acetate	372	1.01	$C_{22}H2_8O_5$	Antioxidant
27.275	Nobiletin	402.39	0.87	$C_{21}H_{22}O_8$	Antioxidant and anti-inflammatory

RT: Retention Time

Table 5: LC-MS mass library results for the ethanolic leaf extract of *Centella asiatica*.

S. No.	Compound name	Molecular weight	Pharmacology
1	Maltol	150.17	Anti gastritis and blood purifier
2	3',5'-Dimethoxyacetophenone	180	Antioxidant
3	Papyriogenin A	466.308	Hepatoprotective
4	Asiatic acid	488.70	Neuro protective, anti-bacterial, anti-fungal, antioxidant, wound healing
5	Asiaticoside	959.12	wound healing
6	Madecassoside	975.1	wound healing
7	Madecassic acid	504.17	Anti-inflammatory

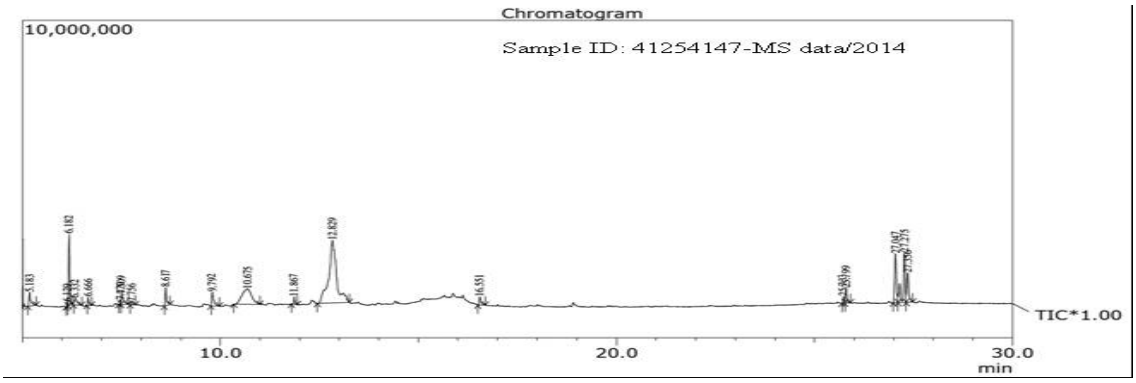

Figure 1: GC-MS spectrum of *Centella asiatica* ethanolic leaf extract.

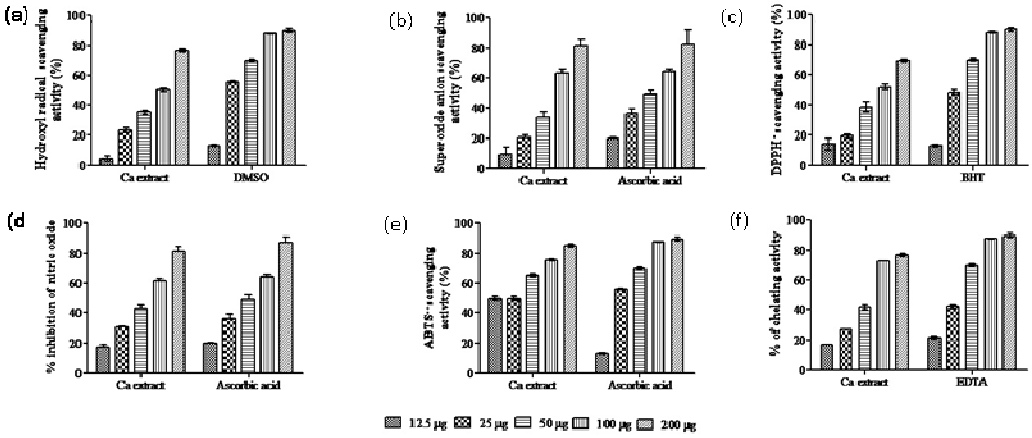

Figure 2: Effect of *Centella asiatica* (Ca) extract on (a): hydroxyl (OH•); (b): superoxide anion (O2•–); (c): DPPH•; (d): Nitric oxide and; e: ABTS•+ radical scavenging ability. (f): Chelating activity of *Centella asiatica* extract. The values are given as mean ± SD of three experiments in each group.

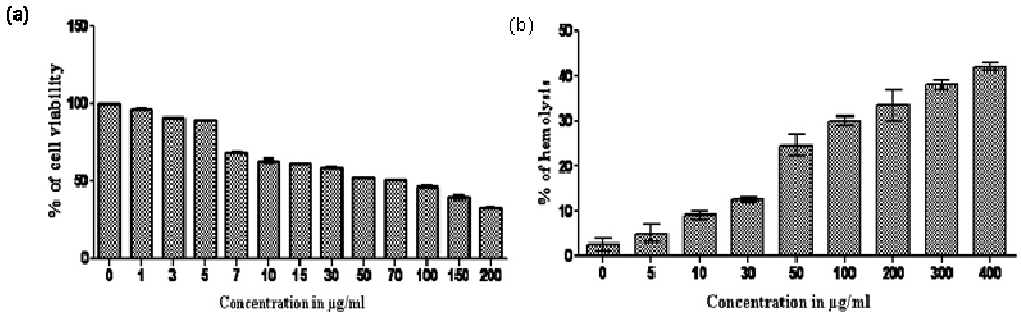

Figure 3: (a) Percentage cell viability of *Centella asiatica* extract in PBMC. Lethal concentration 50 (LC_{50}) value for *Centella asiatica* extract was found to be 69.17 ±3.2 µg/ml. (b) Percentage of hemolysis induced by *Centella asiatica* extract at concentrations ranging from 5-400 µg/ml. Lethal concentration 50 (LC_{50}) value for *Centella asiatica* extract was found to be 476.19 ±5.9 µg/ml. The values are given as mean ± SD of three experiments in each group.

RESULTS AND DISCUSSION

This study was designed to determine the phytocomponents, *in vitro* toxicity, free radical scavenging and chelating activity in the ethanol leaf extract of *Centella asiatica L. (Apiaceae)*. Table 1 shows the percentage yield of *Centella asiatica* (CA) ethanolic leaf extract and was found to be 1.089. The preliminary phytochemical screening of the ethanolic leaf extract of CA showed the presence of triterpenoids, saponins, glycosides, sterols and alkaloids (Table 2). The quantitative analysis of the extract (Table 3) showed the presence of phenols at a concentration of 12.4 GAE µg mg^{-1} dry extract. The concentrations of flavanoids, saponins and alkaloids were found to be 2.70, 11.00 and 3.20 mg/g of crude extract respectively. In GC-MS analysis 7 phytochemical compounds were identified in the ethanolic leaf extract of *Centella asiatica* (CA). The identification of the phytochemical compounds is done by comparing the mass spectrum of the unknown component with the spectrum of the known components stored in the NIST-08 and Wiley-08 libraries. Activities of components identified in ethanolic extract were found from Dr.Duke's phytochemical and ethnobotanical databases [Online database]. Figure 1 shows GC-MS Spectrum of ethanolic leaf extract of *Centella asiatica*. The major components which were found in the leaves of the CA leaf ethanolic extract are (Table 4): Methyl pyromeconic acid (maltol) (RT: 5.183), Methoxy vinyl phenol (RT: 8.617), 3',5'-Dimethoxyacetophenone (RT: 11.867), Beta-D-Ribofuranoside (RT: 10.675), Cyclohexanecarboxylic Acid (RT: 12.825), 5-methoxy-2,2,8,8-tetramethyl-acetate (RT: 25.733) and Nobiletin (RT: 27.275). LC method was used for determining the non-volatile phytochemical contents constituents of *C. asiatica* ethanolic leaf extract. In LC-MS analysis 7 phytochemical compounds were identified in the ethanolic leaf extract of *C. asiatica* (CA). Activities of components identified in ethanolic leaf extract were found from Dr.Duke's phytochemical and ethnobotanical databases [Online database]. The major components which were found in the leaves of the CA leaf ethanolic extract are (Table 5): Maltol, 3', 5'-Dimethoxyacetophenone, Papyriogenin A, Asiatic acid, Asiaticoside, Madecassoside and Madecassic acid. Medicinal plants are rich source of secondary metabolites. The triterpene form the major constituent of *C. asiatica*. Compounds like Asiatic acid, Asiaticoside, Madecassoside and Madecassic acid are already reported to be present in *C. asiatica*.[19] Papyriogenin A, a triterpenoid, was first time identified in *C. asiatica* extract. It is a potent hepato-protective compound.[20]

Figure 2 (a-e) shows the hydroxyl radical, superoxide anion, DPPH•, nitric oxide and $ABTS^{\bullet+}$ scavenging ability of CA ethanolic leaf extract. CA ethanolic leaf extract inhibits radical formation and the percentage of inhibition was observed in a concentration-dependent manner. The effective concentration 50 (EC_{50}) values of CA ethanolic leaf extract in hydroxyl radical, superoxide anion, DPPH•, nitric oxide and $ABTS^{\bullet+}$ were 99.29 ±4.2, 78.98 ±5.4, 95.88 ±8.3, 80.90 ±3.5 and 38.26 ±7.8 µg/ml, respectively and the free radical scavenging property was comparable to standard DMSO (Dimethyl sulfoxide), BHT (Butylated hydroxytoluene) and ascorbic acid, with EC_{50} values 22.34 ± 2.9, 35.67 ± 8.3, 77.72 ± 3.4 for SOD and 77.72 ± 6.9 µg/ml for nitric oxide radical scavenging assays, respectively . Iron binding capacity of CA ethanolic extract was carried out at various concentrations (12.5, 25, 50, 100 and 200 µg/ml). The metal chelator EDTA was used as a standard. Figure 2 (f) shows the percentage of iron chelating activity of CA ethanolic leaf extract compared to standard EDTA. The EC_{50} values of CA extract and EDTA was found to be 68.13±7.9 and 35.67 ±6.8 µg/ml, respectively. Figure 3(a) shows the percentage cell viability of *Centella asiatica* extract in PBMC. Lethal concentration 50 (LC_{50}) value for *Centella asiatica* extract was found to be 69.17 ±3.2 µg/ml. Figure 3(b) shows the percentage of hemolysis induced by *Centella asiatica* extract at concentrations ranging from 5-400 µg/ml. Lethal concentration 50 (LC_{50}) value for *Centella asiatica* extract was found to be 476.19 ±5.9 µg/ml. The presence of various antioxidant and anti-inflammatory compounds may be the reason for the presence of strong antioxidant, free radical scavenging and chelating properties of CA ethanolic leaf extract.

CONCLUSION

The present study revealed the presence of secondary metabolites of various therapeutical active compounds in the *Centella asiatica* ethanolic leaf extract. The presence of various bioactive compounds supports the use of the whole plant for various ailments in Ayurveda. This study further provides the scope of isolating and understanding the characteristics of each compound, alone or in combination, for it pharmacological properties.

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The ameliorating effect of *Centella asiatica* ethanolic extract on albino rats treated with isoniazid

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Abstract

Background: Isoniazid, also called isonicotinyl hydrazine (INH), is a commonly used drug for treating tuberculosis. The main drawback is its toxic side effects. *Centella asiatica* has long been used in the Ayurvedic system of medicine owing to its wide medicinal properties. This study was designed to examine the effectiveness of *C. asiatica* ethanolic leaf extract (CA) on INH-treated albino rats.
Methods: The adverse effects induced by INH (50 mg/kg bw) administration on haematological parameters, oxidative status (thiobarbituric acid-reactive substances, superoxide dismutase, catalase, and reduced glutathione), liver and kidney function markers, and their amelioration by various concentrations of CA (20, 40, 60, and 100 mg/kg bw) or silymarin (SIL) (50 mg/kg bw, administered before 1 h of INH treatment for 30 days to rats) were studied. Moreover, histological studies were carried out in liver and kidney tissues of rats treated with the most effective concentration to further support the possible effectiveness of CA on INH-intoxicated rats.
Results: All the affected parameters returned to near-normal levels, and the effective concentration of extract was found to be 100 mg/kg bw. The histology of both the liver and the kidneys subsequently supported the effectiveness of CA (100 mg/kg bw).
Conclusions: Altogether, the results suggest that CA at 100 mg/kg bw can substantially reduce the toxic effects of INH.

Keywords: antioxidant; *Centella asiatica*; haematological; isoniazid; kidney; liver.

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Introduction

Tuberculosis (TB) is a chronic communicable disease caused by the bacterium *Mycobacterium tuberculosis*. It is second in line as the greatest killer after HIV/AIDS. According to the Global Tuberculosis Report 2013 by the World Health Organization, India had been reported to have the world's quarter burden of TB patients. India and China alone were reported to have accounted for 26% and 12% of the total TB cases, respectively [1]. The two most commonly used first-line anti-TB drugs are isoniazid, also known as isonicotinyl hydrazine (INH) or isotonic acid hydrazide, and rifampicin/rifampin, which are orally administered in combination for >6 months [2–4].

INH is a broad-spectrum antibiotic. It is widely used in the treatment of *M. tuberculosis* bacterial infections [5]. It is the first-line drug, clinically used since 1952, in the treatment and chemoprophylaxis of TB. It is adversely associated with hepatotoxicity [6–8]. INH is readily metabolised by *N*-acetyltransferase, to form acetylisoniazid. It then undergoes hydroxylation by cytochrome P450 enzymes to form hepatotoxic intermediates such as acetylhydrazine and isonicotinic acid. Acetylhydrazine is capable of forming covalent cellular adducts. It is further hydrolysed into hydrazine or acetylated products called diacetylhydrazine. Patients administered with INH exhibit elevated levels of hydrazine in their blood serum [7]. Following administration of a single dose, these hydrazines can rapidly distribute to all tissues without preferential accumulation. Both enzymatic and non-enzymatic pathways are involved in the metabolism of hydrazines. Humans with a slow acetylator genotype tend to accumulate more hydrazine in the serum; this is due to an impaired ability to metabolise and excrete the toxic compounds [6, 7]. Although there are various evidence-based reports on INH hepatotoxicity, INH remains to be a first-line drug for the treatment of TB [8]. A study by Luntz and Smith reported abnormalities in carbohydrate metabolism and an increase in blood glucose level in diabetic and non-diabetic TB patients [9].

Centella asiatica L. (Apiaceae), commonly known as Asiatic pennywort or Indian pennywort, belongs to the family Apiaceae (formerly known as Umbelliferae). It is a slender, prostrate, glabrous, perennial creeping herb rooting at the nodes, with simple petiolate, palmately lobed leaves. It is widely cultivated in Southeast Asia, India, China, Sri Lanka, etc., as a vegetable or spice [10]. *Centella asiatica* has various pharmacological activities like memory-enhancing, anti-inflammatory, antioxidant, immune booster, anti-hypertension, anti-stress, and anti-epilepsy activities; thus, it has been widely used for treating skin diseases, rheumatism, syphilis, hysteria, diarrhoea, wounds, and ulcers [10–14]. These diverse health benefits of *C. asiatica* has led to the increased use of this plant in food and beverages [14]. The *C. asiatica* ethanolic leaf extract (CA) has also been previously reported to have a strong antioxidant property and to act as an iron chelator in an in vitro study [15]. Thus, the above circumstances have directed us to examine the effect of CA on INH-induced toxicity in Wistar rats by evaluating the haematological parameters, oxidative status, liver and kidney function markers, and histological observations of the liver and kidneys.

Materials and methods

Chemicals

INH and SIL were purchased from Sigma-Aldrich Co. Ltd. (USA). Methanol, HCl, sulphanilic acid, sodium nitrate, sodium carbonate, copper sulphate, and bovine serum albumin were purchased from Himedia Laboratories Pvt Ltd. (Mumbai, India). All other chemicals used for the biochemical estimations were of analytical grade and were procured from Himedia Laboratories Pvt Ltd.

Collection and identification of plant material

Centella asiatica used in this study was collected freshly from the outskirts of Chidambaram, Cuddalore District. The plant was identified at the Herbarium of Department of Botany, Annamalai University (herbarium no. DDE/HER/44). The plant materials were washed under running tap water to remove dirt and other debris. It was then spread under a clean shade for drying. The dried plant material was milled to coarse powder using a mechanical grinder and stored in an air-tight container.

Ethanolic leaf extraction of *C. asiatica*

Approximately 1 kg of powdered *C. asiatica* was used for ethanolic extraction using a Soxhlet apparatus. The dark green extract obtained was subjected to ultracentrifugation followed by microfiltration. The final clear dark extract was then concentrated in a rotary evaporator under reduced pressure (10–15 mm Hg) at 40 °C to obtain the crude ethanol extract. The final dried extract was lyophilised and stored in a glass vial at –20 °C for further use.

Percentage yield of CA extract

The percentage yield of the CA ethanolic extract was determined gravimetrically, using the dry weight of the crude extract obtained (X) and dry weight of plant powder used for the extraction (Y) by using the following formula:

$$\text{Percentage yield} = X/Y \times 100.$$

Animals

Male Wistar albino rats of body weight 180–200 g were used for this study. The animals were maintained in the Central Animal House, Department of Experimental Medicine, Rajah Muthaiah Medical College and Hospital, Annamalai University, Annamalainagar, India. The animals were fed on a standard pellet diet and water *ad libitum*. The animals were housed in polycarbonate cages under controlled conditions of temperature (23 ± 2 °C) and humidity (65%–70%) with a 12-h light/dark cycle. All the protocols of this study were approved by the Institutional Animal Ethics Committee and guidelines of Annamalai University (160/1999/CPCSEA; proposal no. 1021, dated 06.08.2013).

Experimental design

A total of 48 rats were randomly divided into eight groups, each comprising six rats. All treatments were administered orally using an intragastric tube daily for a period of 30 days [16, 17]. Group 1 received only vehicle (0.5% dimethyl sulfoxide) and served as a control. Group 2 was administered INH (50 mg/kg bw) alone. Group 3 to group 6 received INH (50 mg/kg bw) and CA at various concentrations (20, 40, 60, and 100 mg/kg bw). Group 7 received CA alone with the highest concentration (100 mg/kg bw) and was assigned as the positive control. Group 8 acted as an internal control and received INH (50 mg/kg bw) and SIL (50 mg/kg bw). Both CA and SIL were treated 1 h prior to INH administration.

The experiment was terminated at the end of 30 days, and the animals were fasted overnight, weighed, and sacrificed by cervical dislocation. Blood was collected in heparinised tubes for the estimation of haematological parameters. Fresh blood was centrifuged to collect serum for glucose and biochemical parameters. The liver and kidney tissues were washed with ice-cold saline and stored in a 10% buffered formalin solution.

Preparation of serum and plasma

Blood samples were collected and centrifuged at ambient temperature for 30 min to separate serum (2000 × g for 10 min). Plasma

samples were collected by centrifuging blood with anticoagulant at 2000 × *g* for 20 min. Then, the supernatant comprising the plasma was carefully separated and utilised for further analysis.

Estimation of blood glucose

Fresh blood was collected and mixed with 10% tricarboxylic acid for glucose estimation. The estimation was done by using the O-toluidine method of Sasaki et al. [18].

Evaluation of haematological parameters

All measurements of haematological parameters were carried out by the method of Dacie and Lewis [19]. The total red blood cells (RBCs), white blood cells (WBC), mean cell haemoglobin (MCH), and mean cell haemoglobin concentration (MCHC), were calculated using the following equations:

$$\text{Number of RBCs/Cu mm} = \frac{\text{Total no. of corpuscles counted}}{\text{Total no. of small squares counted}} \times \text{dilution} \times 100.$$

$$\text{Number of WBCs/Cu mm} = \frac{\text{Total no. of leucocytes counted}}{\text{Total no. of large squares counted}} \times \text{dilution} \times 10.$$

$$\text{MCH (pg)} = \frac{\text{Haemoglobin (Hb) (g/100 mL)} \times 10}{\text{Erythrocyte count (million cells/Cu mm blood)}}.$$

$$\text{MCHC (\%)} = \frac{\text{Hb (g/100 mL)} \times 10}{\text{Haematocrit (Ht) percentage}} \times 100.$$

Hb and Ht were also determined by the method of Dacie and Lewis [19]. The Hb content in the blood was estimated using Sahli's haemometer (Superior, Germany) with permanent glass comparison standards and expressed in g Hb/100 mL blood. Hb was expressed as mg/dL in the final results. To determine the Ht value of blood, blood was centrifuged in heparinised Ht tubes at 2000 *g* for 30 min. The Ht percent was calculated from the volume of blood taken and packed cell volume (PCV) after centrifugation.

Biochemical parameters

Biochemical studies were carried out in blood serum. The concentration of serum thiobarbituric acid-reactive substances (TBARS) was analysed according to the method of Hogberg et al. [20]. The serum superoxide dismutase (SOD) was measured by the method of Kakkar et al. [21]. The serum catalase (CAT) was measured by the method of Sinha [22]. The serum reduced glutathione (GSH) was determined according to the method described by Moron et al. [23].

The total serum protein (TSP) concentration was determined using Folin-Ciocalteau reagent, as described by the method of Lowry et al., and the albumin concentration was determined by the method of Doumas et al., which utilises bromocresol green dye at pH 4.2, to form a green complex [24, 25]. The globulin concentration was calculated as the difference between the albumin concentration and the total protein concentration. The activities of serum alanine transaminase (ALT) and aspartate transaminase (AST) were determined according to the method of Reitman and Frankel [26]. Alkaline phosphatase (ALP) serum activity was determined as described by King and Armstrong [27]. The total serum bilirubin level was measured by using the Van den Bergh reaction, as described by the method of Malloy and Evelyn [28]. The levels of urea and creatinine were determined by the colorimetric method of Patton and Crouch [29].

Histological examination

Liver and kidney tissues from the control and effective-concentration-treated animals were subjected to histological studies. The tissue samples were fixed for 48 h in 10% buffered formalin, followed by dehydrating the tissues by passing through a different mixture of ethyl alcohol and water, and was finally cleaned with xylene and embedded in paraffin for sectioning. Sections of 5–6 μm thickness of tissues were prepared by using a rotary microtome. These sections were fixed on glass slides and stained with haematoxylin and eosin dye, and were observed microscopically.

Statistical analysis

Statistical comparisons were performed by one-way analysis of variance followed by Duncan's multiple range test (DMRT) using SPSS version 16.0 software (SPSS Inc., released 2007; SPSS for Windows, version 16.0; SPSS Inc., Chicago, IL, USA). Values are represented as mean ± SD, and $p < 0.05$ was considered statistically significant.

Results

Percentage yield of plant extract

Table 1 shows the percentage yield of CA ethanolic extract and was found to be 1.089%.

Body weight

Table 2 shows the body weight of control and experimental animals in each group. The mean body weight

Table 1: Percentage yield of plant extract.

Plant	Solvent	Method	Weight of crude extract, g	% Yield
Centella asiatica	Ethanol	Soxhlet extraction	10.89	1.089

Table 2: Effect of CA extract on INH-induced changes in body weight, blood glucose, and haematological parameters in rats of control and experimental groups.

Groups	Control	INH (50 mg/kg bw)	INH+CA (20 mg/kg bw)	INH+CA (40 mg/kg bw)	INH+CA (60 mg/kg bw)	INH+CA (100 mg/kg bw)	CA (100 mg/kg bw)	INH+SIL (50 mg/kg bw)
Body weight, g								
Initial	191.50±19.48^{a}	192.80±20.26^{a}	190.42±15.72^{a}	191.27±20.49^{a}	190.59±15.72^{a}	190.37±18.49^{a}	191.80±20.70^{a}	191.80±16.83^{a}
Final	225.60±19.15^{c}	156.57±18.38^{a}	159.82±13.88^{a,b}	160.95±12.52^{a,b}	181.00±19.09^{b}	223.16±18.49^{c}	223.80±20.26^{c}	208.27±16.88^{c}
Blood glucose, mg/dL								
Initial (7th day)	86.99±4.65^{a}	228.52±2.35^{g}	215.21±3.59^{f}	201.41±4.66^{d}	138.42±3.55^{c}	110.29±1.45^{b}	89.32±3.54^{a}	208.12±2.36^{e}
Final (30th day)	86.97±3.01^{a}	210.42±2.78^{f}	203.41±2.35^{e}	197.93±3.90^{d}	125.82±1.30^{c}	98.71±2.19^{b}	84.68±2.23^{a}	99.69±1.34^{b}
Haematological parameters								
RBC, 10^{6}/mm^{3}	6.88±0.28^{c}	4.81±0.17^{a}	4.92±0.22^{a}	5.02±0.22^{a,b}	5.25±0.13^{b}	6.85±0.23^{c}	6.87±0.23^{c}	6.82±0.29^{c}
WBC, 10^{3}/mm^{3}	5.23±1.66^{a}	8.10±0.96^{c}	7.36±1.46^{b,c}	7.15±1.37^{a,b,c}	6.12±1.68^{a,b}	5.25±1.88^{a}	5.29±1.44^{a}	5.60±1.17^{a,b}
Hb, mg/dL	17.00±0.01^{e}	6.60±0.07^{a}	7.91±0.06^{b}	9.82±0.10^{c}	11.98±0.02^{d}	17.00±0.10^{e}	17.09±0.02^{f}	16.99±0.06^{e}
Ht, %	43.12±3.56^{b}	26.32±2.57^{a}	27.21±2.33^{a}	29.31±1.41^{a}	32.00±1.27^{a}	51.31±20.77^{b}	43.98±3.43^{b}	42.28±2.34^{b}
MCH, pg	21.21±0.08^{e}	15.71±0.09^{a}	16.11±0.04^{b}	17.52±0.03^{c}	18.20±0.02^{d}	21.20±0.04^{e}	21.22±0.08^{e}	21.19±0.03^{e}
MCHC, %	37.15±0.68^{e}	21.21±1.09^{a}	22.59±0.96^{b}	25.80±1.02^{c}	29.29±0.50^{d}	37.15±0.58^{e}	37.19±0.57^{e}	37.11±0.64^{e}

Values are expressed as mean±SD (n=6). Values not sharing a similar superscript letter differ significantly at $p<0.05$ (DMRT).

was significantly decreased ($p<0.05$) in INH-treated rats (group 2) as compared to control animals (group 1). The body weight was found to be increased in INH-treated rats with 1 h pre-treatment with CA ethanolic leaf extract (groups 3–6) in a concentration-dependent manner. Extract at a concentration of 100 mg/kg bw (group 6) was found to be most effective, which was similar to SIL (50 mg/kg bw) (group 8)-treated rats.

Estimation of blood glucose

Table 2 shows the levels of blood glucose in normal and experimental rats. A significant increase ($p<0.05$) in blood glucose level was observed in INH-treated rats (group 2) when compared with control rats (group 1), and it was found to be decreased after 1 h pre-treatment with CA ethanolic extract (groups 3–6) in a concentration-dependent manner and in the reference drug SIL (50 mg/kg bw) (group 8)-treated rats. Extract at a concentration of 100 mg/kg bw (group 6) on the 30th day was found to be most effective in decreasing the blood glucose when compared to INH-treated rats (group 2). Extract at a concentration of 100 mg/kg bw (group 6) was found to be most effective, which was similar to SIL (50 mg/kg bw)-treated rats (group 8).

Evaluation of haematological parameters

The total RBC, Hb, Ht, MCH/MEH, and MCHC were found to be significantly decreased ($p<0.05$) in INH-treated rats (group 2), whereas a significant increase ($p<0.05$) in the WBC level was found in the INH-treated rats (group 2). These levels were found to be restored in the rats pre-treated with CA extract (groups 3–6) in a concentration-dependent manner, with 100 mg/kg bw (group 6) being the most effective concentration. Similar results were observed in SIL (50 mg/kg bw)-treated INH-intoxicated rats (group 8) (Table 2).

Evaluation of serum oxidant status and other biochemical parameters

In blood serum of INH-treated rats (group 2), the concentration of TBARS was found to be significantly increased ($p<0.05$) as compared to control animals (group 1). A significant decrease ($p<0.05$) in the levels of SOD, CAT, and GSH were also found in INH-treated rats (group 2). These

levels were found to be restored in the rats pre-treated with CA extract (groups 3–6) in a concentration-dependent manner, with 100 mg/kg bw (group 6) being the most effective concentration. Similar results were observed in SIL (50 mg/kg bw)-treated INH-intoxicated rats (group 8) (Table 3).

The changes in the level of liver function (TSP; albumin; globulin; activities of serum ALT, AST, and ALP; and total bilirubin) and kidney function markers (urea and creatinine), in INH-treated (group 2) and CA extract-treated rats (groups 3–6) are summarised in Table 4. A significant decrease ($p < 0.05$) in the levels of TSP and albumin, and increase ($p < 0.05$) in the activities of ALT, AST, ALP, globulin, total bilirubin, urea, and creatinine were observed in INH-treated rats (group 2) when compared to control rats (group 1). These levels were found to be restored in the rats pre-treated with CA extract (groups 3–6) in a concentration-dependent manner, with 100 mg/kg bw (group 6) being the most effective concentration. Similar results were observed in SIL (50 mg/kg bw)-treated INH-intoxicated rats (group 8).

Histological examination of the liver and kidneys

The histological examination of liver tissue photomicrograph of INH (50 mg/kg bw)-treated rats (group 2) revealed numerous changes, such as cellular damage, inflammation, vascularisation, hypertrophy, loss of structural integrity of cells, as well as loss in characteristic tissue organisation. Near-normal cellular architecture with normal hepatocyte arrangement, sinusoids, and mild central vein congestion were observed in CA (100 mg/kg bw)-treated INH-intoxicated (group 6) rat liver. Similar results were observed in SIL (50 mg/kg bw)-treated INH-intoxicated rats (group 8) (Figure 1).

The histological examination of kidney renal cortex tissue photomicrograph of INH (50 mg/kg bw)-treated rats (group 2) revealed distorted cellular organisation accompanied with thickened renal basement, swelled glomerulus filled with blood cells and enclosed in uneven spacing of Bowman's capsule. In CA (100 mg/kg bw)-treated INH-intoxicated rats (group 6), the kidney revealed normal renal cortex, and even spacing of Bowman's capsule as well as normal tubular arrangement (proximal and distal tubules) and decrease in the renal basement membrane thickness were observed. Similar results were observed in SIL (50 mg/kg bw)-treated INH-intoxicated rats (group 8) (Figure 2).

Table 3: Effect of CA extract on TBARS, SOD, CAT, and GSH in control and experimental animals.

Groups	Control	INH (50 mg/kg bw)	INH + CA (20 mg/kg bw)	INH + CA (40 mg/kg bw)	INH + CA (60 mg/kg bw)	INH + CA (100 mg/kg bw)	CA (100 mg/kg bw)	INH + SIL (50 mg/kg bw)
TBARS, nmol/mL	2.12 ± 0.17^{a}	3.75 ± 0.43^{d}	3.12 ± 0.23^{c}	3.05 ± 0.17^{c}	2.95 ± 0.13^{c}	$2.41 \pm 0.32^{a,b}$	2.10 ± 0.19^{a}	2.52 ± 0.34^{b}
SOD, 50% NBT reduction/mL	8.45 ± 0.23^{d}	3.27 ± 0.09^{a}	3.29 ± 0.13^{a}	4.79 ± 0.06^{b}	6.23 ± 0.11^{c}	8.43 ± 0.03^{d}	8.47 ± 0.02^{d}	8.41 ± 0.07^{d}
CAT, µm of H_2O_2 utilised/min/mL	1.04 ± 0.10^{e}	0.32 ± 0.02^{a}	0.54 ± 0.01^{b}	0.72 ± 0.03^{c}	0.85 ± 0.04^{d}	1.00 ± 0.14^{d}	1.07 ± 0.23^{d}	$0.98 \pm 0.10^{d,e}$
GSH, mg/dL	$34.98 \pm 3.55^{c,d}$	21.15 ± 2.49^{a}	21.12 ± 3.49^{a}	$25.01 \pm 3.55^{a,b}$	27.44 ± 3.73^{b}	32.72 ± 3.45^{c}	37.72 ± 2.35^{d}	31.81 ± 3.51^{c}

Values are expressed as mean ± SD ($n = 6$). Values not sharing a common superscript letter differ significantly at $p < 0.05$ (DMRT).

Table 4: Effect of CA extract on liver and kidney function tests in control and experimental animals.

Groups	Control	INH (50 mg/kg bw)	INH + CA (20 mg/kg bw)	INH + CA (40 mg/kg bw)	INH + CA (60 mg/kg bw)	INH + CA (100 mg/kg bw)	CA (100 mg/kg bw)	INH + SIL (50 mg/kg bw)
Liver function tests								
AST, IU/L	16.62 ± 3.70^{a}	50.62 ± 6.66^{d}	$45.02 \pm 8.87^{c,d}$	40.98 ± 7.98^{c}	31.26 ± 11.08^{b}	16.88 ± 4.58^{a}	16.38 ± 6.04^{a}	18.32 ± 5.52^{a}
ALT, IU/L	6.24 ± 2.40^{a}	26.23 ± 8.76^{c}	$24.04 \pm 5.25^{b,c}$	$20.19 \pm 7.46^{b,c}$	18.35 ± 5.88^{b}	6.72 ± 3.454^{a}	6.01 ± 2.69^{a}	6.97 ± 2.53^{a}
ALP, IU/L	28.31 ± 4.44^{a}	51.43 ± 6.13^{c}	50.49 ± 9.32^{c}	45.12 ± 4.56^{c}	$34.32 \pm 3.79^{a,b}$	$30.11 \pm 4.78^{a,b}$	27.91 ± 4.68^{a}	35.79 ± 4.34^{b}
Bilirubin, mg/dL	0.39 ± 0.03^{a}	0.54 ± 0.09^{d}	$0.50 \pm 0.04^{c,d}$	$0.46 \pm 0.06^{b,c}$	$0.42 \pm 0.09^{a,b}$	$0.40 \pm 0.06^{a,b}$	0.37 ± 0.02^{a}	$0.41 \pm 0.09^{a,b}$
Albumin, g/dL	3.80 ± 0.02^{e}	2.12 ± 0.06^{a}	2.42 ± 0.13^{b}	2.89 ± 0.15^{c}	3.29 ± 0.20^{d}	3.62 ± 0.24^{e}	3.75 ± 0.19^{e}	3.65 ± 0.20^{e}
Globulin, g/dL	2.50 ± 0.03^{a}	3.48 ± 0.04^{d}	3.12 ± 0.12^{c}	2.86 ± 0.27^{b}	2.53 ± 0.39^{a}	$2.69 \pm 0.13^{a,b}$	$2.61 \pm 0.17^{a,b}$	$2.73 \pm 0.23^{a,b}$
Protein, g/dL	6.60 ± 0.12^{d}	4.51 ± 0.28^{a}	4.68 ± 0.15^{a}	5.03 ± 0.34^{b}	5.37 ± 0.11^{c}	5.58 ± 0.34^{c}	6.58 ± 0.24^{d}	5.55 ± 0.46^{c}
Kidney function tests								
Urea, mg/dL	0.47 ± 0.02^{a}	1.41 ± 0.14^{d}	1.25 ± 0.06^{c}	1.12 ± 0.08^{c}	0.97 ± 0.01^{b}	0.49 ± 0.12^{a}	0.45 ± 0.17^{a}	0.50 ± 0.21^{a}
Creatinine, mg/dL	28.70 ± 1.65^{b}	38.20 ± 1.70^{e}	36.40 ± 1.39^{c}	34.50 ± 1.20^{c}	33.80 ± 1.65^{c}	30.10 ± 1.39^{b}	26.40 ± 1.46^{a}	32.80 ± 1.54^{c}

Values are expressed as mean ± SD ($n = 6$). Values not sharing a common superscript letter differ significantly at $p < 0.05$ (DMRT).

Discussion

The present study was undertaken to understand the efficacy of CA leaf extract on INH-induced toxicity in albino rats. The previous finding by Antony et al. showed that CA alcoholic extract had hepatoprotective effect in chemically (carbon tetrachloride) induced liver injury [30]. Based on the acute toxicity work of Abdulla et al., the ethanolic extract of CA at a dose of 2 g and 5 g/kg bw for 14 days did not manifest any significant visible signs of toxicity [31]. SIL, a flavonolignan from 'milk thistle' (*Silybum marianum*) plant, is a reference drug that is used extensively for hepatoprotection [32].

Oral administration of CA ethanolic leaf extract at a concentration of 100 mg/kg bw normalised ($p < 0.05$) the level of blood glucose in INH-treated rats to near-normal level. Luntz and Smith had reported abnormalities in carbohydrate metabolism and also reported a rise in blood glucose level in diabetic and non-diabetic TB patients [9]. This may be due to the deleterious effect of INH on the pancreas, causing a decreased secretion of insulin, and leading to increased blood glucose level. Drug-induced pancreatitis is not a well-documented event in patients and is thus rare [33]. There had been numerous reports of pancreatitis, either with INH alone or in combination with rifampicin [6, 34].

WBCs are primarily involved in protecting the body at the event of any infection or injury. T and B lymphocyte cells play very important roles in the immune defence system of the body. A recent study by Tousif et al. had reported that the anti-TB drug INH compromises the immunity of mice by inducing activation-induced cell death in activated $CD4^{+}$ T cells, thus resulting in immune impairment of the host by leucopenia [35]. INH is also used as a preventive medicine for healthy individuals who are in close contact with TB patients [36]. Thus, an impaired immune system may lead to increased susceptibility to various pathogens in the healthy individuals. With an increase in infections accompanied with increased oxidative stress in INH-treated rats, the level of neutrophils, eosinophils, and basophils in the host increases. These events collectively lead to the increased levels of total WBCs in INH-treated rats. In the present study, the antioxidant activity of CA extract (100 mg/kg bw) and SIL may help in restoring the oxidant balance and thus prevent the secondary metabolites from compromising the immune system with their toxic effect in the pre-treated INH groups.

A previous study by Yilmaz et al. had reported INH-induced oxidative stress in RBCs of rat. The oxidative stress was a result of increased nitric oxide, adenosine deaminase, and xanthine oxidase, which leads to the

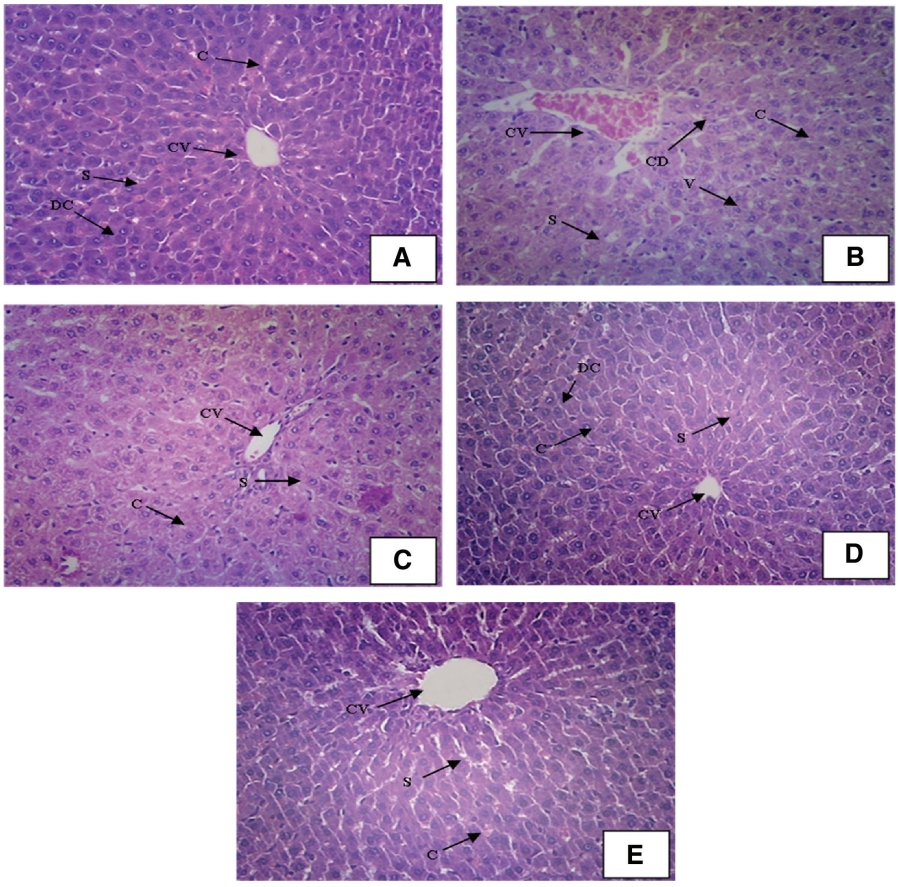

Figure 1: Photomicrographs of histological changes in liver sections of control and experimental rats (×200).
(A, D) Control and CA-treated rats showing normal architecture. (B) INH-treated rats showing distorted cellular organisation accompanied with vacuoles, hypertrophic cells, cellular damage, inflammation, widening of intercellular sinusoids, and congestion in central vein. The nucleoli is not distinct, and the nucleuses appeared to have more condensed chromatin. (C, E) INH-intoxicated rats orally pre-treated with CA or SIL showing near-normal hepatocyte arrangement and sinusoids accompanied with mild central vein congestion. The nucleoli and nucleolus also appeared to be nearly distinct with less condensed chromatin. CV, central vein; S, sinusoids; C, chromatin condensation; DC, division of cells; V, vacuoles; CD, cellular damage.

destruction of RBCs in the blood [37]. A large number of anti-TB drugs, including INH, are known inhibitors of haem biosynthesis [38]. The MCH and MCHC provide the Hb content in RBCs. A decrease in MCH and MCHC indicates INH-induced anaemia in rats. Ht is also known as PCV or erythrocyte volume fraction. It is the volume percentage (%) of RBCs present in the blood. A decrease in Ht indicates that the percentage of total RBCs in the blood is below the normal level [39]. A decrease in Hb synthesis, accompanied by an increase in oxidative stress in INH-treated rats, may be the reason for the decrease in total RBC count and Hb in the blood of INH-treated rats. These conditions were found to be restored to near normal in rats pre-treated with CA extract (100 mg/kg bw) and SIL (50 mg/kg bw).

The liver is the main organ involved in the detoxification of hormones, xenobiotics, and drugs. During xenobiotic detoxification, the metabolites produced can damage the liver cells [40]. In the liver, INH is metabolised into hydrazine and acetylisoniazid, followed by hydrolysis to acetylhydrazine and oxidisation into hepatotoxic intermediaries by CYP 450 [7]. The enzymes CAT and SOD are inactivated by hydrazine, resulting in a significant increase in endogenous H_2O_2 levels in the cells [41]. According to various studies, INH causes cellular damage by inducing oxidative stress. This leads to dysfunction of the hepatic antioxidant defence system [42]. The exhaustion of antioxidant defences and increase in free-radical production leads to imbalance in the prooxidants-antioxidants and cause oxidative stress-induced cell death in the liver. GSH enhances the free-radical scavenging effect of CAT. During this process, GSH is converted into its oxidised form, i.e. glutathione disulphide (GSSG) [43]. There may be an increase in the

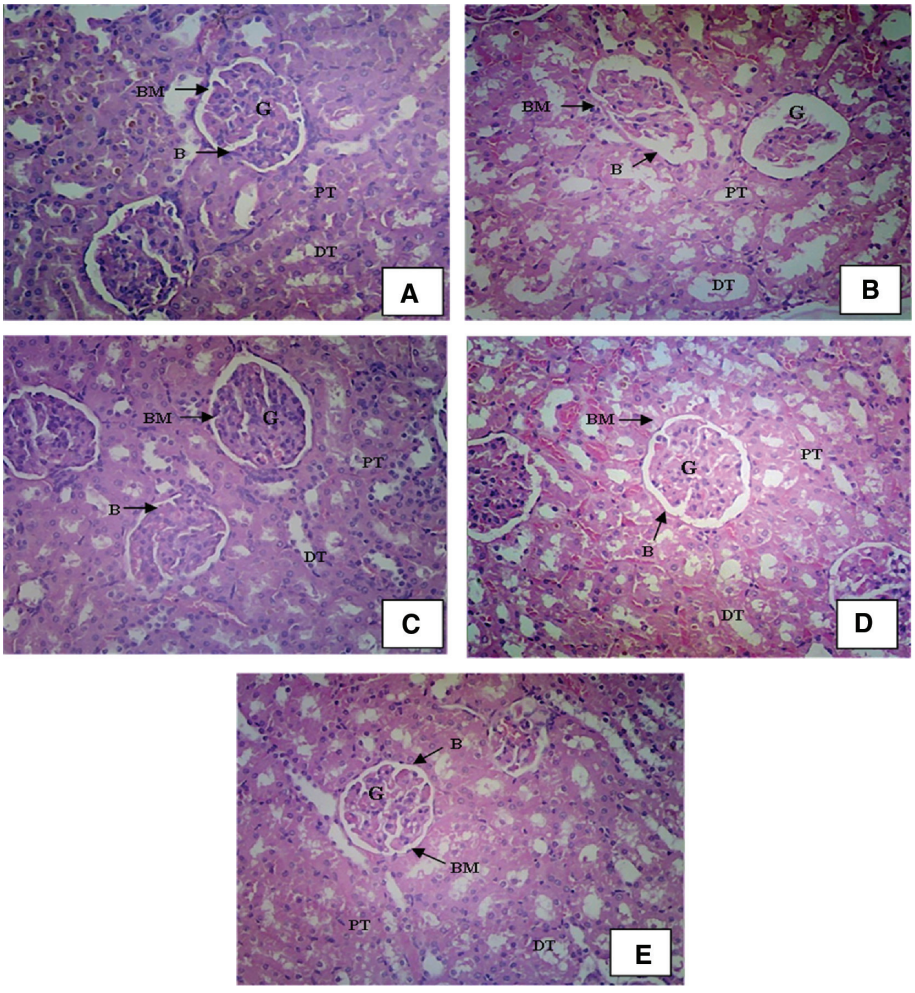

Figure 2: Photomicrographs of histological changes kidney renal cortex sections of control and experimental rats (×200).
(A, D) Control and CA-treated rats showing normal architecture. (B) INH-treated rats showing a distorted cellular organisation accompanied with thickened renal basement, damaged renal tubules, swelled glomerulus filled with blood cells, and uneven spacing of Bowman's capsule. (C, E) Renal cortex of INH-intoxicated rat pre-treated with oral CA extract or SIL showing near-normal glomerulus enclosed in Bowman's capsule, tubular arrangement of proximal as well as distal tubules and decrease in renal basement membrane thickness.
B, Bowman's capsule; PT, proximal tubules; DT, distal tubules; BM, basement membrane; G, glomerulus; BC, blood cells.

utilisation of GSH in INH-treated rats, leading to the depletion of GSH and increase in lipid peroxidation. Based on a previous study, CA ethanolic leaf extract has very good in vitro antioxidant and free-radical scavenging activity. This may be attributed to the presence of various antioxidant compounds present in the extract [15]. Increase in reactive oxygen species (ROS) and reduction in the levels of GSH, SOD, and CAT increase other parameters, like the levels of serum AST and ALT in INH-treated rats. During drug metabolism, INH produces acetylhydrazine and hydrazine, which can cause immense damage to the liver and lead to liver toxicity [7]. Liver-specific enzymes, amino transferases, are involved in the catalytic reactions involving the interconversion of amino acid and α-keto acid, by transferring amino groups. Liver cells are damaged due to increased TBARS, and decreased SOD, CAT, and GSH levels lead to cell membrane damage. This is followed by necrosis of liver cells and release of amino transferase enzymes, AST and ALT, into the blood circulation. The healing of liver cells decreases the levels of AST and ALT in CA extract (100 mg/kg bw)- and SIL (50 mg/kg bw)-treated INH groups to near normal. Hepatotoxicity causes a cirrhotic liver condition, leading to defective biliary functioning and increased bilirubin release in blood circulation [44]. Pre-treatment with CA ethanolic extract (100 mg/kg bw) as well as the reference drug SIL (50 mg/kg bw) restored the level of bilirubin to near-normal levels. This may be due to the stabilisation of biliary dysfunction of INH-treated rat liver, thus improving the functions of the rat liver to near normal. ALP, a marker

enzyme, is generally increased in liver disorders. Elevated levels of serum ALP are indicative of cellular leakage and loss of functional integrity of liver cell membrane [16, 45]. In the present study, the damage to the membrane of liver cells by ROS and increase in lipid peroxidation might have caused leakage of cellular enzymes, such as ALP, into the serum. This was found to be decreased in CA extract (100 mg/kg bw)- and SIL (50 mg/kg bw)-treated INH groups to near normal.

Albumin is the main protein found in blood and is biosynthesised in the liver. A decrease in the levels of albumin indicates liver dysfunction [46]. Various studies have indicated a decrease in albumin level, and increase in serum total protein and serum globulin in TB drugs treated rats [40–42, 45]. The estimation of TSP helps in differentiating between normal and damaged liver function. This is because most of the serum proteins like albumins and globulins are biosynthesised in the liver [46]. During hepatocellular injury, total protein may be reduced a little, but there is always a sharp decrease in albumin level and increase in globulin level [40]. In the current study, a decrease in the TSP and serum albumin, and an increase in serum globulin were observed. This may be due to damage and dysfunction of the liver by INH administration. The increase in the antioxidative status of the liver in the CA extract (100 mg/kg bw)- and SIL (50 mg/kg bw)-treated INH groups might be the reason for the restored levels of TSP, serum albumin, and globulin to near normal.

After the liver, the toxic secondary metabolites produced by phase I and II metabolising enzymes in the liver gets excreted out of the body through the kidneys. The second organ that gets affected by toxic metabolites of TB drugs are the kidneys. The nephrotoxicity causes an incomplete filtration of urea and creatinine, leading to increased levels of these toxicants in the blood circulation [47]. In this study, the kidney damage caused by INH treatment in rats led to increased serum levels of urea and creatinine. Nephrotoxicity might be the result of increased ROS and decreased antioxidant enzyme levels in the renal system [48]. Our previous study stated that CA ethanolic leaf extract, by gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) analysis, contains various pharmacologically active compounds and showed in vitro antioxidant and free-radical scavenging activity, which may be due to the synergic effect of various compounds present in the extract [15]. Thus, the increase in the antioxidative status of the kidney in the CA extract (100 mg/kg bw)-treated INH groups might be the reason for the restored levels of urea and creatinine to near normal.

Severe distortion in hepatocellular organisation was observed in INH (50 mg/kg bw for 30 days)-treated rats. This observation was in agreement with the results of Jadhav and Mateenuddin and those of Sankar et al. [49, 50]. Similarly, distortion in overall histological arrangement was also observed in the kidney cortex sections in INH-treated rats. These findings were in agreement to those of Hussein et al. [51]. These adverse effects on INH-administered liver and kidney sections were found to be restored by CA extract (100 mg/kg bw) and SIL (50 mg/kg bw) pre-treatment. This may be due to the presence of various antioxidant and anti-inflammatory compounds present in the extract.

Conclusions

The present study collectively demonstrates that the pre-treatment of INH-treated rats with CA leaf ethanolic extract (100 mg/kg bw) can reduce the possible side effects caused by INH, a specific drug for TB treatment. This also provides future avenues to study the liver and kidney ultrastructure and the molecular mechanisms involved in the protective mechanism of the CA ethanolic extract. Further, the isolation and characterisation of potent molecules responsible for this protective effect would prove advantageous in the search of new lead compounds for pharmacological companies in producing more effective drugs, which would help in reducing the side effects of commercially available drugs.

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Research Article

THE AMELIORATING EFFECT OF *CENTELLA ASIATICA* ETHANOLIC EXTRACT ON LIVER OF ISONIAZID INTOXICATED ALBINO RATS

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ABSTRACT

Objective: Isoniazid (INH) is one of the commonly used drugs for treating tuberculosis. The main drawback of this drug is its toxic side effects on the liver. *Centella asiatica* (CA) has been used since time immemorial in the Ayurvedic system of medicine due to its wide range of medicinal properties.

Methods: This study was designed to examine the protective effect of CA ethanolic leaf extract on the liver of INH intoxicated albino rats. The adverse effects induced by INH (50 mg/kg bw) administration on liver function markers (aspartate transaminase, alanine transaminase, alkaline phosphatase, total bilirubin, albumin, globulin, and protein) and their amelioration on treatment with various concentrations of CA (20, 40, 60, 100 mg/kg bw) or silymarin (50 mg/kg bw, administered before 1 hr of INH treatment for 30 days to rats) was studied. Moreover, the lipid peroxidation (thiobarbituric acid-reactive substances [TBARSs]), antioxidant status (TBARS, superoxide dismutase, catalase and glutathione-S-transferase), glycogen level, histological and ultrastructural (transmission electron microscopy) studies were carried out in liver tissues of rats treated with the most effective concentration to further support the possible effectiveness of CA on the liver of INH intoxicated rats.

Results: All the affected parameters were brought back to near normal levels, and the effective concentration of extract was found to be 100 mg/kg bw. The oxidant status, glycogen level, histology and ultrastructure of liver also subsequently supported the effectiveness of CA (100 mg/kg bw).

Conclusions: Thus, the overall results suggest that CA at 100 mg/kg bw can considerably reduce the toxic effects inflicted by INH intoxication on rat liver.

Keywords: Isoniazid, Antioxidant, *Centella asiatica*, Liver, Glycogen.

INTRODUCTION

Tuberculosis (TB) is a communicable chronic disease inflicted by the infection caused by the bacterium *Mycobacterium* TB. It is second in the line of mortality rate after HIV/AIDS. According to the 2013 Global TB Report by the World Health Organization, India had been accounted to comprise the world's quarter TB patient's burden. India and China alone were stated to have 26% and 12% of the total TB cases, respectively [1]. The most commonly administered first-line anti-TB drugs are isoniazid (INH), which is also known as INH or isotonic acid hydrazide, and rifampicin or rifampin. These drugs are orally administered either in combination or alone for more than 6 months [2-4]. INH is a known broad-spectrum antibiotic. It is commonly administered for treating *Mycobacterium* TB bacterial infections [5]. This first-line drug is clinically used since the year 1952 and is being used in treating as well as in chemoprophylaxis of TB. It is adversely correlated with hepatotoxicity [6-8]. INH is readily metabolized by *N*-acetyltransferase, forming acetylisoniazid. This then undergoes hydroxylation by cytochrome P450 enzymes, forming hepatotoxic intermediates like acetylhydrazine as well as isonicotinic acid. Acetylhydrazine is known for forming covalent cellular adducts. Acetylhydrazine gets further hydrolyzed to form hydrazine or acetylated products, called as diacetylhydrazine. Patients administered with INH are known to exhibit elevating levels of hydrazine in their blood serum [7]. Administration of a single dose of INH, the hydrazines produced can rapidly disseminate to all the organs without any favorable accumulation in a precise organ. Metabolism of hydrazines involves both enzymatic and nonenzymatic pathways. Humans possessing a slow acetylator genotype have a tendency to accumulate more hydrazine in their serum. This effect may be due to an impaired ability to metabolize and excrete the toxic secondary metabolites of INH [6,7]. Although several evidence-based reports exist on INH-induced hepatotoxicity, INH still remains to be a first-line drug for treating TB [8]. A study by Luntz and Smith also reported irregularities in carbohydrate metabolism accompanied with an increased blood glucose level in diabetic as well as in nondiabetic TB patients [9]. Thus, INH administered patients not only face the loss of normal hepatic function but are also challenged with abnormal carbohydrate metabolism, leading to the weakening of the overall health status of TB patients. Steps to ensure overall well-being must be prioritized in treating TB patients, ensuring both effective treatment and healthy metabolism for a speedy recovery.

Centella asiatica L. (Apiaceae) (CA) is commonly known as Asiatic pennywort or the Indian pennywort. It belongs to the Apiaceae (formerly known as Umbelliferae) family. It is characterized by slender, prostrate, glabrous, perennial creeping herb rooting at the nodes, with simple petiolate, palmately lobed leaves. It is extensively cultivated in Southeast Asia, India, China, and Sri Lanka as a vegetable and/or spice [10]. CA has been reported as possessing various pharmacological activities such as memory-enhancing, anti-inflammatory, antioxidant, immune boosting, antihypertension, antistress, and antiepilepsy activities. These properties have influenced the plant's utilization for treating diseases associated with skin, rheumatism, syphilis, hysteria, diarrhea, wounds, as well as ulcers [10-14]. Diverse health benefits of CA have led to the augmented usage of this plant in various foods and beverages [14]. An *in vitro* study has reported that CA ethanolic leaf extract possesses a strong antioxidant property and suggested that this plant can also act as an iron chelator [15]. Thus, therapeutic potential of CA has motivated us to scrutinize the effect of CA on INH-induced liver toxicity in Wistar albino rats, for evaluating the liver function markers, oxidative status, glycogen, histological as well as ultrastructural changes in the liver.

METHODS

Chemicals and reagents

INH and silymarin (SIL) were purchased from Sigma-Aldrich® Co. Ltd., USA. Methanol, HCL, sulfanilic acid, sodium nitrate, sodium carbonate,

copper sulfate and bovine serum albumin were procured from Himedia Laboratories Pvt. Ltd., Mumbai, India. All other chemicals used for the biochemical estimations were of analytical grade and were procured from Himedia Laboratories Pvt. Ltd., Mumbai, India.

Collection and identification of plant material

CA used in this study was collected freshly from the outskirts of Chidambaram, Cuddalore District. The plant was then identified at the Herbarium of Department of Botany, Annamalai University (Herbarium No. DDE/HER/44). All the plant materials were washed thoroughly under running tap water for removing any dirt and/or other debris. The cleaned plant materials were then spread under a clean shade for drying. The final dried plant material was milled to a coarse powder using a mechanical grinder and stored in an air-tight container.

Ethanolic leaf extraction of CA

Approximately, 1 kg of powdered CA was used for ethanolic extraction using a Soxhlet apparatus. The dark green extract was subjected to an ultracentrifugation, followed by microfiltration. The final clear dark extract was then concentrated in a rotary evaporator under reduced pressure (10-15 mmHg) at 40°C to obtain the crude ethanol extract. The final dried extract was lyophilized and was stored in a glass vial at −20°C for further use.

Percentage yield of CA extract

The percentage yield of the CA ethanolic extract was determined gravimetrically, i.e., by measuring the dry weight of the final crude extract (X) and also the dry weight of plant powder utilized for the extraction (Y), using the following formula,

Percentage yield = X/Y × 100.

Experimental animals

Male Wistar albino rats of body weight 180-200 g were used for this study. The animals were maintained at Central Animal House, Department of Experimental Medicine, Rajah Muthiah Medical College and Hospital, Annamalai University, Annamalainagar, India. The animals were fed on a staple standard pellet diet and water *ad libitum*. The animals were housed in polycarbonate cages under controlled conditions of temperature (23±2°C) and humidity (65-70%) with a 12 hrs light/dark cycle. All the protocols of this study were approved by the Institutional Animal Ethics Committee and Guidelines of Annamalai University (160/1999/CPCSEA; Proposal number: 1021, dated 06.08.2013).

Experimental design

A total of 48 rats were randomly divided into eight groups, each comprising six rats. All the treatments were oral and test drug was administered using an intragastric tube daily for 30 days [16,17]. Group 1 received only vehicle (0.5% dimethyl sulfoxide) and served as a control. Group 2 was administered INH (50 mg/kg bw) alone. Group 3-6 received INH (50 mg/kg bw) and CA at various concentrations (20, 40, 60 and 100 mg/kg bw). Group 7 received CA alone with the highest concentration (100 mg/kg bw) and was assigned as a positive control. Group 8 acted as an internal control and received INH (50 mg/kg bw) and SIL (50 mg/kg bw). Both CA and SIL were administered 1 hr before INH administration.

The experiment was terminated at the end of 30 days, and the animals were fasted overnight, weighed and sacrificed by cervical dislocation. Fresh blood was centrifuged to collect serum for biochemical parameters. A portion of freshly dissected rat liver from each group was stored in 3% glutaraldehyde solution (prepared in 0.1 M phosphate buffer; pH 7.2). A portion of liver tissue from each was washed with ice cold saline and stored in a 10% buffered formalin solution.

Preparation of serum and plasma

Blood samples were collected and centrifuged at ambient temperature for 30 minutes to separate serum (2000 ×*g* for 10 minutes). Plasma samples were collected by centrifuging blood with anticoagulant at 2000 ×*g* for 20 minutes. Then the supernatant comprising the plasma was carefully separated and utilized for further analysis.

Biochemical parameters

The total serum protein (TSP) concentration was determined using Folin-Ciocalteu reagent, as described by the method of Lowry *et al*. and the albumin concentration was carried out by the method of Doumas *et al*., which utilizes bromocresol green dye at pH 4.2, to form green colored complex [18,19]. The globulin concentration was derived from the difference between albumin concentration and the total protein concentration. Activities of serum alanine transaminase (ALT) and aspartate transaminase (AST) were determined according to the method of Reitman and Frankel [20]. Alkaline phosphatase (ALP) serum activity was determined as described by King and Armstrong [21]. The total serum bilirubin level was measured using Van den Bergh reaction, as described by the method of Malloy and Evelyn [22].

Biochemical studies were carried out in blood serum. The concentration of serum thiobarbituric acid-reactive substances (TBARS) was analyzed according to the method of Hogberg *et al*. [23]. The serum superoxide dismutase (SOD) was carried out by the method of Kakkar *et al*. [24]. The serum catalase (CAT) was measured by the method of Sinha [25]. The serum glutathione-S-transferase (GST) was determined according to the method described by Habig *et al.* [26]. Glycogen content in liver was determined as described by Morales *et al*. [27].

Histological examination

The liver tissues for the control and effective concentration treated animals were subjected to histological studies. The tissue samples were fixed for 48 hrs in 10% buffered formalin, followed by dehydrating the tissues by passing through a different mixture of ethyl alcohol and water, and was finally cleaned with xylene and embedded in paraffin for sectioning. Sections of the 5-6 µm thickness of tissues were prepared using a rotary microtome. These sections were fixed on glass slides and stained with hematoxylin and eosin dye and were observed microscopically.

Transmission electron microscopic (TEM) study of liver tissues

A portion of freshly dissected rat liver was sliced into 1 mm^3 and stored in 3% glutaraldehyde (EM grade) in 0.1 M phosphate buffer (pH 7.2) for fixing (48 hrs at 2-4°C). The samples were washed with 0.1 M phosphate buffer (pH 7.2), post fixed in 1% osmium tetroxide prepared in 0.1 M phosphate buffer (pH 7.2), dehydrated in a graded ethanol series, and embedded in epoxy resin. Ultrathin sections with 40-60 nm thickness were cut using Leica ultramicrotome with a diamond knife (DiATOME). The ultrathin sections were taken on a copper grid and stained with (double metallic) uranyl acetate and Reynold's solution (sodium citrate and lead mitrate). The sections were examined using a Philips Tecnai T12 (120 kV) Electron Microscope (Netherland). The images were acquired using a Gatan Image Filter (GIF; Ultrascan 10000 slow scan) with CCD camera 4 K × 4 K chip and were processed using Gatan software (T12).

Statistical analysis

Statistical comparisons were performed by one-way analysis of variance followed by the Duncan's multiple range test, SPSS software version 16.0 (SPSS Inc. Released 2007, SPSS for Windows, Version 16.0, SPSS Inc., Chicago, Ill, USA). Values are represented as mean ± standard deviation and $p<0.05$ was considered statistically significant.

RESULTS

Percentage yield of plant extract

Table 1 shows that the percentage yield of CA ethanolic extract and was found to be 1.089%.

Evaluation of serum and plasma biochemical parameters (liver function test)

The changes in the level of liver function (TSP, albumin, globulin, activities of serum ALT, AST and ALP and total bilirubin) in INH

(Group 2) and CA extract treated rats (Group 3-6) are summarized in Fig. 1. A significant decrease ($p<0.05$) in the levels of TSP and albumin and increase ($p<0.05$) in the activities of ALT, AST, ALP, globulin, and total bilirubin was observed in INH-treated rats (Group 2) when compared to control rats (Group 1). These levels were found to be restored in the CA extract pretreated rats (Group 3-6) in a concentration-dependent manner, with 100 mg/kg bw (Group 6) being the most effective concentration. Similar results were observed in SIL (50 mg/kg bw) treated INH intoxicated rats (Group 8).

Evaluation of liver oxidant status and glycogen levels

In INH-treated rats (Group 2), the concentration of TBARS in liver was found to be significantly increased ($p<0.05$) as compared to control animals (Group 1). A significant decrease ($p<0.05$) in the levels of SOD, CAT, and GST were also observed in the liver of INH-treated rats (Group 2). These levels were found to be restored in the CA extract pretreated rats with a concentration of 100 mg/kg bw (Group 6). Similar results were observed in SIL (50 mg/kg bw) treated INH intoxicated rats (Group 8) (Table 2). Further, the glycogen reserve in liver was found to be significantly depleted ($p<0.05$) in INH-treated rats (Group 2) compared to control animals (Group 1). The glycogen reserve was restored in the CA extract pretreated rats with a concentration of 100 mg/kg bw (Group 6). Similar results were observed in SIL (50 mg/kg bw) treated INH intoxicated rats (Group 8) (Fig. 2).

Histological and ultrastructural examination of liver tissues

The histological examination of liver tissue photomicrograph of INH (50 mg/kg bw) treated rats (Group 2) revealed the numerous changes such as cellular damage, inflammation, vascularization, hypertrophy, loss of structural integrity of cells as well as loss in characteristic tissue organization. Near normal cellular architecture with normal hepatocyte arrangement, sinusoids, mild central vein congestion were observed in CA (100 mg/kg bw) treated INH intoxicated (Group 6) rat liver. Similar results were observed in SIL (50 mg/kg bw) treated INH intoxicated rats (Group 8) (Fig. 3).

The analysis of INH-treated rat (Group 2) liver ultramicrographs obtained from TEM revealed the presence of cell membrane enfolding, damaged nuclear membrane, nuclear chromatin condensation, regression of mitochondrial cisternae, and the presence of fat droplets with vacuoles. Near normal hepatocyte ultrastructure with euchromatic nucleus, prominent nuclear membrane, prominent nucleolus, many mitochondria, rough endoplasmic reticulum, appearance of glycogen granules and less vacuolated cytoplasm were observed in CA ethanolic extract administered (100 mg/kg bw) INH intoxicated rat hepatocytes (Group 6). Similar effects were observed in SIL (50 mg/kg bw) treated INH intoxicated rat (Group 8) hepatocyte (Fig. 4).

Table 1: Percentage yield of plant extract

Plant	Solvent	Method	Weight of crude extract (g)	% yield
CA	Ethanol	Soxhlet extraction	10.89	1.089

CA: *Centella asiatica*

DISCUSSION

The present experiment was carried out to evaluate the effectiveness of CA leaves extract on INH intoxicated albino rats. The previous finding by Antony *et al*. confirmed that CA alcoholic extract has hepatoprotective effect in carbon tetrachloride and cadmium-induced liver injuries [28,29]. Abdulla *et al*. acute toxicity work revealed that the ethanolic extract of CA at a dose ranging from 2 to 5 g/kg bw for 14 days showed no manifestation of any significantly noticeable signs of toxicity [30]. SIL is a flavonolignan obtained from the "milk thistle" plant, *Silybum marianum*, which is a reference drug used widely for hepatoprotection [31].

The liver is the central organ engaged in detoxification of various products such as hormones, xenobiotics, toxins, and drugs. During detoxification of xenobiotics, the metabolites generated can lead to hepatocyte damage [32]. INH is metabolized in the liver into hydrazine and acetylisoniazid, which is followed by hydrolysis

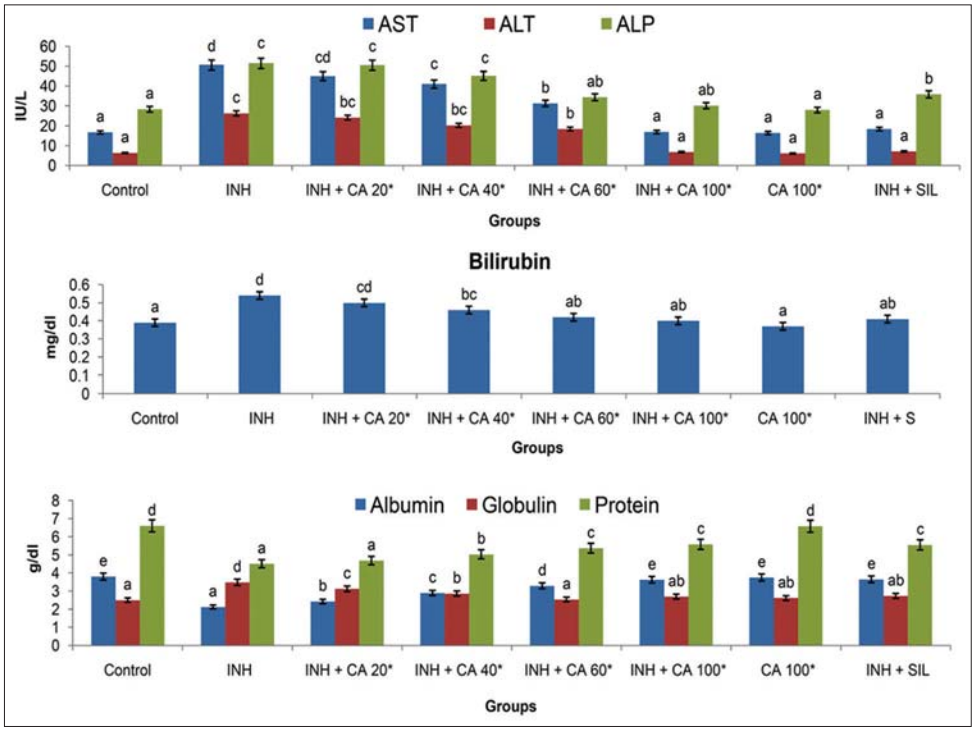

Fig. 1: Effect of extract on liver function tests in control and experimental animals. Values are expressed as mean±standard deviation (n=6). Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT). (INH: isoniazid 50mg/kg/bw/day; CA: *Centella asiatica* ethanolic leaves extract; SIL: Silymarin 50mg/kg/bw/day; *mg/kg bw/day)

Table 2: Effect of CA on TBARS, SOD, CAT, and GST in liver of control and experimental groups of rats

Groups	Control	INH (50 mg/kg bw)	INH (50 mg/kg bw) + CA (100 mg/kg bw)	CA (100 mg/kg bw)	INH (50 mg/kg bw) + SIL (50 mg/kg bw)
TBARS[1]	0.76±0.13[a]	4.38±0.27[d]	1.32±0.23[c]	0.78±0.05[a]	1.09±0.07[b]
SOD[2]	7.90±0.25[c]	3.82±0.30[a]	6.93±0.60[b]	8.05±0.41[c]	6.94±0.47[b]
CAT[3]	78.14±3.62[c]	43.24±2.80[a]	56.57±4.64[b]	80.94±1.46[c]	59.00±2.65[b]
GST[4]	8.81±0.67[cb]	6.31±0.47[a]	7.49±0.62[b]	9.01±0.34[d]	8.27±0.45[c]

Values are expressed as mean±SD (n=6). Values not sharing a common superscript letter differ significantly at P<0.05 (DMRT). DMRT: Duncan's multiple range test, SD: Standard deviation, [1]TBARS in tissues were expressed as nmoles/100 g wet tissue, [2]SOD for tissues were expressed as 50% inhibition of nitroblue tetrazolium reduced in 1 minute/mg protein, [3]CAT for tissues were expressed as µmoles of H_2O_2 consumed/minute/mg protein, [4]GST for tissue were expressed as CDNB-GSH conjugate formed/minute/mg protein. TBARS: Thiobarbituric acid-reactive substances, SOD: Superoxide dismutase, CAT: Catalase, GST: Glutathione-S-transferase, INH: Isoniazid 50 mg/kg/bw/day, CA: *Centella asiatica* ethanolic leaves extract, SIL: Silymarin 50 mg/kg/bw/day

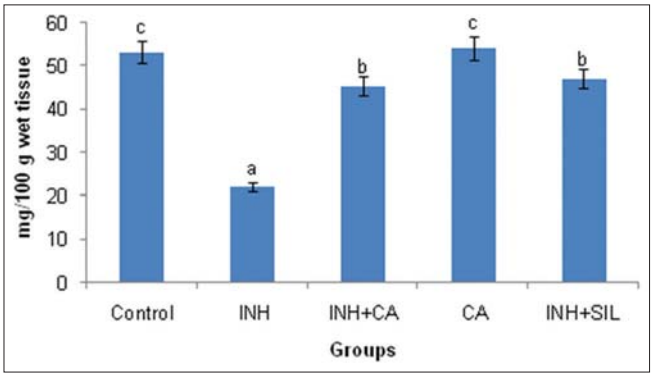

Fig. 2: Effect of *Centella asiatica* leaves ethanolic extract on liver glycogen in control and experimental rats. Values are expressed as mean±standard deviation (n=6). Values not sharing a common superscript letter differ significantly at p<0.05 (DMRT). (INH: Isoniazid 50 mg/kg/bw/day; CA: *Centella asiatica* ethanolic leaves extract; SIL: Silymarin 50 mg/kg/bw/day; *mg/kg bw/day)

forming acetylhydrazine and undergoing oxidization by cytochrome 450 to produce hepatotoxic intermediaries [7]. These hepatotoxic intermediates such as acetylhydrazine and hydrazine can inflict cellular damages in liver causing hepatotoxicity [7]. Liver specific enzymes such as aminotransferases are engaged in the catalytic reactions facilitating the interconversion of amino acid and α-keto acid, by transferring amino groups. Damage to hepatocytes leads to the release of aminotransferases enzymes, AST and ALT, into the blood circulation. Hepatotoxicity also causes cirrhotic liver conditions, leading to defects in biliary functioning and elevated bilirubin release in the blood circulation [33]. ALP is a marker enzyme, which is found increased in the liver disorders. Elevation in the levels of serum ALP is an indication of cellular leakage and also the loss of functional integrity of hepatocyte cell membrane [34]. The healing of liver cells may be the reason for the decrease in the levels of AST, ALT, ALP, and bilirubin in pretreated CA extract (100 mg/kg bw) and SIL (50 mg/kg bw) treated INH intoxicated groups to near normal levels. These effects may be owed to the improvement in the functioning of the rat liver and stabilization of biliary dysfunction of INH-treated rat liver to near normal. The increase in reactive oxygen species (ROS) accompanied by a reduction in the levels of SOD, CAT, and GST in liver tissues also directly influence the elevation of parameter levels of serum such as AST, ALT, and ALP in INH-treated rats. Liver cells face damage due to increase in TBARS level and decrease in SOD, CAT and GST, leading to an increased cell membrane damage and leakage of cellular contents into the circulatory system The enzymes CAT and SOD are previously reported to get inactivated by hydrazine, which results in significant increase in the endogenous levels of H_2O_2 in the liver cells [35]. INH is also well known to inflict cellular damage by aggravating oxidative stress in the organ system. These effects also cause dysfunction of the native hepatic antioxidant defense system [36]. Thus, the depletion of the antioxidant defenses accompanied with an increase in free radical production, leads to an imbalance in the overall prooxidant-antioxidant status, leading to oxidative stress-induced cell death in the liver in INH-treated rats [37]. CA ethanolic leaf extract has previously been reported to possess very good *in vitro* antioxidant as well as free radical scavenging activity. This may be attributed due to the presence of various antioxidant compounds present in the extract [15]. The elevated levels of TBARS and decreased levels of SOD, CAT, and GST in liver tissues were found to be restored in CA extract (100 mg/kg bw) and SIL (50 mg/kg bw) pretreated INH groups to near normal levels. Thus, from the findings of this study, it is evident that INH cause damage to the membrane of liver cells by ROS lead to increase in lipid peroxidation (TBARS), causing depletion of cellular antioxidants (SOD, CAT, and GST) and leading to cell membrane damage and leakage of cellular enzymes such as like AST, ALT, and ALP into the serum. These adverse effects were successfully contemplated by pretreatment with CA extract (100 mg/kg bw) and SIL (50 mg/kg bw) in INH intoxicated rats.

Albumin is biosynthesized in the liver and is also the main protein found in the blood. Decrease in the albumin level indicates dysfunction of the liver [38]. Various studies have indicated a decrease in albumin level, and an increase in serum total protein and serum globulin in TB-treated rats [32,34-36]. TSP level helps in differentiating between normal and damaged liver condition, as most of the serum proteins such as albumins and globulins are biosynthesized in the liver [38]. During any hepatocellular injury, the total protein may decline a little, but always a sharp decrease in albumin and increase in globulin levels are always observed [25]. The current findings reveal a decrease in the TSP and serum albumin, and an increase in serum globulin. This may be the result of damage as well as dysfunction of liver by administration of INH. Increase in the antioxidative status of the liver observed on pretreatment with CA extract (100 mg/kg bw) and SIL (50 mg/kg bw) in INH intoxicated groups might be the reason behind the restored levels of TSP, serum albumin, and globulin to near normal. A decrease in glycogen level was observed in INH (50 mg/kg bw) treated rats and was in agreement with the findings of Jadhav and Mateenuddin [39], and Sankar *et al*. [40]. This result reflects that the carbohydrate metabolism of the rats is affected by INH administration. The pretreatment of CA extract (100 mg/kg bw) in INH intoxicated groups successfully restored the depleted glycogen level in hepatic tissues to near normal. Pretreatment with standard drug SIL (50 mg/kg bw) in INH intoxicated rats also considerably elevated glycogen level but was not as effective as CA extract (100 mg/kg bw) pretreatment. The effectiveness of CA extract may be due to the synergic effect of various phytochemicals, which help in the restoration of carbohydrate metabolism in INH-treated rats.

Sever distortion in the hepatocellular organization was observed in INH (50 mg/kg bw) treated rats. This observation was in agreement with the results of Jadhav and Mateenuddin [39], and Sankar *et al*. [40]. These histological damages were clearly reflected in ultrastructural damages in liver section along with depletion of glycogen granules in INH-treated rats. These adverse effects on histology and/or ultrastructural changes in INH administered liver sections were found to be restored on CA extract (100 mg/kg bw) and SIL (50 mg/kg bw) pretreatment. This may be due to the presence of various antioxidant and anti-inflammatory compounds present in the extract, which could restore the normal functioning of hepatocytes.

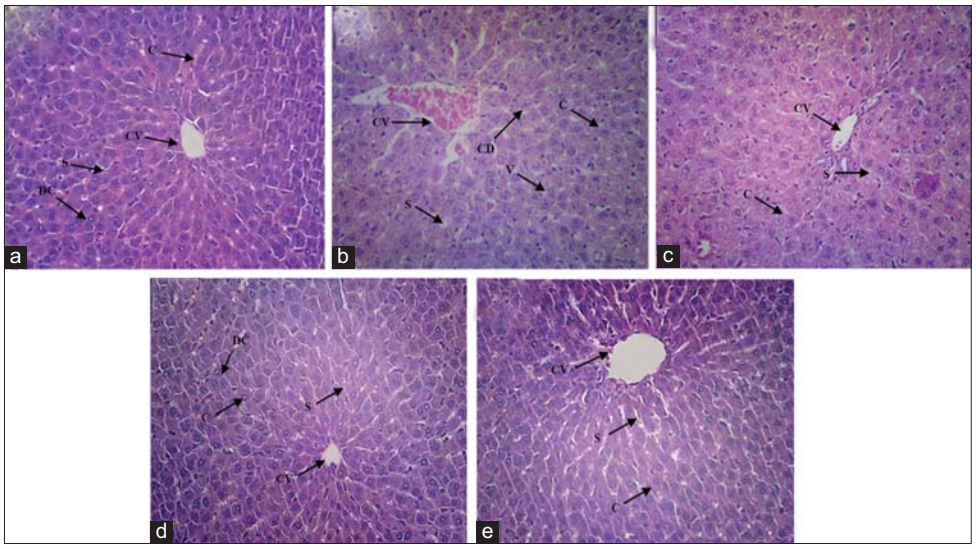

Fig. 3: Photomicrographs of histological changes in H and E stained liver sections of control and experimental rats (×200). (a and d) Control and *Centella asiatica* ethanolic treated rat liver showed clear central vein (CV) with hexagonal cells, embedded in connective tissue. All hepatocytes are radiantly arranged as trabecules from the central portal vein, separated by sinusoids (S). The hepatocytes contain clear spheroidal nucleus with distinct nucleolus as well as peripheral chromatin condensation (C). Few cells contain two nucleuses (cells undergoing division) was also observed (DC), (b) Isoniazid (INH) treated rat liver showed distorted cellular organization accompanied with vacuoles (V), hypertrophic cells, cellular damage (CD), inflammation, widening of inter cellular sinusoids (S) and congestion in central vein (CV). The nucleoli are not distinct and the nucleuses appeared to have more condensed chromatin (C), (c and e) CA ethanolic extract or silymarin oral pretreated INH intoxicated rats liver showed near normal hepatocyte arrangement and sinusoids (S) accompanied with mild central vein congestion (CV). The nucleoli and nucleolus also appeared to be nearly distinct with less condensed chromatin (C)

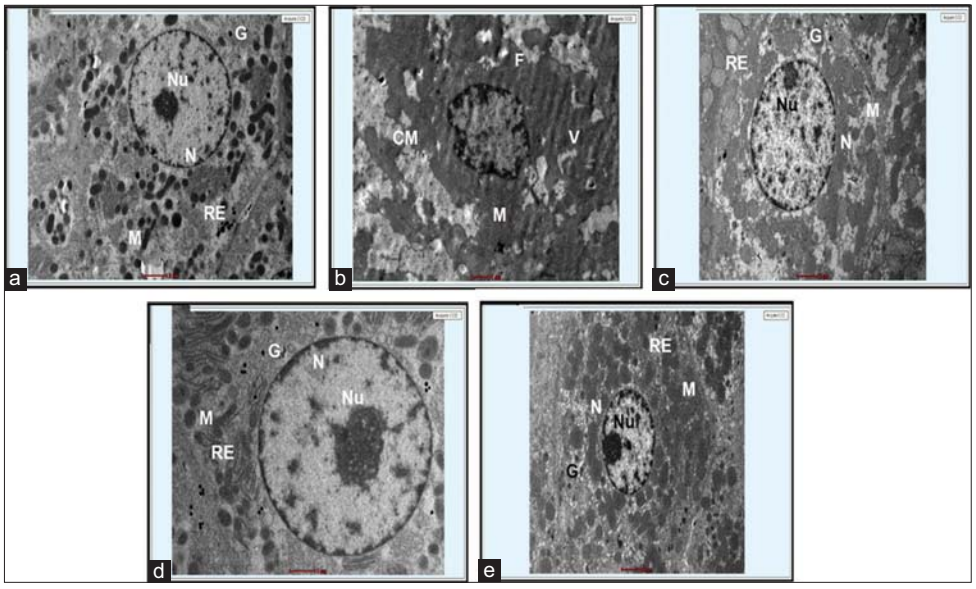

Fig. 4: Liver ultramicrographs of control and experimental rats. (a and d) Control and *Centella asiatica* ethanolic treated rats liver showed a normal hepatocyte with euchromatic nucleus (N) with prominent nuclear membrane, prominent nucleolus (Nu), many mitochondria (M) having normal cristae, rough endoplasmic reticulum (RE), and glycogen granules (G). Transmission electron microscopic (TEM) magnification for A, ×12,000; TEM magnification for D, ×15,000, (b) Isoniazid (INH) treated rat liver showed hepatocyte with enfolding of cell membrane (CM), damaged nuclear membrane, condensation of nuclear chromatins and pyknotic nucleus (N), regression of mitochondrial cisternae (M), and presence of fat droplets (F) with vacuole (V). TEM magnification, ×15,000, (c and e) CA ethanolic extract or silymarin oral pretreated INH intoxicated rats liver showed hepatocyte with euchromatic nucleus (N) with prominent nuclear membrane, prominent nucleolus (Nu), many mitochondria (M), rough endoplasmic reticulum (RE), glycogen granules (G) with less vacuolated cytoplasm. TEM magnification, ×12,000

CONCLUSION

This study jointly reveals that the pretreatment of INH-treated rats with CA leaves ethanolic extract at a concentration of 100 mg/kg bw can substantially reduce the side effects caused by INH, which is one of the first-line drugs for TB patients. This also provides future avenues to study the molecular mechanisms associated with the protective mechanism of the CA ethanolic extract on INH intoxication. Isolation and characterization of the potent molecules responsible for this protective effect can help in developing new lead compounds which

could be useful in pharmaceutical companies in producing more effective drugs that can contribute in reducing the side effects of commercially available drugs and also enhance overall human health.

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