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Short Communication

In vitro hepatoprotective activity of *Eichhornia Crassipes* flowers against CCl_4 induced toxicity in BRL3A cell line

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The present study was carried out to determine the *in vitro* hepatoprotective activity of ethanolic extract from *Eichhornia crassipes* (EEEC) flowers using the CCl_4-challenged BRL3A cell model. Hepatoprotective activity of EEEC (at concentrations of 50, 100 and 200 μg/mL) and standard drug silymarin (200 μg/mL) was evaluated against CCl_4 induced toxicity using BRL3A cell line by measuring the cell viability, aspartate aminotransferase (AST), alanine aminotransaminase (ALT), lactate dehydrogenase (LDH) leakage, lipid peroxidation (LPO) and glutathione level (GSH). Treatment with CCl_4 produced a significant decrease in cell viability. In addition, hepatotoxicity was revealed by increased hepatic marker enzymes like AST, ALT and LDH paralleled with elevated lipid peroxidation and decline in GSH levels. The toxicity induced by CCl_4 in the BRL3A cells was significantly recovered by treatment with EEEC. The tested doses (100 and 200 μg/mL) significantly (P <0.01) reduced the CCl_4 induced elevation of AST, ALT and LDH and also restored the altered biochemical parameters. These findings provide a basis for confirming the traditional uses of *E. crassipes* in treating liver ailments.

Keywords: BRL3A cell line, CCl_4, *Eichhornia crassipes*, Hepatoprotective.

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Introduction

Liver injury is a widespread medical ailment across the world and the most common causes of injury are viral infection, metabolic disorders, chemicals, medications, and/or alcohol abuse[1,2]. The hepatocyte death can eventually result in liver fibrosis and cirrhosis[3,4]. Despite tremendous progress in hepatology research, no significant and safe hepatoprotective drugs are available in the modern system of medicine[5]. Because of the undesirable side effects of synthetic drugs, there is a growing focus on the scientific evaluation of medicinal plants using systematic research methodology.

Eichhornia crassipes (Mart.) Solms, also known as water hyacinth, is a free-floating aquatic plant that is native to South America[6]. The ‘beautiful blue devil’, water hyacinth is recognized by its lavender flowers and shining bright leaves[7]. In Bangladesh, roots and flowers are used in the treatment of hepatic disorders and Pandu (abdominal swelling on one side)[8]. Previous studies have documented the hepatoprotective effect of *E. crassipes* leaves[9]. The hepatoprotective activity of *E. crassipes* flowers is not yet known. Our previous study revealed that ethanolic extract of *E. crassipes* flowers has potent antioxidant activity when compared to other extracts tested[10]. Keeping the folkloric claims and previous reports, an attempt was taken to evaluate the hepatoprotective effect of ethanolic extract of *E. crassipes* flowers.

Materials and Methods

Plant material and extraction

The flowers of *E. crassipes* were collected in the month of May, from the local pond near Budampadu village of Guntur district, Andhra Pradesh, India. The flowers are authenticated by the botanist Dr Madhava Chetty, Professor, Department of Botany, Sri Venkateswara University, Tirupathi, and a voucher specimen was deposited in the herbarium of the institution with reference number 2148 for future reference. The flowers were shade dried and were extracted in the Soxhlet extractor successively using solvents from non-polar to polar (petroleum ether, ethyl acetate, and ethanol). All the extracts were vacuum dried to obtain petroleum ether extract (PEEC), ethyl acetate extract (EAEC), and ethanol extract (EEEC) respectively.

Drugs and Chemicals

Phosphate buffered saline (PBS), Fetal bovine serum (FBS), Dulbecco's modified eagle medium (DMEM), and trypsin were procured from Hi-Media Labs Pvt. Ltd (Mumbai, India). 3-(4, 5-dimethylthiazol-2-yl)-5-diphenyltetrazolium bromide (MTT) and Silymarin were procured from Sigma Aldrich (MO, USA). All other chemicals and solvents used were of analytical grade.

Hepatoprotective activity using BRL3A cell line

Cell culture

In the present study BRL3A (Buffalo Rat liver cell line) cell line was used to study the hepatoprotective

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activity of EEEC. BRL3A was obtained from National Centre for Cell Sciences (NCCS), Pune, India. It was cultured in DMEM (Dulbecco's modified eagles medium), supplemented with 10% inactivated fetal bovine serum (FBS), 100 IU/mL of penicillin, 100 μg/mL of streptomycin and 5 μg/mL amphotericin in a humidified atmosphere of 5% CO_2 at 37 ºC until confluent. Later the cells were dissociated using TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). Stock cultures were grown in 25 cm^2 culture flasks and the study was carried out in 96 microtitre plates.

***In vitro* cytotoxicity assay**

MTT assay was carried out for EEEC to assess its nontoxic doses. After 24 hours of incubation, the hepatocytes were exposed to the fresh medium containing 100 µL of toxicant (medium containing 1% (v/v) CCl_4) along with/without various concentrations of ethanolic extract (62.5-1000 µg/mL) or the medium alone (as normal) and the plates were incubated at 37 °C in a humidified incubator with 5% CO_2 for 72 hours. After appropriate incubation, test samples in the wells were discarded and 50 µL of MTT in PBS was added to each well and then further incubated at the same growth parameters for 3 h. The supernatant was removed and 100 µL MTT solubilization solution was added to each well and the absorbance was read with a microplate reader at 540 nm. The 50% cytotoxic concentration $(CTC)_{50}$ is the concentration of test drug that needed to inhibit cell growth by 50% is generated from the dose-response curves for test samples[11].

% Growth Inhibition = 100 - (Mean OD of individual test group / Mean OD of the control group) × 100

CCl_4 induced toxicity in BRL3A cell lines

The non-toxic or safe concentration of the extract was chosen to assess the antihepatotoxic property. The hepatoprotective activity of EEEC was evaluated using well maintained BRL3A cell lines. Cells were treated with 100 µL each of culture medium containing 0.25% (v/v) DMSO; 1% (v/v) CCl_4; 1% (v/v) CCl_4 + silymarin (200 µg/mL); 1% (v/v) CCl_4 + EECT (50, 100, 200 µg/mL); all the incubations were continued for 24 hours. Then, media and cell lysates were collected and stored at -20 ºC until further analysis. The activities of AST, ALT, and LDH in the supernatant medium were measured by using diagnostic kits according to the manufacturer's instructions. The level of MDA and GSH in the cell lysates was measured using standard procedures[12,13]. Protein estimation was estimated using the Bradford protein assay, using bovine serum albumin as a protein standard[14]. The viability (%) was calculated using the below formula:

% cell viability = (Mean OD of individual test group/ Mean OD of the control group) × 100

Statistical analysis

Results were expressed as mean±standard error mean and statistical significance was analyzed by one-way ANOVA followed by Dunnett's test using GraphPad Instat statistical software. Values of P <0.05 were considered significant in all cases.

Results and Discussion

In recent years, *in vitro* assays such as cell-based models are gaining importance due to their low cost, quick and reliability. The liver cell lines such as BRL3A and HepG2 are routinely used for preliminary screening of hepatoprotective agents, as they retain many characteristics of normal liver cells[15]. CCl_4 is a hepatotoxic haloalkane whose mechanism has been studied intensively over the past years. It is well known that CCl_4 undergoes metabolic activation by a cytochrome P-450 dependent step to free radical products which can initiate lipid peroxidation[16].

In this study, the hepatoprotective effect of *E. crassipes* flowers was investigated using CCl_4 induced hepatotoxicity in the BRL3A cell lines. Before evaluating the hepatoprotective activity of various concentrations of EEEC, it is mandatory to demonstrate that they are non-toxic. MTT test was used as an indicator of cytotoxicity induced by EEEC in infected BRL3A cells and a non-toxic dose was determined. This colorimetric assay involves the conversion of MTT to a purple formazan derivative by mitochondrial succinate dehydrogenase, which is present only in viable cells[17]. The % cytotoxicity of EEEC was found to be dose-dependent and increases with increased concentrations. The % inhibition of cell proliferation at 62.5, 125, 250, 500, and 1000 µg/mL was found to be 11.40±0.5, 18.36±0.3, 28.16±0.6, 41.70±1.1, 52.70±1.0 respectively, with an IC_{50} value of 887 µg/mL (Table 1). The

Table 1 — Cytotoxic properties of ethanolic extract of *Eichhornia crassipes* flowers against BRL3A cell line

Test concentration	% Cytotoxicity	CTC_{50} (µg/mL)
62.5 µg/mL	11.40±0.52	
125 µg/mL	18.36±0.38	
250 µg/mL	28.16±0.69	887
500 µg/mL	41.70±1.11	
1000 µg/mL	52.70±1.09	

Values are expressed as mean±SEM (n=3)

Table 2 — Effect of ethanolic extract of *Eichhornia crassipes* flowers on biochemical parameters in CCl_4 induced toxicity in BRL3A cell line

Experimental groups	% Viability	AST (U/L)	ALT (U/L)	LDH (U/L)	MDA (nmoL/ mg protein)	GSH (nmoL/ mg protein)
Normal control	100	15.95±0.21	10.90±0.34	108.45±0.42	3.53±0.15	25.76±0.51
DMSO control (0.25%)	97.68±0.32	15.07±0.18	10.26±0.16	107.61±0.52	2.99±0.19	24.87±0.42
CCl_4 (1% v/v)	24.08±0.43	51.44±0.51	25.61±0.76	212.87±1.86	7.92±0.22	11.39±0.30
CCl_4 + EEEC (50 µg/mL)	32.64±0.60^{a,b}	48.69±0.52^{b}	25.19±0.97^{b}	209.51±3.15^{b}	7.87±0.42^{b}	13.20±0.50^{b}
CCl_4 + EEEC (100 µg/mL)	51.29±0.52^{a,b}	32.44±1.36^{a,b}	18.10±0.46^{a,b}	187.73±2.29^{a,b}	5.74±0.27^{a,b}	19.61±0.59^{a,b}
CCl_4 + EEEC (200 µg/mL)	65.74±1.04^{a,b}	26.65±0.96^{a,b}	14.72±0.61^{a,b}	173.29±2.40^{a,b}	4.50±0.31^{a,b}	21.66±0.58^{a,b}
CCl_4 + Silymarin (200 µg/mL)	84.21±1.39^{a,b}	19.57±0.93^{a,b}	13.29±0.57^{a,b}	156.30±2.67^{a,b}	4.27±0.28^{a,c}	22.51±0.41^{a,b}

Values are expressed as mean±SEM (n=6); ^{a}P <0.01 when compared with toxicant cells, ^{b}P <0.01, when compared with DMSO, treated cells and ^{c}P <0.05, when compared with DMSO, treated cells

concentrations used to study the hepatoprotective activity are found to be non-toxic for the BRL3A cell line.

Table 2 depicts the results of cell viability and leakage parameters such as AST, ALT and LDH, MDA and GSH levels in all experimental groups. A significant decrease in cell viability and GSH content was observed in the BRL3A cells exposed to CCl_4 when compared with the DMSO control group. Similarly, toxin treatment caused a significant rise in the levels of AST, ALT, LDH, and MDA in BRL3A cells. It was found that treatment with various concentrations (50, 100, and 200 µg/mL) of EEEC significantly (P <0.01) increased the cell viability when compared to toxicant treated cells. The maximum protection observed with the extract was 65.74% (200 µg/mL concentration) whereas the standard drug Silymarin at 200 µg/mL exhibited 84.21%. EEEC at 100 and 200 µg/mL significantly (P <0.01) prevented the CCl_4 induced elevation of AST, ALT, LDH, and MDA levels. Similarly, EEEC at 100 and 200 µg/mL significantly protected the decline in GSH activity when compared to toxicant treated cells (P <0.01). The higher concentrations of EEEC (100 and 200 µg/mL) are found to be more effective when compared to 50 µg/mL.

Cells exposed to the toxic agents lose cell viability, release liver enzymes into the culture medium, do not metabolize the tetrazolium salt, and exhibit significantly changed total antioxidant capacity (TAOxC) and levels of MDA, SOD, and GSH[18,19]. The hepatoprotective mechanism was ascertained by evaluating the relevant parameters like enzyme leakage, quantification of intracellular malondialdehyde and glutathione levels. The possible underlying mechanism for the hepatoprotective effect of EEEC is due to its ability to inhibit lipid peroxidation and maintenance of glutathione in a reduced state by virtue of its antioxidative powers.

Conclusion

In conclusion, the obtained experimental data strongly support the view that *E. crassipes* can be used as a promising hepatoprotective agent. Detailed *in vivo* hepatoprotective studies are in progress in our laboratory which may further strengthen the protective nature. This study provides the first scientific evidence about the hepatoprotective nature of *E. crassipes* flowers.

Conflicts of interest

No potential conflicts of interest were reported by the authors.

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