**In-vitro Cytoprotective Activity of Eichhornia crassipes Flowers Against Hydrogen Peroxide-induced Oxidative Stress in BRL 3A Rat Liver Cells**

S. Rajarajan*, S. Sivakrishnan and V. Ganesan

1Department of Pharmacy, Annamalai University, Annamalainagar, Chidambaram, Tamilnadu, 608002-India.

2Department of Pharmaceutics the Erode College of Pharmacy Veppampalayam Vallipurathanpalayam Post, Tamilnadu-638112 India.

Authors’ contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The aim of this study was to investigate the cytoprotective effect of ethanol extract of *Eichhornia crassipes* flowers and its fractions against hydrogen peroxide induced oxidative stress in BRL 3A liver cells. Powdered flowers of *Eichhornia crassipes* were subjected to hot continuous extraction in soxhlet extractor using ethanol as solvent material. Initially, the solvent extracts were subjected to qualitative, quantitative analysis and assessed for *in-vitro* free radical scavenging activity and antioxidant activity. The ethanol extract was fractionated using benzene, chloroform and n-butanol. The crude ethanol extract and its fractions were evaluated for its potential cytoprotective effect against hydrogen peroxide (H$_2$O$_2$) induced oxidative stress in BRL 3A cell lines. Biochemical assays were carried out to determine the cytoprotective activity, including cell viability, lipid peroxidation by determining the formation of malondialdehyde, lactate dehydrogenase leakage into culture medium, the catalase activity and the content of reduced glutathione (GSH) in the cells. Exposure
of BRL 3A to 2mM H$_2$O$_2$ reduced the cell viability, increased the malondialdehyde (MDA) level, increased the leakage of lactate dehydrogenase (LDH) and caused reduction in antioxidant activities. Pretreatment of cultured cells with crude ethanol extract of *Eichhornia crassipes* flowers and different solvent fractions at concentrations 0.01, 0.1, 1, 10, 100 μg/ml for 30 minutes before H$_2$O$_2$ exposure attenuated the oxidative injury in dose-dependent manner. It was observed that crude ethanol extract of *Eichhornia crassipes* flowers exhibited a strong cytoprotective by increasing cell viability, decreasing lipid peroxidation and LDH leakage. Further increase in catalase and reduced glutathione activity was noted in the cells pre-treated with ethanol extract of *Eichhornia crassipes* flowers. These findings suggest that ethanol extract of *Eichhornia crassipes* flowers has a strong cytoprotective activity against oxidative injury caused by reactive oxygen species.

**Keywords:** *Eichhornia crassipes* flowers; Cytoprotective; Oxidative stress; BRL 3A; Rat liver cells.

1. **INTRODUCTION**

Oxidative stress induced cell damage has been implicated in pathogenesis of various diseases including cancer, cardiovascular diseases, inflammation and neurodegenerative diseases [1]. It is mediated by reactive oxygen species (ROS), including hydrogen peroxide (H$_2$O$_2$), superoxide, hydroxyl radicals, peroxy radicals and singlet oxygen which are generated as by products of normal and aberrant metabolic processes that utilize molecular oxygen [2] [3]. The prolonged production of large amounts of ROS may lead to changes in signal transduction and gene expression resulting in disease development and progression [4]. Further, ROS can attack proteins, deoxynucleic acid and lipid membranes, thereby disrupting cellular functions and integrity. It has been demonstrated that ROS cause cell death via apoptosis. There are many types of chemical and physiological inducers of oxidative stress which are able to cause apoptotic cell death. Induced oxidative stress can be achieved *in-vitro* through the delivery of H$_2$O$_2$, the major component of ROS [5]. The biologically significant reaction of H$_2$O$_2$ is its spontaneous conversion, catalyzed by the Fenton reaction, to the highly reactive hydroxyl radicals that react instantaneously with any biological molecule from which it can abstract hydrogen atom to cause lipid peroxidation and DNA damage in cells [6]. Antioxidants are found to have preventive and therapeutic effects on oxidative stress induced cell damage. Therefore, substantial efforts have been made in recent years to identify both natural and synthetic antioxidants. Several synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tert-butyldihydroquinone (TBHQ), are commercially available, but each has been shown to cause side effects in humans [7]. These substances have been suspected to be responsible for carcinogenic effects in living organisms [8]. Therefore, much attention has been focused on natural antioxidants. In recent years, there has been considerable interest in natural products with antioxidant property. Antioxidant supplements have attracted the focus of attention as potentials in prevention of diseases caused by oxidative damage [9]. Traditionally, many herbal medicines and medicinal plants have been used for treatment of complicated diseases/disorders. Among the herbal resources is *Eichhornia crassipes* (Mart.) Solms belonging to family Pontederiaceae. *Eichhornia crassipes* (Mart.) Solms, commonly known as water hyacinth, is one of the aquatic plants that have attracted most scientific interest in the last decade. Originally from South America, its ornamental appeal led to its introduction into Africa, Asia, the South Pacific, North America and Europe, where it has become invasive [10] [11]. Plant can grow to 3 ft in height. The leaves are oval to elliptical, thick, up to 6 in. (15 cm) wide and waxy with spongy petioles, curved inwards at the edges. Flowers are blue-purple on upright spikes. Each flower has six petals with uppermost having a yellow patch [12]. Its great capacity to multiply has become a real problem in the tropics, where high temperatures and lack of predators has led to its uncontrolled development. However, *Eichhornia crassipes* is not just an invasive and harmful plant; it is also a useful plant with remarkable metal pollutant phytoaccumulation capacities. *Eichhornia crassipes* is capable of bio-concentrating toxic metals such as Cr, Cu, Co, Ni, Zn, Pb, Cd and As in its root system [13] [14] [15]. It is also reported to possess valuable phytochemicals which are of medicinal importance [16]. The fresh juice of this weed is used by tribes to treat wounds, to ease swelling, burning and to stop bleeding [17]. *Eichhornia*
**Figure 1. Eichhornia crassipes flowers**

**2. MATERIALS AND METHODS**

**2.1 Materials**

RPMI-1640 medium, fetal bovine serum, penicillin G, streptomycin, 3-[4,5-dimethylthiazol-2-yl]-2,5-dephenyl tetrazolium bromide (MTT), dimethylsulfoxide (DMSO), 2-thiobarbituric acid (TBA), trichloroacetic acid (TCA), Triton X-100, 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), ethylenediaminetetraacetic acid (EDTA), diithiothreitol (DTT), nicotinamide adenine dinucleotide reduced (NADH), *dinitrophenylhydrazine* (DNPH), quercetin, tannic acid, gallic acid, Folin–Ciocalteu reagent, Folin–Denis reagent were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sodium carbonate, aluminium chloride, sodium nitroprusside were procured from SD Fine Chemicals Ltd., Mumbai, India. Benzene, chloroform, N-butanol, ethanol was from E-Merck (India) Ltd., Mumbai, India. All other chemicals used were of analytical grade.

**2.2 Materials and Methods**

**2.2.1 Plant Material**

The fresh flowers of *Eichhornia crassipes* were collected in the month of June from Bhavani River, Tamilnadu, India. The plant material was taxonomically indentified, confirmed and authenticated by Dr. A. Balasubramanian, Executive Director and Former Siddha Research Consultant (AYUSH), ABS Herbal gardens, Salem and the authentication was retained in our laboratory for further reference. The collected flowers were shade dried and the dried materials were crushed to coarse powder with mechanical grinder. The powder was stored in an airtight container for extraction. The image of *Eichhornia crassipes* flower is shown in Fig. 1.

The crude methanol extract of *Eichhornia crassipes* macrophytes was reported to possess a potential cytotoxic and antioxidant property against cancer cell lines [19]. Based on the widespread use of *Eichhornia crassipes* in traditional medicine, it has been decided to select the plant for investigation. Although previous research suggested that the ethanol extract of *Eichhornia crassipes* flowers possess free radical scavenging and antioxidant activity, no study has been published yet at the cellular level, using markers of cellular stress and antioxidant enzymes against H₂O₂-induced oxidative stress. In the present study, an effort was made to ascertain the cytoprotective effect of *Eichhornia crassipes* flower extract against H₂O₂-induced cytotoxicity in rat liver cells (BRL 3A).

**2.2.2 Extraction**

The powdered flowers of *Eichhornia crassipes* were subjected to hot continuous extraction using ethanol as a solvent material in soxhlet apparatus for 72 hours. After extraction, the solvents were removed by distillation and evaporated under reduced pressure in a rotary evaporator to obtain crude extract of *Eichhornia crassipes* flowers. The collected extract was then transferred to a clean glass vessel and covered with a foil paper in which slits are made for evaporation of solvent traces. The dried extract thus obtained was stored in air tight glass container for further investigation.

**2.2.3 Phytochemical screening**

The various solvent extracts obtained were subjected to preliminary phytochemical screening [20].

**2.2.4 Quantitative estimation of bioactive compounds in crude ethanol extract of *Eichhornia crassipes* flowers**

**Determination of phenolic content**: The total phenolic content was determined spectrophotometrically using the Folin-Ciocalteu method. It is based on the oxidation of phenolic groups by phosphomolybdic and phosphotungstic acids (FC reagent). This method, based on the [21] and the early work of Singleton & Rossi [22] is a colorimetric oxidation/reduction method for phenolic compounds. A blue colour which is the product of
metal oxidation exhibits a broad light absorption with a maximum at 764 nm. The intensity of light absorption is proportional to the concentration of phenols. 20 μL of the diluted sample was added to 100 μL of Folin–Ciocalteu reagent. After 8 min, 300 μL of saturated sodium carbonate solution (25%) was added. The absorbance was measured at 764 nm. The calibration curve was prepared with gallic acid solutions ranging from 10 to 1000 μg/ml and the results are given as gallic acid equivalents (GAE).

Determination of total tannin content: The total tannin content was determined by modified method of Polshelttiwar et al. [23]. Different concentrations of extract (0.1 ml) were mixed with 0.5 ml of Folin-Denis reagent and followed by 1 ml of sodium carbonate (0.5% w/v) solution and distilled water (up to 5 ml). The absorbance was measured at 755 nm within 30 min of the reaction against the blank. The total tannin in the extract was expressed as the equivalent to tannic acid (g TAE/g extract).

Determination of total flavonoids: Flavanoid content was measured using aluminium chloride colorimetric method. Various concentrations of extract were mixed with 0.1 ml of 10 % aluminium chloride (w/v), 0.1 ml of 1 M potassium acetate and 2.8 ml distilled water. The mixture was allowed to stand at room temperature for 30 minutes. The absorbance of reaction mixture was measured at 415 nm. Results are expressed as mg/g quercetin equivalent [20].

Evaluation of total antioxidant capacity by phosphomolybdenum method: The antioxidant activities of various solvent extracts were evaluated by the phosphomolybdenum method according to the procedure described by Prieto et al.[24]. The assay is based on the reduction of Mo (VI) – Mo (V) by the extract and the subsequent formation of a green phosphate/Mo (V) complex at acidic pH. 0.3 ml different concentrations of extract (10μg/ml to 200 μg/ml) were mixed with 3 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were incubated in a boiling water bath at 95°C for 90 min. The absorbance of the solution was measured at 695 nm after cooling to room temperature. The antioxidant capacity of each sample was expressed as ascorbic acid equivalent.

2.2.5 Determination of free radical scavenging activity

DPPH radical scavenging assay: Free radical scavenging activity was determined spectrophotometrically using the method of Blois [25] This method is based on the measurement of the reducing ability of antioxidants toward the DPPH radical. Briefly, 100 μl of various concentrations of the flower extract (1.95 μg/ml to 1000 μg/ml) in methanol were added to 10 ml of a methanol solution of DPPH (1.01.10^-2 M). The mixture was allowed to stand at room temperature for 30 min in the dark after a vigorous shake. The absorbance was measured at 517 nm. The control mixture consists of 100 μl of methanol and 10 ml of DPPH solution. The scavenging activity on the DPPH radical was calculated as inhibition percentage using the following equation:

\[
\% \text{ Inhibition} = \left( \frac{AB - AS}{AB} \right) \times 100
\]

where AB is the absorbance of the control reaction (containing all reagents except the test compound), and AS is the absorbance of the test compound. Ascorbic acid was used as reference standard. The tests were carried out in triplicate. The extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph of inhibition percentage plotted against extract concentration.

Lipid peroxidation inhibitory activity: The lipid peroxidation inhibitory activity of the flower extract was determined according to the method of Duh and Yen [26]. Egg lecithin (3 mg/ml phosphate buffer, pH 7.4) was sonicated in an ultrasonic sonicator for 10 min to ensure proper liposome formation. Test samples of flower extract (100 μl) of different concentrations (1.95 μg/ml to 1000 μg/ml) were added to liposome mixture (1 ml). Ferric chloride (10 μl, 400 mM) and L-ascorbic acid (10 μl, 200 mM) were added to induce lipid peroxidation. After 1 h incubation at 37°C the reaction was stopped by adding hydrochloric acid (2 ml, 0.25 N) containing trichloroacetic acid (150 mg/ml) and thiobarbituric acid (3.75 mg/ml). The reaction mixture was boiled for 15 min, cooled, centrifuged at 1000 rpm for 15 min and the absorbance of the supernatant was measured at 532 nm. Tocopherol was used as reference standard. The control was without extract.

The scavenging effect was measured using the following equation
Scavenging effect (%) = [(C - T/C) X 100] / C

Nitric oxide scavenging activity: This method is based on principle that sodium nitroprusside in aqueous solution at physiological pH spontaneously generate nitric oxide which further interacts with oxygen to produce nitrite ion that can be estimated using Griess reagent. Scavenger of nitric oxide competes with oxygen leading to reduced production of nitrite ion. For experimental, sodium nitroprusside (10 mM) in phosphate buffer saline (PBS pH 7.4) was mixed with different concentration of extract (1.95 μg/ml to 1000 μg/ml) dissolved in respective solvent and incubated at 25°C for 150 minutes. The same reaction mixture without extract but equivalent amount of solvent served as control. After incubation period 1.5 ml of the incubated solution was removed and diluted with 1.5 ml of Griess reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% naphthyl ethylene diamine dihydrochloride). The absorbance of the chromophore formed during diazotization of the nitrite with sulphanilamide and subsequent coupling with naphthyl ethylene diamine was measured at 546 nm. Quercetin was used as positive control [27]. The tests were carried out in triplicate. The nitric oxide scavenging activity is calculated as

Scavenging activity = [(C - T/C) X 100] / C

where C = absorbance of control and T = absorbance of test solution.

Ferric reducing antioxidant capacity: According to the method described by Oyaizu [28] the reducing powers of extracts were determined. Different concentrations of flower extracts (1.95 μg/ml to 1000 μg/ml) in 1 ml of distilled water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide \([K_3Fe(CN)_{6}]\) (2.5 mL, 1%). After incubation at 50°C for 20 min 2.5 ml of trichloroacetic acid (10%) was added to the mixture and centrifuged at 3000 rpm for 10 min. The supernatant layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and FeCl₃ (0.5 ml, 0.1%) and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicates increased reducing power. Ascorbic acid was used as reference standard.

Cupric ions (Cu²⁺) reducing antioxidant capacity (CUPRAC assay): By the method of [29], the cupric reducing antioxidant capacity (CUPRAC) was determined. 1 ml of CuCl₂ solution (1.0x10⁻²M), 1 ml ethanolincuconeprune solution (7.5x10⁻² M) and 1 ml NH₄CH₂COO (1M, pH 7.0) were added to a test tube and mixed. The extract at concentration (500 μg/ml) was added to the initial mixture to make the final volume 4.1 ml and after 1 h, the absorbance was measured at 450 nm against a reagent blank. The cupric ions (Cu²⁺) reducing capacity was expressed as trolox equivalent (μg/ml).

Fractionation of crude extract of *Eichhornia crassipes* flowers: About 4 g of the dried ethanol extract was dissolved in 20 ml water and was successively partitioned with benzene, chloroform and n-butanol. The percentage yield of the resulting extracts was calculated. The extract and the fractions obtained were freeze dried and stored at 4°C [30].

2.2.6 In-vitro cytoprotective study of crude extract and fractions of *Eichhornia crassipes* flowers against H₂O₂ induced oxidative stress in BRL 3A rat liver cells

Cell lines and cell culture: Rat liver cells BRL 3A was cultured in RPMI-1640 medium containing 10 % fetal bovine serum, 100 U/ml penicillin G and 100 mg/ml streptomycin at 37°C in an incubator of humidified air with 5 % CO₂.

Cytoprotective activity of crude extract and fractions of *Eichhornia crassipes* flowers: Cytoprotective study of crude extract and fractions of *Eichhornia crassipes* flowers on H₂O₂-induced cell injury was investigated by MTT assay [31]. A total of 2x10⁴ cells were plated per well in 96-well plates with 200 μl culture medium for 16 h and were exposed to various concentrations of crude extract and fractions of *Eichhornia crassipes* flowers(0.01, 0.1, 1, 10 and 100 μg/ml) for 30 min before exposure to 2mM H₂O₂ for 3 hours. At the time of incubation 20 μl of MTT solution (2 mg/ml) in phosphate buffered saline (PBS) was added to each well. After 4h of incubation, the supernatant was discarded and 200 μl of dimethyl sulfoxide was added to each well to terminate the reaction. At 550 nm the absorbance was measured using an enzyme-linked immunosorbent assay (ELISA) plate reader. Cell viability was expressed as a percentage of control, untreated cells.

MDA assay: A total of 7x10⁵ cells were plated per well in 6-well plates with 2ml culture medium.
for 18h and exposed to crude extract and fractions of *Eichhornia crassipes* flowers(0.01, 0.1, 1, 10 and 100 µg/ml) for 30min before exposure to 2mM H₂O₂ for 3h. To the cell samples two volumes of 2-thiobarbituric acid reagent (0.375% 2-thiobarbituric acid, 15% trichloroacetic acid, and 0.1mM EDTA) were added and boiled at 100°C for 40 min. The absorbance of each supernatant was measured at 532 nm after cooling and centrifugation at 3000×g for 10 min. The results were expressed as percentage of absorbance relative to control absorbance (100%). The lower the absorbance percentage, higher the protection against the induced lipid peroxidation [32].

**LDH assay:** A total of 7×10⁵ cells were plated per well in 6-well plates with 2ml culture medium for 18h and exposed to crude extract and fractions of *Eichhornia crassipes* flowers(0.01, 0.1, 1, 10 and 100 µg/ml) for 30 min before exposure to 2mM H₂O₂ for 3 hours. One milligram of NADH/ml in 0.75mM sodium pyruvate was maintained at 37 °C for 5min. 100µl of NADH/ml in 0.75mM sodium pyruvate mixture was added to 10µl of cell culture medium and kept at 37 °C for 30 min. To each tube, the colour reagent (0.2 mg/ml 2,4-dinitrophenylhydrazine in 1M hydrochloric acid, 100µl) was added and incubated for 20 min at room temperature. By addition of 1ml 0.4M NaOH the reaction was terminated. Absorbance was read in a microplate reader at 450 nm. LDH leakage was expressed as the percentage percentage of the total LDH activity (LDH in the medium +LDH in the cell), according to the equation Percentage LDH released = LDH activity in the medium/total LDH activity× 100 [33].

**Catalase assay:** A total of 7×10⁵ cells plated per well in 6 well plates with 2 ml culture medium for 18 hours were exposed to various concentrations of crude extract and fractions of *Eichhornia crassipes* flowers(0.01, 0.1, 1, 10 and 100 µg/ml) for 30 minutes before exposure to 2mM H₂O₂ for 3 hours. The homogenates were centrifuged at 14,000 rpm at 4°C for 15 min. In the soluble supernatants the enzyme activities were measured. The measurement of catalase activity is based on H₂O₂ breakdown using spectrophotometer at 240 nm and expressed as percentage of untreated control [34].

**GSH assay:** In 2ml culture medium a total of 7×10⁵ cells were plated per well in 6-well plates with for 18h and exposed to crude extract and fractions of *Eichhornia crassipes* flowers at different concentrations for 30 min before exposure to 2mM H₂O₂ for 3h. The cells were washed and harvested in 0.5 ml of PBS with 0.1% of Triton X - 100. The mixture was centrifuged (3000 rpm, 10 min, 4°C) after 10 minutes of incubation and 0.3 ml of the supernatant was mixed with 1.0 ml of Tris-base0.8M, EDTA 0.02M buffer, pH 8.9. After the addition of 0.1 ml of DTNB 0.01M in methanol, the content of reduced GSH in the cells was measured at 412 nm and expressed as percentage of untreated control [35].

### 2.3 Statistical Analysis

All tests were carried out independently in triplicate (n=3) and the results are expressed as mean ± S.D. The results of *in-vitro* cytoprotective activity were analysed for statistical significance by one way ANOVA followed by Dunnett’s test (Graphpad Software Inc,La Jolla, CA. Trial version ). The criterion for statistical significance was set at P< 0.05.

### 3. RESULTS AND DISCUSSION

#### 3.1 Percentage Yield

The percentage yield of extract obtained from powdered *Eichhornia crassipes* flowers using ethanol as solvent was 7.25 % w/w.

#### 3.2 Preliminary Phytochemical Studies

Preliminary qualitative investigation performed in the ethanol extract of *Eichhornia crassipes* flowers revealed the presence of major phytocompounds alkaloids, phenolic compounds, tannins, flavonoids, sterols, terpenoids, glycosides, carbohydrates, proteins and amino acids

#### 3.3 Quantitative Estimation of Bioactive Compounds

The total phenolic content in EEEC was found to be 237.60 ± 2.36 µg GAequivalentsper mg of dry extract. The total tannin content in EEEC was found to be 365.35 ± 2.85 µg of TAEequivalentper mg of dry extract. The total flavonoid content in EEEC was found to be 247.60 ± 2.45 µg of quercetinequivalentper mg of extract (Table 1).
Table 1. Total phenolic content (TPC), Total tannin content (TTC), Total flavonoid content (TFC) and Total antioxidant capacity (TAC) in ethanol extract of Eichhornia crassipes (EEEC) flowers

| Extract | TPC (μg of GAE/mg of extract) | TTC (μg of TAE/mg of extract) | TFC (μg of quercetin/mg of extract) | TAC (μg of ascorbic acid/mg of extract) |
|---------|-----------------|-----------------|-----------------|-----------------|
| EEEC    | 237.60 ± 2.36   | 365.35 ± 2.85   | 247.602.45      | 441.011.32      |

Total phenolic content (TPC) is expressed as microgram of gallic acid equivalent per milligram of sample. Total tannin content (TTC) is expressed as microgram of tannic acid equivalents per milligram of dry extract. Total flavonoid content (TFC) is expressed as micrograms of quercetin equivalents per milligram of dry extract. Total antioxidant capacity is expressed as micrograms of ascorbic equivalents per milligram of dry extract.

Table 2. Cupric ions (Cu^{2+}) reducing capacity (CUPRAC assay) of ethanol extract of Eichhornia crassipes flowers (EEEC)

| Extract | CUPRAC μg of Trolox/mg of extract |
|---------|----------------------------------|
| EEEC    | 159.68 ± 2.50                    |

3.4 Total Antioxidant Capacity

The total antioxidant activity of EEEC flowers was evaluated by phosphomolybdenum method and the results were expressed as ascorbic acid equivalents. The ethanol extract showed a potent antioxidant activity with an antioxidant capacity of 441 ± 1.32 μg ascorbic acid equivalents per mg of dry extract (Table 1).

3.5 Free Radical Scavenging Activity

The free radical scavenging activity of EEEC was investigated by DPPH scavenging activity, nitric oxide scavenging activity, lipid peroxidation scavenging activity, cupric ion reducing assay, ferric reducing assay and metal chelating activity.

3.6 Radical Scavenging Activity

The DPPH radical scavenging activity of EEEC flowers is shown in Fig. 2. The ethanol extract showed a significant dose-dependent inhibition of DPPH activity with a 50 % inhibition (IC_{50}) at a concentration of 79 ± 0.20 μg/ml which was comparable to reference standard ascorbic acid with IC_{50} value of 15.52 ± 0.18 μg/ml.

3.7 Lipid Peroxidation Inhibitory Activity

The lipid peroxidation inhibitory activity of EEEC flowers is shown in Fig. 3. It was observed that EEEC flowers showed a weak inhibitory effect on ultrasound induced lipid peroxidation in liposome prepared from egg lecithin. The IC_{50} value of EEEC was found to be above 1000 μg/ml, whereas standard drug tocopherol showed an IC_{50} value of 2.13 ± 0.11 μg/ml.

3.8 Nitric Oxide Scavenging Activity

The nitric oxide scavenging activity of EEEC flowers is shown in Fig. 4. The EEEC flowers showed nitric oxide scavenging activity by reducing the amount of nitrite generated from the decomposition of sodium nitroprusside in-vitro. The IC_{50} value of EEEC was found 389 ± 2.24 μg/ml and the IC_{50} value of standard compound quercetin was found to be 12.0 ± 0.16 μg/ml respectively.

3.9 Ferric Reducing Power Assay

The ferric reducing power of EEEC flowers is shown in Fig. 5. It was observed that the reducing ability of the extracts increased with the concentration.

3.10 CUPRAC Assay

CU^{2+} ion reducing capacity of EEEC flowers is shown in Table 2. EEEC flowers showed CUPRAC reducing capacity of 159.68 ± 2.50 μg of Trolox/mg of extract.

Cytoprotective activity of fractions and crude ethanol extract of Eichhornia crassipes flowers Cell viability: H_{2}O_{2} induced oxidative injury to BRL 3A cells was quantified by MTT assay. As illustrated in and Fig. 6, exposure to 2 mM H_{2}O_{2} for 3 hours significantly (p<0.001) reduced the cell viability of BRL 3A cells. The percentage cell viability was reduced to 35.27 ± 0.58% that of the control cells. Pre-treatment of
cells with benzene fraction of *Eichhornia crassipes* flowers at various concentrations (0.01, 0.1, 1, 10 and 100 µg/ml) showed viability percentage of 39.17 ± 0.60, 40.60 ± 0.92, 41.90 ± 0.92, 41.27 ± 1.73 and 44.20 ± 1.17 % respectively, while chloroform fraction of *Eichhornia crassipes* flowers at various concentrations (0.01, 0.1, 1, 10 and 100 µg/ml) restored cell survival to 39.43 ± 1.65, 40.47 ± 1.42, 42.07 ± 0.85, 50.40 ± 1.20 and 52.37 ± 1.12 % respectively. The percentage cell viability in cell pre-treated with n-butanol fraction of *Eichhornia crassipes* flowers at various concentrations (0.01, 0.1, 1, 10 and 100 µg/ml) was found to be 39.60 ± 0.96, 40.87 ± 1.10, 42.75 ± 0.86, 52.36 ± 1.20 and 54.58 ± 1.10 respectively. The percentage cell viability in cell pre-treated with ethanol extract of *Eichhornia crassipes* flowers at various concentrations (0.01, 0.1, 1, 10 and 100 µg/ml) was found to be 40.60 ± 0.94, 42.17 ± 1.18, 46.57 ± 1.14, 62.17 ± 1.46 and 69.17 ± 1.20 respectively. From the cell viability assay it was observed that ethanol extract was highly significant (p<0.001) in protecting the BRL 3A cells at various concentrations examined. The concentrations 1 µg/ml, 10 µg/ml and 100 µg/ml were highly significant (p<0.001) in protecting the BRL 3A cells from oxidative injury with greater cell survival percentage compared to other solvent extract.

![Fig. 2](image2.png)

**Fig. 2.** DPPH scavenging activity of ethanol extract of *Eichhornia crassipes* (EEEC) flowers

![Fig. 3](image3.png)

**Fig. 3.** Lipid peroxidation inhibitory activity of ethanol extract of *Eichhornia crassipes* flowers using tocopherol as assay reference. Each value is expressed as mean ± standard deviation (n=3)
Fig. 4. Nitric oxide scavenging activity of ethanol extract of *Eichhornia crassipes* flowers using quercetin as assay references. Each value is expressed as mean ± standard deviation (n=3)

Lipid peroxidation: The formation of MDA in the cells is used as an index of membrane lipid peroxidation and marker of oxidative stress. The effect of ethanol extract and various solvent fractions of *Eichhornia crassipes* flower extract on MDA levels in BRL 3A cells against H$_2$O$_2$ induced oxidative stress was evaluated. As illustrated in Fig. 7, BRL 3A cells exposed to 2mM H$_2$O$_2$ for 3 hours significantly increased (p<0.001) the absorbance percentage which indicate the increased MDA levels compared to that of control grouped cells. The percentage increase in absorbance was found to be 270.4 ± 10.56 compared to control cells. In contrast, the cells pre-treated with each benzene fraction of *Eichhornia crassipes* flowers at various concentrations (0.01, 0.1, 1, 10 and 100 µg/ml) showed absorbance with percentage decrease of about 269.1 ± 9.18, 248.5 ± 10.86, 226.1 ± 8.77, 220.8 ± 2.62 and 219.6 ± 11.39 % respectively, while chloroform fraction of *Eichhornia crassipes* flowers at various concentrations (0.01, 0.1, 1, 10 and 100 µg/ml) showed absorbance with percentage decrease of about 254.1 ± 7.82, 241.6 ± 9.95, 242.4 ± 2.68, 226 ± 8.77 and 210.1 ± 21.3 % respectively. The percentage decrease in absorbance noted in cells pre-treated with n-butanol fraction at various concentrations (0.01,
0.1, 1, 10 and 100 µg/ml) was 250.6 ± 6.84, 240.2 ± 8.21, 238.3 ± 2.45, 224.4 ± 6.77 and 200.6 ± 1.56 % respectively. The percentage decrease in absorbance in cells pre-treated with ethanol extract of *Eichhornia crassipes* flowers at various concentrations (0.01, 0.1, 1, 10 and 100 µg/ml) was found to be 239.3 ± 11.84, 229.2 ± 8.99, 195.7 ± 7.35, 152.8 ± 3.11 and 124.1 ± 3.11 % respectively.

There was no significant difference in absorbance percentage in cells pre-treated with lower concentrations of benzene and chloroform fractions (0.01, 0.1, and 1 µg/ml) compared to H2O2 control grouped cells. Pre-treatment with ethanol extract at concentrations 1, 10 and 100 µg/ml significantly reduced the absorbance percentage. The percentage absorbance at various concentrations was found to 195.7 ± 7.35 (1 µg/ml) (p<0.01), 152.8 ± 3.11 (10 µg/ml) (p<0.001) and 124.1 ± 3.11 (100 µg/ml) (p<0.001). Pre-treatment with benzene, chloroform and n-butanol fractions at concentrations 10 µg/ml and 100 µg/ml also

**H2O2 control cell viability:** 35.27 ± 0.58 %

**Fig. 6.** Effect of various solvent fractions and crude ethanol extract of *Eichhornia crassipes* flowers on cell viability in H2O2 injured BRL 3A cells. Cell viability was evaluated by the MTT assay. Data are mean ± S.E.M. values and expressed as percentage of control. Data are expressed as percentage of control cells and presented as mean ± S.E.M. of three independent experiments. Values are significantly different from H2O2 control cells; ns-non significant; P < 0.05; **P < 0.01; ***P < 0.001 (one-way ANOVA followed by Dunnett’s test)

**H2O2 control Lipid peroxidation:** 255.4 ± 10.56 %

**Fig. 7.** Effect of various solvent fractions and crude ethanol extract of *Eichhornia crassipes* flowers on lipid peroxidation in H2O2 injured BRL 3A cells. Cells were pretreated with extract for 30min before exposure to 2mM H2O2 for 3h. The results were expressed as percentage of absorbance relative to control absorbance (100 %) and presented as mean ± S.E.M. of three independent experiments. Values are significantly different from H2O2 control cells; ns-non significant; *P < 0.05; **P < 0.01; ***P < 0.001 (one-way ANOVA followed by Dunnett’s test)
showed significant difference (p<0.5 to p<0.01) in absorbance percentage compared to H₂O₂ control cells but the significance is comparatively low in comparison to H₂O₂ control and ethanol extract ((p<0.01) to p<0.001). From the results, it was observed that the ethanol extract showed a significant protection against lipid peroxidation compared to fractions by reducing the percentage absorbance to significant levels.

**LDH leakage:** The effect of various solvent fractions and crude ethanol extract of *Eichhornia crassipes* flowers on LDH leakage in BRL 3A cells against H₂O₂ induced oxidative stress was evaluated. As shown in Fig. 8, there was a significant increase (p<0.001) in LDH leakage into medium in H₂O₂ treated cells compared to control cells. The percentage increase was found to be 74.70 ± 0.57 compared to control cells with percentage release of 11.97 ± 2.68. There was no significant decrease in LDH leakage in group of cells pre-treated with benzene fraction at different concentrations and untreated H₂O₂ treated cells. The percentage LDH release in cells pre-treated with benzene fraction of *Eichhornia crassipes* flowers at various concentrations (0.01, 0.1, 1, 10 and 100 µg/ml) was 71.00 ± 1.2, 74.20 ± 3.34, 72.97 ± 1.44, 68.50 ± 0.83 and 70.27 ± 1.38 % respectively. Pre-treatment with chloroform and n-butanol fractions at concentrations 1 µg/ml, 10 µg/ml and 100 µg/ml also showed significant difference (p<0.5) in LDH release percentage compared to H₂O₂ control cells but the significance is comparatively low in comparison to ethanol extract and H₂O₂ control (p<0.5 to p<0.001).

**Catalase activity:** The effect of various solvent fractions and crude ethanol extract of *Eichhornia crassipes* flowers on antioxidant enzymes in BRL 3A cells against H₂O₂ induced oxidative injury was evaluated. Fig. 9 illustrates the activity of catalase in BRL 3A cells. After exposure of BRL 3A cells to 2mM H₂O₂ for 3 hours, the activity of H₂O₂ control LDH release: 74.70 ± 0.57 %

Fig. 8. Effect of various solvent fractions and crude ethanol extract of *Eichhornia crassipes* flowers on LDH release in H₂O₂ injured BRL 3A cells. Cells were pretreated with extract for 30min before exposure to 2mM H₂O₂ for 3h. Data are expressed as percentage of the total LDH activity of control cells and presented as mean ± S.E.M. of three independent experiments. Values are significantly different from H₂O₂ control cells; ns-non significant; *P < 0.05; **P < 0.01; ***P< 0.001 (one-way ANOVA followed by Dunnett’s test)
intracellular catalase significantly decreased (p<0.001) to 40.17 ± 2.81 % of control value. In cells pre-treated with benzene fraction, there was no significant increase in catalase activity. The percentage catalase activity at concentrations 0.01, 0.1, 1, 10 and 100 µg/ml was found to be 46.47 ± 3.19 (ns), 42.77 ± 1.88 (ns), 42.53 ± 2.78 (ns), 42.10 ± 2.42 (ns) and 43.47 ± 2.22 % (ns) respectively, whereas the cells pre-treated with chloroform fraction at concentrations 0.01, 0.1, 1, 10 and 100 µg/ml showed a percentage increase of 41.07 ± 2.92 (ns), 40.27 ± 1.48 (ns), 44.23 ± 1.82 (ns), 46.93 ± 2.29 (ns) and 52.07 ± 2.17 % (p<0.05) respectively. BRL 3A cells pre-treated with n-butanol fraction at concentrations 0.01, 0.1, 1, 10 and 100 µg/ml showed the catalase activity of 42.06 ± 1.84 (ns), 42.18 ± 1.26 (ns), 42.45 ± 1.21 (ns), 49.46 ± 1.45 (p<0.05) and 54.58 ± 1.65 % (p<0.05) respectively. Whereas cells pre-treated with ethanol extract at concentrations 0.01, 0.1, 1, 10 and 100 µg/ml increased the catalase activity to 45.73 ± 1.33 (ns), 46.70 ± 3.27 (ns), 47.67 ± 3.26 (ns), 54.30 ± 1.61 (p<0.01) and 65.40 ± 2.20 (p<0.001) % respectively. It was observed that ethanol extract at concentrations 10 µg/ml and 100 µg/ml was highly significant (p<0.01 to p<0.001) in protecting BRL 3A cells against H$_2$O$_2$ oxidative injury by restoring the catalase activity significantly compared to other solvent fractions.

**GSH activity:** The effect of various solvent fractions and crude ethanol extract of *Eichhornia crassipes* flowers on GSH levels in BRL 3A cells against H$_2$O$_2$ induced oxidative injury was evaluated. Fig. 10 illustrates the glutathione levels in BRL 3A cells. After exposure of BRL 3A cells to 2mM H$_2$O$_2$ for 3 hours, the activity of intracellular glutathione significantly decreased (p<0.001) to 44.87± 1.46 % of control value. There was no significant difference in glutathione levels in BRL 3A cells pre-treated with benzene fraction. The percentage GSH activity cells pre-treated with benzene fraction at concentrations 0.01, 0.1, 1, 10 and 100 µg/ml was 44.97 ± 3.28 (ns), 42.18 ± 1.26 (ns), 42.45 ± 1.21 (ns), 49.46 ± 1.45 (p<0.05) and 54.58 ± 1.65 % (p<0.05) respectively. Whereas cells pre-treated with ethanol extract at concentrations 0.01, 0.1, 1, 10 and 100 µg/ml increased the percentage GSH activity of 44.97 ± 3.28 (ns), 48.06 ± 2.7 (ns), 59.4 ± 3.25 (p<0.01), 79.43 ± 2.13 (p<0.001) and 84.7 ± 2.80 (p<0.001) % respectively compared to H$_2$O$_2$ control cells. It was observed that ethanol extract at concentrations 1 µg/ml, 10 µg/ml and 100 µg/ml was found to be significantly compared to other solvent fractions.

**4. DISCUSSION**

The oxidative stress induced tissue damage has been implicated in number of diseases including cancer, diabetes and neurodegenerative diseases [36] [37] [38]. There are numerous inducers of oxidative stress such as hydrogen peroxide, superoxide anion and hydroxyl radical which cause damage to biological molecules and induce cytotoxicity and apoptosis in many different cell types [2] [[39]. To protect the cells from oxidative damage, antioxidants play an important role in health care system. In recent years, antioxidant supplements have attracted the focus of attention as potentials in prevention of diseases caused by oxidative damage. Many synthetic antioxidants such as butylated hydroxyl anisole (BHA) and butylated hydroxyl toluene (BHT) are very effective and are used for industrial processing but they possess potential health risk and toxic properties to human health and should be replaced with natural antioxidants [40]. Therefore, it is of great interest to health care community to search for potential antioxidants from natural products and herbal preparations for therapeutic applications. In the present study, *Eichhornia crassipes* flowers were evaluated for cytoprotective activity against H$_2$O$_2$ induced oxidative stress in BRL 3A (in-vitro rat liver cell line). In preliminaryphytochemical investigation and quantification of bio-active components, the ethanol extract of *Eichhornia crassipes* flowersshowed the presence of phenolics, flavonoids and tannins. Free radical scavenging and antioxidant activity was carried out for extract by in-vitro assays. The extract showed a dose dependent effect free radical scavenging and anti-oxidant activity. The extract was subjected to fractionation using solvents of increasing polarity. The ethanol extract and its fractions were evaluatedfor in-vitro cytoprotective activity against H$_2$O$_2$ induced oxidative damage in BRA 3A cells.
**H$_2$O$_2$ control catalase activity: 40.17 ± 2.81 %**

Fig. 9. Effect of various solvent fractions and crude ethanol extract of *Eichhornia crassipes* flowers on catalase activity in H$_2$O$_2$ injured BRL 3A cells. Cells were pretreated with extract for 30min before exposure to 2mM H$_2$O$_2$ for 3h and catalase activity was determined in BRL 3A cellular lysate preparation. Data are expressed as percentage of control cells and presented as mean ± S.E.M. of three independent experiments. Values are significantly different from H$_2$O$_2$ control cells; ns-non significant; *P < 0.05; **P < 0.01; ***P < 0.001 (one-way ANOVA followed by Dunnett’s test)

**H$_2$O$_2$ control GSH activity: 44.87± 1.46 %**

Fig. 10. Effect of various solvent fractions and crude ethanol extract of *Eichhornia crassipes* flowers on glutathione content in H$_2$O$_2$ injured BRL 3A cells. Cells were pretreated with extract for 30min before exposure to 2mM H$_2$O$_2$ for 3h and the content of reduced GSH in the cells was measured via spectrophotometry. Data are expressed as percentage of control cells and presented as mean ± S.E.M. of three independent experiments. Values are significantly different from H$_2$O$_2$ control cells; ns-non significant; *P < 0.05; **P < 0.01; ***P < 0.001 (one-way ANOVA followed by Dunnett’s test)

H$_2$O$_2$ is a well known genotoxic agent able to induce oxidative damage, including DNA strand breakage and base modification [41]. It is one of the main ROS generated during redox process [42] and can be produced from nearly all sources of oxidative stress [43]. Hence, H$_2$O$_2$ induced
oxidative injury was selected as a model to screen the cytoprotective role of *Eichhornia crassipes* flower extract and its fraction. H$_2$O$_2$ induced cytotoxic injury to BRA 3A cells was quantified by MTT assay. Active mitochondria of living cells can cleave MTT to produce formazan, the amount of which is directly related to the number of living cells. A dose-dependent increase in cell viability was noted in cells pre-treated with ethanol extract *Eichhornia crassipes flowers*. In comparison with control, the percentage increase in cell viability in group treated with ethanol extract was highly significant and ethanol extract showed a noticeable dose-dependent increase in cell viability in comparison to other solvent fractions. To further investigate the protective effect of extracts, LDH leakage assay was performed. LDH is a cytosolic enzyme used to evaluate cell viability and membrane integrity [44]. Upon damage to the plasma membrane, LDH is rapidly released into the cell culture medium. An increase in number of dead cells or damage in plasma membrane results in increase in LDH activity in the culture medium [33]. In the present study it was noted that ethanol extract *Eichhornia crassipes flowers* significantly reduced the LDH leakage from BRA 3A cells into the culture medium in comparison to H$_2$O$_2$ control. The percentage decrease in LDH release with ethanol extract was dose dependent and compared to other solvent fractions, ethanol extract *Eichhornia crassipes flowers* effectively reduced the LDH leakage from BRA 3A cells into cell culture medium.

Hydroxyl radicals are generated by a fenton-like reaction between H$_2$O$_2$ and transition metal ions such as copper and iron in biological system [45]. The generated hydroxyl radical reacts with number of target molecules, including proteins, membrane lipids and DNA. Moreover, hydroxyl radicals cause oxidation of lipids which results in formation of end products such as MDA and unsaturated aldehydes that can generate a variety of mutagenic adducts by reacting with DNA [46]. Since MDA acts as an index of membrane lipid peroxidation and marker of oxidative stress, MDA assay was performed to quantify the extent of lipid peroxidation in BRL 3A cells exposed to H$_2$O$_2$ with or without ethanol extract *Eichhornia crassipes flowers* and its fractions and the results are compared with respective controls. From the results of MDA assay, it was noted that ethanol extract *Eichhornia crassipes flowers* significantly reduced the percentage of lipid peroxidation in comparison to H$_2$O$_2$ control. The percentage decrease in lipid peroxidation in group pre-treated with ethanol extract was dose dependent. Compared to other solvent fractions, BRA 3A cells pre-treated with ethanol extract *Eichhornia crassipes flowers* showed reduced the lipid peroxidation levels.

Augmented GSH levels and catalase activity was observed in cells pre-treated with ethanol extract of *Eichhornia crassipes flowers*. GSH plays a major role in xenobiotic metabolism and quenches ROS directly. When the cells are exposed to xenobiotics, more amount of GSH is utilised for conjugation making it less available to serve as antioxidant [47] [48]. When intracellular GSH content is reduced, the oxidative stress enhances and eventually results in cell death [49] [50]. In the present study, the levels of GSH reduced in BRL 3A cells exposed to H$_2$O$_2$. Pre-treatment with ethanol extract of *Eichhornia crassipes flowers* significantly elevated the levels of GSH. Ethanol extract showed a marked increase in GSH levels compared to other solvent extracts. Moreover, ethanol extract also enhanced the activities of catalase in BRL 3A cells which is involved in decomposition of H$_2$O$_2$ to O$_2$ and H$_2$O [51]. These results suggest [52] that ethanol extract of *Eichhornia crassipes flowers* is very effective in protecting the cells against oxidative stress.

5. CONCLUSION

In conclusion, our present study clearly demonstrates that the ethanol extract of *Eichhornia crassipes flowers* was able to protect BRL 3A cells against hydrogen peroxide by stabilizing and increasing the antioxidant defense which are disturbed during induced oxidative stress. The potential cytoprotection by the ethanol extract of *Eichhornia crassipes flowers* might be due to presence of phenolics, tannins and flavonoids.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.
CONSENT

It is not applicable.

ETHICAL APPROVAL

Animal Ethic committee approval has been taken to carry out this study.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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