In-vitro cytoprotective activity of Eichhornia crassipes flowers against hydrogen peroxide induced oxidative stress in BRL 3A rat liver cells

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ABSTRACT

The aim of this study was to see whether an ethanol extract of Eichhornia crassipes flowers and its fractions could protect BRL 3A liver cells from hydrogen peroxide-induced oxidative stress. Eichhornia crassipes powdered flowers were subjected to a hot continuous extraction in a soxhlet extractor using ethanol as the solvent material. The solvent extracts were first tested for in-vitro free radical scavenging and anti-oxidant activity using qualitative and quantitative methods. Benzene, chloroform, and n-butanol were used to fractionate the ethanol extract. In BRL 3A cell lines, the crude ethanol extract and its fractions were tested for their possible cytoprotective effect against hydrogen peroxide (H2O2) induced oxidative stress. Cell viability, lipid peroxidation by measuring the formation of malondialdehyde, lactate dehydrogenase leakage into culture medium, catalase activity, and the content of reduced glutathione (GSH) in the cells were all tested in biochemical assays to determine the cytoprotective activity. BRL 3A cells were exposed to 2mM H2O2, which decreased cell viability, increased malondialdehyde (MDA) levels, increased lactate dehydrogenase (LDH) leakage, and reduced antioxidant activities. Pretreatment of cultured cells for 30 minutes with crude ethanol extract of Eichhornia crassipes flowers and various solvent fractions at concentrations of 0.01, 0.1, 1, 10, 100 g/ml attenuated oxidative injury in a dose-dependent manner until H2O2 exposure. The crude ethanol extract of Eichhornia crassipes flowers was found to have a strong cytoprotective impact, raising cell viability while decreasing lipid peroxidation and LDH leakage. In the cells pretreated with ethanol extract of Eichhornia crassipes flowers, there was a further increase in catalase and a decrease in glutathione activity. These results indicate that an ethanol extract of Eichhornia crassipes flowers has potent cytoprotective properties against reactive oxygen species-induced oxidative injury.

INTRODUCTION

The pathogenesis of numerous diseases, including cancer, cardiovascular diseases, inflammation, and neurodegenerative diseases, has been linked to oxidative stress-induced cell damage (Sasaki et al., 2007). Reactive oxygen species (ROS), such as hydrogen peroxide (H2O2), superoxide, hydroxyl radicals, peroxy radicals, and singlet oxygen, are generated as byproducts of natural and abnormal metabolic processes that use molecular oxy-
H2O2. The delivery of H2O2, the major component of ROS, can be used to induce oxidative stress in vitro (Ji and Gao, 2008). The Fenton reaction catalyses H2O2’s spontaneous conversion to highly reactive hydroxyl radicals, which react instantly with any biological molecule from which it can abstract hydrogen atom, causing lipid peroxidation and DNA damage in cells (Halliwell, 2008). Antioxidants have been discovered to have both protective and therapeutic effects on cell damage caused by oxidative stress. As a result, in recent years, significant attempts have been made to classify both natural and synthetic antioxidants. Commercially available synthetic antioxidants include butylatedhydroxyanisole (BHA), butylatedhydroxytoluene (BHT), and tert-butylhydroquinone (TBHQ), but each has been linked to human side effects (Valentão et al., 2002). These compounds have been accused of having carcinogenic effects in living organisms (Sarikaya and Arica, 2005). As a result, natural antioxidants have received a lot of publicity. Natural products with antioxidant properties have sparked a lot of interest in recent years. Antioxidant supplements have seen a lot of press as alternative treatments for diseases caused by oxidative stress (Uttara et al., 2009). Many herbal remedies and medicinal plants have traditionally been used to treat complex diseases and disorders. Eichhornia crassipes is one of the herbal resources (Mart.) Solms is a member of the Pontederiaceae family. Water hyacinth, Eichhornia crassipes (Mart.) Solms, is one of the aquatic plants that has sparked the most scientific interest in the last decade. Its ornamental appeal led to its introduction into Africa, Asia, the South Pacific, North America, and Europe, where it has since become invasive (Jones et al., 2018; Kriticos and Brunel, 2016). The plant can reach a height of 3 feet. The leaves are waxy, oval to elliptical, thick, up to 6 in. (15 cm) wide, and oval to elliptical, with spongy petioles that curve inwards at the edges. On upright spikes, blue-purple flowers bloom. Each flower has six petals, the highest of which has a yellow patch (Gopal, 1987). Its enormous capacity for reproduction has become a major issue in the tropics, where high temperatures and a lack of predators have resulted in uncontrolled development. Eichhornia crassipes, on the other hand, is not only an invasive and toxic weed; it’s also a useful plant with impressive metal pollutant phytoaccumulation abilities. In its root system, Eichhornia crassipes is capable of bio-concentrating toxic metals such as Cr, Cu, Co, Ni, Zn, Pb, Cd, and As (Ali et al., 2018; Saha et al., 2017; Ug Show the full text
June from the Bhavani River in Tamilnadu, India. Dr. A. Balasubramanian, Executive Director and Former Siddha Research Consultant (AYUSH), ABS Herbal Gardens, Salem, taxonomically identified, validated, and authenticated the plant content, and the authentication was held in our laboratory for future reference. The flowers were sun-dried, and the dried materials were ground into a coarse powder using a mechanical grinder. For extraction, the powder was held in an airtight jar. The image of *Eichhornia crassipes* flower is shown in Figure 1.

Figure 1: *Eichhornia crassipes* flowers

**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.

**Phytochemical screening**

The various solvent extracts obtained were subjected to preliminary phytochemical screening (Khandelwal and Kokate, 1995).

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

**Phenolic content determination**

**Principle**

This process is based on the phenolic compound reducing the mixture of heteropolysphosphotungstates-molybdates, resulting in the formation of blue chromogen. Only under simple conditions, such as those provided by a sodium carbonate solution, do phenolic compounds react with the folin-ciocalteu reagent. It has been discovered that under simple conditions, phenolic compounds dissociate to form a phenolate anion, which reduces the folin-ciocalteu reagent, a mixture of tungstates and molybdates, resulting in a blue-colored solution. The absorbance reading of a spectrophotometer can be used to determine the colour intensity of the formulated blue chromogen.

**Procedure**

Using the Folin-Ciocalteu process, the total phenolic content was calculated spectrophotometrically. It is based on phosphomolybdic and phosphotungstic acids oxidising phenolic classes (FC reagent). This method is a colorimetric oxidation/reduction method for phenolic compounds based on Slinkard and Singleton (1977) and Singleton and Rossi (1965) early work. A blue coloured compound created by metal oxidation has a strong light absorption spectrum with a maximum at 764 nm. The amount of light absorbed is proportional to the amount of phenols present. A diluted sample of 20 litres was applied to 100 litres of Folin–Ciocalteu reagent. After 8 minutes, 300 litres of 25% saturated sodium carbonate solution were applied. At 764 nm, the absorbance was calculated. Gallic acid solutions ranging from 10 to 1000 g/ml were used to create the calibration curve, and the results are expressed as gallic acid equivalents (GAE).

**Determination of total tannin content**

**Principle**

In an alkaline medium, tannin and tannin-like compounds reduce phosphotungstic molybdic acid, resulting in a highly coloured blue solution. The intensity of which is proportional to the amount of tannin as measured at 775nm against normal tannic acid.

**Procedure**

The total tannin content was calculated using Polsheltiwar et al. updated’s process (2007). Different concentrations of extract (0.1 ml) were combined with 0.5 ml Folin-Denis reagent, 1 ml sodium carbonate (0.5 percent w/v) solution, and distilled water (up to 5 ml). Within 30 minutes of the reaction against the blank, the absorbance was estimated at 755 nm. The total tannin in the extract was calculated as the tannic acid equivalent (g TAE/g extract).

**Complete flavonoids were measured**

**Principle**

The formation of the flavonoid aluminium complex, which has a maximum absorption at 415nm, is the basis for this process. The method for determin-
DPPH is a purple colour dye with a maximum absorption wavelength of 517 nm. When it reacts with hydrogen donor, it converts to 2, 2-diphenyl-1-picryl hydrazine, which results in a decrease in absorbance. Antioxidants and therefore radical scavengers are substances that are capable of performing the reaction.

**Procedure**

Blois’ approach was used to assess free radical scavenging behaviour spectrophotometrically (Blois, 1958). This approach is focused on determining the antioxidants’ ability to reduce the DPPH radical. In a nutshell, 100 l of different concentrations of leaf extract in methanol (1.95 g/ml to 1000 g/ml) were applied to 10 ml of a DPPH methanol solution (1.01.10-2 M). After a vigorous shake, the mixture was allowed to sit at room temperature for 30 minutes in the dark. At 517 nm, the absorbance was calculated. 100 l of methanol and 10 ml of DPPH solution make up the power mixture. The DPPH radical scavenging operation was measured as an inhibition percentage using the following equation:

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

The absorbance of the control reaction (which contains all reagents except the test compound) is AB, and the absorbance of the test compound is AS. The reference norm was ascorbic acid. The experiments were performed three times. From the graph of inhibition percentage plotted against extract concentration, the extract concentration providing 50% inhibition (IC50) was determined.

**Lipid peroxidation inhibitory activity**

**Principle**

The most commonly used tool for determining lipid peroxidation is TBARS (Thiobarbituric acid reactive substance). This approach is focused on the ability of malondialdehyde, one of the secondary products of lipid peroxidation, to react with thiobarbituric acid, as the name suggests (TBA). This method works by forming an MDA-(TBA)2 complex by reacting MDA with thiobarbituric acid in an acidic environment at a higher temperature, which can be measured spectrophotometrically at 532nm.

**Procedure**

The leaf extract’s lipid peroxidation inhibitory activity was calculated using the Duh and Yen (1997). To ensure proper liposome formation, egg lecithin (3 mg/ml phosphate buffer, pH 7.4) was sonicated for 10 minutes in an ultrasonic sonicator. Leaf extract (100 l) samples of various concentrations (1.95 g/ml to 1000 g/ml) were applied to the liposome mixture as test samples (1 ml). To induce lipid peroxidation, ferric chloride (10 l, 400
mM) and L-ascorbic acid (10 l, 200 mM) were added. Hydrochloric acid (2 ml, 0.25 N) containing trichloroacetic acid (150 mg/ml) and thiobarbituric acid (3.75 mg/ml) was added to avoid the reaction after 1 hour of incubation at 37°C. The reaction mixture was boiled for 15 minutes, cooled, and centrifuged for 15 minutes at 1000 rpm, with the supernatant’s absorbance estimated at 532 nm. The reference norm was tocopherol. There was no extract in the test group. The scavenging effect was measured using the following equation:

\[
\text{Scavenging effect (\%) = } \left( \frac{A \text{ control} - A \text{ sample}}{A \text{ control}} \right) \times 100
\]

**Activation of nitric oxide scavenging enzymes**

The theory behind this approach is that sodium nitroprusside in aqueous solution at physiological pH produces nitric oxide, which then reacts with oxygen to create nitrite ion, which can be measured using the Griess reagent. Nitric oxide scavenger competes with oxygen, resulting in lower nitrite ion emissions. For the experiment, different concentrations of extract (1.95 g/ml to 1000 g/ml) dissolved in respective solvents were combined with sodium nitroprusside (10 mM) in phosphate buffer saline (PBS pH 7.4) and incubated at 25°C for 150 minutes. As a control, the same reaction mixture was used without the extract but with the same amount of solvent. 1.5 ml of the incubated solution was after the incubation time and diluted with 1.5 ml of Griess reagent (1 percent sulphanilamide, 2 percent phosphoric acid and 0.1 percent naphthyl ethylene diamine dihydrochloride). The chromophore developed during nitrite diazotization with sulphanilamide and subsequent coupling with naphthyl ethylene diamine had an absorbance of 546 nm. As a positive regulation, quercetin was used (Green et al., 1982). The experiments were performed three times. The nitric oxide scavenging activity is calculated as:

\[
\text{Scavenging activity} = \left( \frac{C - T}{C} \right) \times 100
\]

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

**Ferric’s antioxidant potential is reduced by ferric**

**Principle**

By reducing Fe([CN]6)3 to Fe([CN]6), the compound’s reducing potential can be determined. 2. When free Fe3+ is added to the reduced substance, an extreme Perl’s prussian blue complex, Fe4([CN]6)3, forms with a high absorbance at 700 nm. An rise in the reaction mixture’s absorbance will suggest an increase in the reducing potential due to increased complex formation. Depending on the reducing capacity of antioxidant samples, the yellow colour of the test solution transitions to different shades of green and blue in this assay.

**Procedure**

The reducing powers of extracts were calculated using the method defined by Oyaizu (1986). In 1 ml of distilled water, different concentrations of leaf extracts (1.95 g/ml to 1000 g/ml) were combined with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [K3Fe(CN)6] (2.5 mL, 1 percent ). After 20 minutes of incubation at 50°C, 2.5 ml of 10% trichloroacetic acid was applied to the mixture and centrifuged for 10 minutes at 3000 rpm. The absorbance of the solution’s supernatant layer (2.5 ml) was measured at 700 nm after it was combined with distilled water (2.5 ml) and FeCl3 (0.5 ml, 0.1 percent). Increased reaction mixture absorbance suggests increased reducing strength. The reference norm was ascorbic acid.

**The antioxidant potential of cupric ions (Cu2+) is reduced (CUPRAC assay)**

**Principle**

The basic concept of reducing cupric ions to cuprous ions (cu2+ to cu+) is used to calculate antioxidant potential in the cu2+ reduction assay. Matrices containing antioxidants are combined with cu2+ solution, and the antioxidants in the matrices reduce the Cu2+ ions to Cu+, which then reacts with the chromatic solution (neocuprine). The absorbance at a wavelength of 450 nm can be used to track the reaction with chromatic solution. It is simple to measure the antioxidant power.

**Procedure**

The cupric reducing antioxidant potential (CUPRAC) was calculated using the method of Apak et al. (2004). A test tube was filled with 1 ml CuCl2 solution (1.0x10-2 M), 1 ml ethanolicneocuprione solution (7.5x10-3 M), and 1 ml NH4CH3COO (1M, pH 7.0). The extract was applied to the initial mixture at a concentration of 500 g/ml to make the final volume 4.1 ml, and the absorbance was measured at 450 nm against a reagent blank after 1 hour. The reducing potential of cupric ions (Cu2+) was measured in trolox equivalents (g/ml).

**Eichhornia crassipes flower crude extract fractionation**

Approximately 4 g of dried ethanol extract was dissolved in 20 ml water and partitioned with benzene, chloroform, and n-butanol in that order. The extracted extracts’ percentage yield was determined. The collected extract and fractions were

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freeze dried and stored at 4°C (Magaji et al., 2007).

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

**Cell lines and cell culture**

BRL 3A rat liver cells were cultured at 37°C in RPMI-1640 medium containing 10% foetal bovine serum, 100 U/ml penicillin G, and 100 mg/ml streptomycin in a humified air incubator with 5% CO2.

**Cytoprotective activity of crude extract and fractions of *Eichhornia crassipes* flowers**

MTT assay was used to examine the cytoprotective effects of crude extract and fractions of *Eichhornia crassipes* flowers on H2O2-induced cell injury (Hu and Wang, 2009). For 16 hours, a total of 2104 cells were plated per well in 96-well plates with 200 l culture medium and exposed to different concentrations of crude extract and fractions of *Eichhornia crassipes* flowers (0.01, 0.1, 1, 10, and 100 g/ml) for 30 minutes before being exposed to 2mM H2O2 for 3 hours. Every well received 20 l of MTT solution (2 mg/ml in phosphate buffered saline (PBS)) at the time of incubation. The supernatant was discarded after 4 hours of incubation, and 200 l of dimethyl sulfoxide was applied to each well to finish the reaction. An enzyme-linked immunoosorbent assay (ELISA) plate reader was used to test the absorbance at 550 nm. Cell viability was calculated as a percentage of untreated control cells.

**MDA assay**

7105 cells were plated per well in 6-well plates with 2ml culture medium for 18 hours, then exposed for 30 minutes to crude extract and fractions of *Eichhornia crassipes* flowers (0.01, 0.1, 1, 10, and 100 g/ml) before being exposed to 2mM H2O2 for 3 hours. Two volumes of 2-thiobarbituric acid reagent (0.375 percent 2-thiobarbituric acid, 15% trichloroacetic acid, and 0.1mM EDTA) were applied to the cell samples and boiled for 40 minutes at 100°C. After cooling and centrifugation at 3000g for 10 minutes, the absorbance of each supernatant was measured at 532 nm. The findings were expressed as a percentage of absorbance compared to that of the control group (100 percent). The higher the defence against induced lipid peroxidation, the lower the absorbance percentage (Sakanaka and Ishihara, 2008).

**LDH assay**

7105 cells were plated in 6-well plates with 2ml culture medium for 18 hours before being exposed to crude extract and fractions of *Eichhornia crassipes* flowers (0.01, 0.1, 1, 10, and 100 g/ml) for 30 minutes before being exposed to 2mM H2O2 for 3 hours. For 5 minutes, one milligramme of NADH/ml in 0.75mM sodium pyruvate was held at 37°C. 10l of cell culture medium was mixed with 100l of NADH/ml in a 0.75mM sodium pyruvate solution and held at 37°C for 30 minutes. The colour reagent (0.2 mg/ml 2,4-dinitrophenylhydrazine in 1M hydrochloric acid, 100l) was applied to each tube and incubated at room temperature for 20 minutes. The reaction was stopped by adding 1 ml of 0.4 M NaOH. At 450 nm, the absorbance was measured in a microplate reader. According to the equation, LDH leakage was calculated as a percentage of total LDH activity (LDH in the medium + LDH in the cell).

Percentage LDH released = LDH activity in the medium/total LDH activity × 100 (Hong and Liu, 2004).

**Catalase assay**

For 18 hours, 7105 cells were plated per well in 6 well plates with 2 ml culture medium and exposed to different concentrations of crude extract and fractions of *Eichhornia crassipes* flowers (0.01, 0.1, 1, 10, and 100 g/ml) for 30 minutes before being exposed to 2mM H2O2. The cells were homogenised at 4°C in 200 M of 0.05 M phosphate buffer, pH 7.0, containing 1mM EDTA, 0.5mM DTT, and protease and phosphatase inhibitor cocktails. The homogenates were centrifuged for 15 minutes at 14,000 rpm at 4°C. The enzyme activities in the soluble supernatants were assessed. Catalase activity is measured using a spectrophotometer set to 240 nm and expressed as a percentage of the untreated control (Yoo et al., 2008).

**GSH assay**

7105 cells were plated per well in 6-well plates with 2ml culture medium for 18 hours and then exposed to crude extract and fractions of *Eichhornia crassipes* flowers at different concentrations for 30 minutes before being exposed to 2mM H2O2 for 3 hours. In 0.5 ml of PBS with 0.1 percent Triton X -100, the cells were washed and harvested. After 10 minutes of incubation, the mixture was centrifuged (3000 rpm, 10 min, 4°C) and 0.3 ml of the supernatant was combined with 1.0 ml of Tris-base 0.8M, EDTA 0.02M buffer, pH 8.9. The content of reduced GSH in the cells was calculated at 412 nm after the addition of 0.1 ml of DTNB 0.01M in methanol and expressed as a percentage of the untreated control (Dvořák et al., 2003).

**Analytical statistics**

All experiments were performed in triplicate (n=3) and the results are expressed as mean standard deviation. The statistical significance of the in-vitro cytoprotective activity results was determined...
using a one-way ANOVA followed by Dunnett’s test (Graphpad Software Inc, La Jolla, CA. Trial version). The statistical significance criterion was set at P 0.05.

RESULTS AND DISCUSSION

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

Phytochemical research in its early stages
Preliminary qualitative investigation performed in the ethanol extract of *Eichhornia crassipes* flowers revealed the presence of major phytoconstituents alkaloids, phenolic compounds, tannins, flavonoids, sterols, terpenoids, glycosides, carbohydrates, proteins and amino acids (Table 1).

Estimation of bioactive substances in a quantitative manner
In EEEC, the total phenolic content was found to be 237.60 ± 2.36 g GAE equivalents per mg dry extract. The total tannin content of EEEC was determined to be 365.35 ± 2.85 g TAE equivalent per mg dry extract. In EEEC, the total flavonoid content was found to be 247.60 ± 2.45 g of quercetin equivalent per mg of extract (Table 2).

Total antioxidant capacity
The phosphomolybdenum method was used to assess the overall antioxidant activity of EEEC flowers, and the findings were expressed as ascorbic acid equivalents. With an antioxidant potential of 441 ± 1.32 g ascorbic acid equivalents per mg of dry extract, the ethanol extract showed potent antioxidant activity (Table 2).

Radical scavenging action of DPPH
Figure 2 depicts the DPPH radical scavenging activity of EEEC flowers. The ethanol extract inhibited DPPH activity in a dose-dependent manner, with a 50% inhibition (IC50) of 79 ± 0.20 g/ml, which was comparable to the IC50 value of 15.52 ± 0.18 g/ml for the reference norm ascorbic acid.

Inhibitory action against lipid peroxidation
Figure 3 depicts the lipid peroxidation inhibitory behaviour of EEEC flowers. EEEC flowers were found to have a minor inhibitory effect on ultrasound-induced lipid peroxidation in egg lecithin liposomes. The IC50 value of EEEC was found to be above 1000 μg/ml, whereas standard drug to copherol showed an IC50 value of 2.13 ± 0.11 μg/ml.

Activation of nitric oxide scavenging enzymes
Figure 4 depicts the EEEC flowers’ nitric oxide scavenging action. In vitro, the EEEC flowers displayed nitric oxide scavenging behaviour by reducing the amount of nitrite generated by sodium nitroprusside decomposition. The IC50 value of EEEC was found to be 389.24 ± 2.24 g/ml, while the IC50 value of the regular compound quercetin was 12.0 ± 0.16 g/ml.

Assay for ferric reducing strength
Figure 5 depicts the ferric reducing strength of EEEC flowers. It was discovered that the extracts’ reducing capacity increased with concentration.

CUPRAC assay
Cu²⁺ ion reducing capacity of EEEC flowers is shown in Table 3. EEEC flowers showed CUPRAC reducing capacity of 159.68 ± 2.50 μg of Trolox/mg of extract.
Table 1: Phytochemical screening of ethanol extract of Eichhornia crassipes (EEEC) flowers

<table>
<thead>
<tr>
<th>Phytochemicals</th>
<th>Test</th>
<th>EECC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>a. Mayer’s test</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>b. Wagner’s test</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>c. Dragendorff’s test</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>d. Hager’s test</td>
<td>+</td>
</tr>
<tr>
<td>Phytosterols and Triterpenoids</td>
<td>a. Leibermann’s test</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>b. Leiberman-Burchard test</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>c. Salkowski test</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>a. Alkaline reagent test</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>b. Shinoda test</td>
<td>+</td>
</tr>
<tr>
<td>Saponins</td>
<td>a. Froth Test</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>b. Foam test</td>
<td>-</td>
</tr>
<tr>
<td>Proteins and aminoacids</td>
<td>a. Millon’s test</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>b. Ninhydrin test</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>c. Biuret test</td>
<td>+</td>
</tr>
<tr>
<td>Fixed oils and fats</td>
<td>a. Oily spot test</td>
<td>-</td>
</tr>
<tr>
<td>Phenolics and Tannins</td>
<td>a. Ferric chloride test</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>b. Lead acetate test</td>
<td>+</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>a. Molisch’s test</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>b. Fehling’s test</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>c. Benedict’s test</td>
<td>+</td>
</tr>
<tr>
<td>Glycosides</td>
<td>a. Modified Borntrager’s test</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>b. Legal’s test</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>c. Keller-Killiani test</td>
<td>+</td>
</tr>
</tbody>
</table>

Presence (+) Negative (-)

Table 2: In an ethanol extract of Eichhornia crassipes (EEEC) flowers, total phenolic content (TPC), total tannin content (TTC), total flavonoid content (TFC), and total antioxidant potential (TAC) were measured

<table>
<thead>
<tr>
<th>Extract</th>
<th>TPC (µg of TAE/mg of extract)</th>
<th>TTC (µg of quercetin/mg of extract)</th>
<th>TFC (µg of ascorbic acid/mg of extract)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EEEC</td>
<td>237.60 ± 2.36</td>
<td>365.35 ± 2.85</td>
<td>441.01 ± 11.32</td>
</tr>
</tbody>
</table>

Table 3: Cupric ions (Cu2+) reducing potential of ethanol extract of Eichhornia crassipes (CUPRAC assay) (EEEC)

<table>
<thead>
<tr>
<th>Extract</th>
<th>CUPRAC (µg of Trolox/mg of extract)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EEEC</td>
<td>159.68 ± 2.50</td>
</tr>
</tbody>
</table>

79 µg/ml in comparison with the IC50 value of ascorbic acid 15.52 µg/ml.

Cytoprotective activity of fractions and crude ethanol extract of Eichhornia crassipes flowers

Cell viability

The MTT assay was used to measure the oxidative injury caused by H2O2 in BRL 3A cells. The viability of BRL 3A cells was significantly reduced (p<0.001) after exposure to 2mM H2O2 for 3 hours, as shown in and Figure 6. The viability of the cells was reduced to 35.27 ± 0.58 percent of the control cells. Pre-treatment of cells with benzene fractions of Eichhornia crassipes flowers at various concentrations (0.01, 0.1, 1, 10, and 100 g/ml) resulted in viability percentages of 39.17 ± 0.60, 40.60 ± 0.92, 41.90 ± 0.92, 41.27 ± 1.73, and 44.20 ± 1.17 percent, respectively, while chloroform fractions of Eichhornia cras-
flowers at various concentrations (0.01, 0.1, 1, 10, and 100 g/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. The percentage cell viability in cells pretreated with ethanol extract of *Eichhornia crassipes* flowers at different concentrations (0.01, 0.1, 1, 10, and 100 g/ml) was 40.60 ± 0.94, 42.17 ± 1.18, 46.57 ± 1.14, 62.17 ± 1.46, and 69.17 ± 1.20, respectively. The cell viability assay revealed that at different concentrations, methanol extract was highly important (p<0.001) in protecting BRL 3A cells. When compared to other solvent extracts, the concentrations of 1 g/ml, 10 g/ml, and 100 g/ml were highly important (p<0.001) in protecting BRL 3A cells from oxidative damage, with a higher cell survival percentage.

In Figure 6, the MTT assay was used to determine cell viability. Data is expressed as a percentage of control and is expressed as mean ± standard error of the mean (S.E.M.). The data is viewed as the mean ± S.E.M. of three separate experiments and expressed as a percentage of control cells. The results vary greatly from the H2O2 control cells. ns-not significant; P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001 (one-way ANOVA with Dunnett’s test).

### Peroxidation of lipids

MDA production in cells is used as a marker of oxidative stress and an indicator of membrane lipid peroxidation. The effect of *Eichhornia crassipes* flower extract ethanol extract and various solvent fractions on MDA levels in BRL 3A cells exposed to H2O2 induced oxidative stress was investigated. Figure 7

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**Figure 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)

**Figure 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)

**Figure 5:** Ferric reducing antioxidant capacity of ethanol extract of *Eichhornia crassipes* flowers using ascorbic acid as assay references. Each value is expressed as mean ± standard deviation (n=3)

**Figure 6:** Effects of different solvent fractions and a crude ethanol extract of *Eichhornia crassipes* flowers on cell viability in BRL 3A cells injured by H2O2

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shows that BRL 3A cells exposed to 2mM H2O2 for 3 hours significantly increased (p<0.001) the absorbance percentage, indicating higher MDA levels, as compared to control grouped cells. As compared to control cells, the percentage increase in absorbance was found to be 270.4 ± 10.56%. The cells pre-treated with benzene fraction of *Eichhornia crassipes* flowers at various concentrations (0.01, 0.1, 1, 10, and 100 g/ml) showed absorbance decreases of around 269.1 ± 9.18, 248.5 ± 10.86, 226.1 ± 8.77, 220.8 ± 2.62, and 219.6 ± 11.39 percent, respectively, while the cells pre-treated with chloroform fraction of *Eichhornia crassipes* flowers at various concentrations (0.01, 0.1, 1, 10, and 100 g/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 percent, respectively.

In comparison to H2O2 control grouped cells, there was no substantial difference in absorbance percentage in cells pre-treated with lower concentrations of benzene and chloroform fractions (0.01, 0.1, and 1 g/ml). The absorbance percentage was significantly reduced after pretreatment with ethanol extract at concentrations of 1, 10, and 100 g/ml. At different concentrations, the percentage absorbance was found to be 195.7 ± 7.35 (1 g/ml) (p<0.001), 152.8 ± 3.11 (10 g/ml) (p<0.001), and 124.1 ± 3.11 (100 g/ml) (p<0.001). Pre-treatment with benzene, chloroform, and n-butanol fractions at concentrations of 10 g/ml and 100 g/ml resulted in a substantial difference in absorbance percentage (p<0.5 to p<0.01) when compared to H2O2 control cells, but the significance is relatively low when compared to H2O2 control and ethanol extract (p<0.01 to p<0.001). According to the findings, the ethanol extract provided substantial defence against lipid peroxidation as compared to the fractions by significantly lowering the percentage absorbance.

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

In Figure 7, before being exposed to 2mM H2O2 for 3 hours, cells were pretreated with extract for 30 minutes. The findings were described as the mean S.E.M. of three independent experiments and expressed as a percentage of absorbance compared to control absorbance (100 percent). The results vary greatly from the H2O2 control cells. *P 0.05; **P 0.01; ***P 0.001 (one-way ANOVA accompanied by Dunnett’s test); ns-not relevant.

**Leakage of LDH**

The impact of different solvent fractions and a crude ethanol extract of *Eichhornia crassipes* flowers on LDH leakage in BRL 3A cells exposed to H2O2 induced oxidative stress was investigated. Figure 8 shows that LDH leakage into medium was significantly increased (p<0.001) in H2O2 treated cells relative to control cells. When compared to control cells, which had a percentage release of 11.97 ± 2.68, the percentage increase was found to be 74.70 ± 0.57. In a group of cells pre-treated with benzene fraction at various concentrations and untreated H2O2 treated cells, there was no substantial reduction in LDH leakage. 71.00 ± 1.2, 74.20 ± 3.34, 72.97 ± 1.44, 68.50 ± 0.83, and 70.27 ± 1.38 percent LDH release was observed in cells pre-treated with benzene fraction of *Eichhornia crassipes* flowers at different concentrations (0.01, 0.1, 1, 10, and 100 g/ml). In comparison to H2O2 control cells, pre-treatment with chloroform fraction of *Eichhornia crassipes* flowers at different concentrations (0.01, 0.1, 1, 10, and 100 g/ml) resulted in percentage LDH release of 74.13 ± 5.35, 72.63 ± 3.32, 62.23 ± 4.35, 60.2 ± 5.11, and 47.8 ± 1.23 percent. The percentage LDH release in cells pretreated with the n-butanol fraction of *Eichhornia crassipes* flowers at different concentrations (0.01, 0.1, 1, 10, and 100 g/ml) was 78.20 ± 3.48, 72.82 ± 3.21, 62.24 ± 2.45, 59.45 ± 1.74, and 55.32 ± 1.26, respectively. Pretreatment of BRL 3A cells with ethanol extract at concentrations of 0.01, 0.1, 1, 10, and 100 g/ml resulted in percentage LDH release of 74.57 ± 3.51, 72.70 ± 3.05, 60.40 ± 3.00, 44.4 ± 0.15, and 35.77 ± 1.56 percent, respectively. In comparison to H2O2 con-
control cells, BRL 3A pretreated with ethanol extract at concentrations of 10 and 100 g/ml significantly decreased (p<0.001) LDH leakage into medium. Pretreatment with chloroform and n-butanol fractions at concentrations of 1 g/ml, 10 g/ml, and 100 g/ml resulted in a substantial difference (p<0.5) in LDH release percentage when compared to H2O2 control cells, but the significance is poor when compared to ethanol extract and H2O2 control (p<0.001).

Figure 8: Effects of different solvent fractions and a crude ethanol extract of Eichhornia crassipes flowers on LDH release in BRL 3A cells injured by H2O2

H2O2 control LDH release: 74.70 ± 0.57 %

In Figure 8, Before being exposed to 2mM H2O2 for 3 hours, cells were pretreated with extract for 30 minutes. The data was viewed as the mean S.E.M. of three independent experiments and expressed as a percentage of total LDH activity in control cells. *P 0.05; **P 0.01; ***P 0.001 (one-way ANOVA followed by Dunnett’s test); ns-non significant; *P 0.05; **P 0.01; ***P 0.001 (one-way ANOVA followed by Dunnett’s test).

Catalase activity

The antioxidant enzymes in BRL 3A cells were tested in response to H2O2 mediated oxidative injury using various solvent fractions and crude ethanol extract of Eichhornia crassipes flowers. Catalase activity in BRL 3A cells is shown in Figure 9. After 3 hours of exposure to 2mM H2O2, the activity of intracellular catalase decreased significantly (p<0.001) to 40.17 ± 2.81 percent of the control value. There was no substantial increase in catalase activity in cells that had been pre-treated with benzene fraction. At concentrations of 0.01, 0.1, 1, 10, and 100 g/ml, the percentage catalase activity was 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 percent (ns), respectively. The cells pre-treated with chloroform fraction at concentrations of 0.01, 0.1, 1, 10, and 100 g/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 percent (p<0.05), respectively. The catalase activity of BRL 3A cells pre-treated with n-butanol fraction at concentrations of 0.01, 0.1, 1, 10, and 100 g/ml was 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 percent (p<0.05), respectively. Catalase activity was increased by 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) percent in cells pre-treated with ethanol extract at concentrations of 0.01, 0.1, 1, 10, and 100 g/ml, respectively. In comparison to other solvent fractions, ethanol extract at concentrations of 10 g/ml and 100 g/ml was found to be highly important (p<0.01 to p<0.001) in protecting BRL 3A cells against H2O2 oxidative injury by significantly restoring catalase function.

Figure 9: The effect of various solvent fractions and a crude ethanol extract of Eichhornia crassipes flowers on catalase activity in BRL 3A cells injured by H2O2

H2O2 control catalase activity: 40.17 ± 2.81 %

In Figure 9, Catalase activity was measured in BRL 3A cellular lysate preparation after cells were pretreated with extract for 30 minutes before being exposed to 2mM H2O2 for 3 hours. The data is viewed as the mean S.E.M. of three separate experiments and expressed as a percentage of control cells. The results vary greatly from the H2O2 control cells. *P 0.05; **P 0.01; ***P 0.001 (one-way ANOVA accompanied by Dunnett’s test); ns-not relevant.

Activity of GSH

The effect of different solvent fractions and a crude ethanol extract of Eichhornia crassipes flowers on GSH levels in BRL 3A cells exposed to H2O2 medi-
ated oxidative injury was investigated. The glutathione levels in BRL 3A cells are shown in Figure 10. The activity of intracellular glutathione decreased significantly (p<0.001) after BRL 3A cells were exposed to 2mM H2O2 for 3 hours, falling to 44.87 ± 1.46 percent of the control value. In BRL 3A cells pre-treated with benzene fraction, there was no substantial difference in glutathione levels. In comparison to H2O2 control cells, the percentage GSH activity cells pre-treated with benzene fraction at concentrations of 0.01, 0.1, 1, 10, and 100 g/ml were 44.97 ± 1.82 (ns), 49.0 ± 3.72 (ns), 51.97 ± 2.11 (ns), 48.03 ± 1.33 (ns), and 47.23 ± 2.21 percent (ns). Glutathione content was 43.67 ± 2.02 (ns), 41.57 ± 2.77 (ns), 44.83 ± 2.03 (ns), 56.37 ± 1.81 (p<0.05), and 59.57 ± 1.06 percent (p<0.05) after pretreatment of BRL 3A cells with chloroform fraction 0.01, 0.1, 1, 10, and 100 g/ml, respectively. In comparison to H2O2 control cells, glutathione levels in cells pre-treated with n-butanol fraction 0.01, 0.1, 1, 10, and 100 g/ml showed a percentage GSH activity of 48.45 ± 1.24 percent (ns), 44.96 ± 1.12 percent (ns), 48.52 ± 2.45 percent (ns), 49.25 ± 1.02 percent (ns), and 69.12 ± 2.28 percent (p<0.01). In comparison to H2O2 control cells, cells pre-treated with ethanol extract at concentrations of 0.01, 0.1, 1, 10, and 100 g/ml increased percentage GSH activity by 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) percent. In comparison to other solvent fractions, ethanol extract at concentrations of 1 g/ml, 10 g/ml, and 100 g/ml was found to be highly important (p<0.01 to p<0.001) in protecting BRL 3A cells against H2O2 oxidative injury by significantly restoring catalase function.

**Figure 10**: Effects of different solvent fractions and a crude ethanol extract of *Eichhornia crassipes* flowers on glutathione content in BRL 3A cells injured by H2O2

**H2O2 control GSH activity: 44.87 ± 1.46 %**

In Figure 10, the content of reduced GSH in the cells was determined using spectrophotometry after cells were pretreated with extract for 30 minutes before being exposed to 2mM H2O2 for 3 hours. The data is viewed as the mean S.E.M. of three separate experiments and expressed as a percentage of control cells. The results vary greatly from the H2O2 control cells. *P < 0.05; **P < 0.01; ***P < 0.001 (one-way ANOVA accompanied by Dunnett’s test); ns-not relevant.

The tissue damage caused by oxidative stress has been linked to a variety of diseases, including cancer, diabetes, and neurodegenerative diseases (Guyton and Kensler, 1993; West, 2000; Gorman et al., 1996). Various oxidative stress inducers, such as hydrogen peroxide, superoxide anion, and hydroxyl radical, destroy biological molecules and cause cytotoxicity and apoptosis in a variety of cell types (Gardner et al., 1997; Fiers et al., 1999). Antioxidants are essential in the health-care system because they protect cells from oxidative harm. Antioxidant supplements have received a lot of attention in recent years as possible preventative for diseases caused by oxidative harm. Many synthetic antioxidants, such as butylated hydroxyl anisole (BHA) and butylated hydroxyl toluene (BHT), are extremely efficient and are used in industrial manufacturing, but they pose a health risk and should be substituted with natural antioxidants (Anagnostopoulou et al., 2006). As a result, the quest for potential antioxidants in natural products and herbal preparations for medicinal applications is of considerable interest to the medical community. Flowers of *Eichhorniacrassipes* were tested for their ability to protect BRL 3A cells from oxidative stress caused by H2O2 (in-vitro rat liver cell line). The ethanol extract of *Eichhorniacrassipes* flowers contained phenolics, flavonoids, and tannins, according to preliminary phytochemical analysis and quantification of bioactive components. In vitro assays were used to test the extract’s free radical scavenging and antioxidant function. Free radical scavenging and anti-oxidant activity is dose dependent in the extract. The extract was fractionated using solvents of varying degrees of polarity. In BRA 3A cells, the ethanol extract and its fractions were tested in vitro for cytoprotective activity against H2O2-induced oxidative damage.

H2O2 is a well-known genotoxic agent that can cause oxidative damage to DNA strands and base modifications (Halliwell and Aruoma, 1991). It’s one of the most common ROS formed during the redox process (Rhee, 1999), and it can come from almost any source of oxidative stress (Li et al., 2003). As a result, the cytoprotective function of *Eichhornia crassipes* flower extract and its fraction was tested using H2O2 induced oxidative injury as a model. The MTT assay was used to measure the cytotoxic injury caused by H2O2 in BRA 3A cells. MTT can be cleaved by active mitochondria in living cells to cre-
ate formazan, the volume of which is proportional to the number of living cells. In cells pre-treated with ethanol extract *Eichhornia crassipes* flowers, a dose-dependent increase in cell viability was observed. The percentage increase in cell viability in the group treated with ethanol extract was highly important as compared to the control group, and ethanol extract showed a dose-dependent increase in cell viability when compared to other solvent fractions. The LDH leakage assay was used to further explore the protective effect of extracts. LDH is a cytosolic enzyme that is used to determine the viability of cells and the integrity of their membranes (Issa et al., 2004). LDH is rapidly released into the cell culture medium when the plasma membrane is damaged. An rise in LDH activity in the culture medium is caused by an increase in the number of dead cells or damage to the plasma membrane (Hong and Liu, 2004). In contrast to the H2O2 regulation, ethanol extract *Eichhornia crassipes* flowers significantly reduced LDH leakage from BRA 3A cells into the culture medium in the current study. The percentage decrease in LDH release with ethanol extract was dose-dependent, and ethanol extract *Eichhornia crassipes* flowers effectively decreased LDH leakage from BRA 3A cells into cell culture medium as compared to other solvent fractions.

In biological systems, a fenton-like reaction between H2O2 and transition metal ions such as copper and iron produces hydroxyl radicals (Song et al., 2006). Proteins, membrane lipids, and DNA are among the molecules that the produced hydroxyl radical reacts with. Furthermore, hydroxyl radicals induce lipid oxidation, resulting in the formation of end products such as MDA and unsaturated aldehydes, which can react with DNA to form a variety of mutagenic adducts (Termini, 2000). Since MDA is a marker of oxidative stress and an indicator of membrane lipid peroxidation, the MDA assay was used to quantify the level of lipid peroxidation in BRL 3A cells exposed to H2O2 with or without ethanol extract *Eichhornia crassipes* flowers and fractions, and the results were compared to respective controls. In contrast to the H2O2 control, the ethanol extract *Eichhornia crassipes* flowers significantly reduced the percentage of lipid peroxidation, according to the results of the MDA assay. The percentage reduction in lipid peroxidation in the ethanol extract-treated community was dose dependent. BRA 3A cells pre-treated with ethanol extract *Eichhornia crassipes* flowers display lower levels of lipid peroxidation as compared to other solvent fractions.

In cells pre-treated with ethanol extract of Eichhorniacrassipes flowers, increased GSH levels and catalase activity were observed. GSH is involved in xenobiotic metabolism and directly quenches ROS. As cells are exposed to xenobiotics, more GSH is used for conjugation, leaving less usable for antioxidant functions (Meister, 1994; Anderson, 1996). As the amount of GSH in the cells is decreased, oxidative stress rises, ultimately leading to cell death (Merad-Boudia et al., 1998; Ibi et al., 1998). GSH levels in BRL 3A cells exposed to H2O2 were found to be lower in this study. The levels of GSH were substantially increased after pretreatment with an ethanol extract of *Eichhornia crassipes* flowers. When compared to other solvent extracts, ethanol extract showed a significant increase in GSH levels. Furthermore, ethanol extract increased catalase activity in BRL 3A cells, which is involved in the decomposition of H2O2 to O2 and H2O (Paes et al., 2001). These findings indicate that an ethanol extract of *Eichhornia crassipes* flowers can effectively protect cells from oxidative stress.

**CONCLUSION**

To summarise, our findings show that an ethanol extract of *Eichhornia crassipes* flowers can protect BRL 3A cells from hydrogen peroxide by stabilising and increasing antioxidant defences that are disrupted during induced oxidative stress. The presence of phenolics, tannins, and flavonoids in the ethanol extract of *Eichhornia crassipes* flowers may explain the possible cytoprotection.

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**Conflict of Interest**

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