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Zebrafish-Based Evaluation of *Cymbopogon flexuosus* Extract in Acrylamide-Induced Neurodegeneration

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ABSTRACT

The primary aim of this study was to investigate the neuroprotective potential of leaves extract from *Cymbopogon flexuosus* against neurotoxicity in an acrylamide (ACR)-induced zebrafish model. Following ACR exposure, zebrafish exhibited neurotoxic effects characterized by a threefold reduction in glutathione reductase activity, a 3.4-fold increase in lipid peroxidation, a 1.7-fold elevation in nitrite levels, a 3.9-fold increase in acetylcholinesterase activity, and a 1.4-fold decrease in total protein levels compared to wild-type controls. Administration of the reference drug Donepezil demonstrated significant neuroprotective effects in ACR-treated zebrafish, with glutathione reductase activity increasing 2.7-fold, lipid peroxidation decreasing 3.4-fold, nitrite levels decreasing 1.5-fold, acetylcholinesterase activity decreasing 3.2-fold, and total protein levels increasing 1.4-fold, approaching values observed in wild-type fish. Treatment with aqueous leaf extracts of *Cymbopogon flexuosus* following ACR exposure restored glutathione reductase, lipid peroxidation, nitrite, total protein, and acetylcholinesterase activities to levels comparable to the control group. Among the tested dosages, the 440mg/ L extract exhibited superior neuroprotective efficacy in ACR-induced zebrafish. Specifically, treatment with 440mg/ L extract resulted in a 2.7-fold increase in glutathione reductase activity, a 3.1-fold reduction in lipid peroxidation, a 1.4-fold decrease in nitrite levels, a 2.7-fold decrease in acetylcholinesterase activity, and a 1.3-fold increase in total protein levels. These outcomes were comparable to those observed in wild-type zebrafish.

INTRODUCTION

A wide range of oxidative stress-related diseases in both humans and animals have been linked to exposure to various environmental chemicals, many of which are

byproducts of industrial and anthropogenic activities **(Grajek *et al.*, 2015)**. Acrylamide (ACR), a water-soluble, white crystalline alkene, is one such compound of concern. Human exposure to acrylamide occurs primarily through industrial contact, contaminated drinking water, and the consumption of thermally processed starchy foods such as potato chips and cereal products. At temperatures exceeding 120°C, acrylamide is formed via the Maillard reaction, where free amino groups react with carbonyl-containing compounds **(Erkekoglu *et al.*, 2014; Albalawi *et al.*, 2018)**. Extensive toxicological studies in rodents and other animal models have demonstrated that monomeric acrylamide exhibits carcinogenic, genotoxic, neurotoxic, and developmental toxic properties. Mechanistically, acrylamide interacts with sulfhydryl groups on cysteine residues of proteins involved in membrane fusion, thereby disrupting neurotransmitter release. Clinically, acrylamide poisoning may manifest as central nervous system impairment, with symptoms such as lethargy, ataxia, skeletal muscle weakness, and peripheral numbness **(Kim *et al.*, 2015; Singh *et al.*, 2020)**. In recent years, there has been increasing interest in the use of natural products, particularly plant-derived compounds, for neuroprotection. Many medicinal plants possess bioactive phytochemicals with antioxidant, anti-inflammatory, and anti-apoptotic properties that may mitigate neuronal damage. Among these, *Cymbopogon flexuosus* (lemongrass) has been traditionally used in folk medicine and is known to possess a wide range of pharmacological activities. However, its neuroprotective potential remains underexplored. Investigating the *in vitro* neuroprotective and antioxidant effects of *Cymbopogon flexuosus* could offer new insights into the development of plant-based therapeutics for neurodegenerative disorders **(Dubey *et al.*, 2025a, b)**. The purpose of this research was to analyze the changes in several biomarkers addressing the adult zebrafish brain to learn more about acrylamide-induced neurotoxicity and the neuroprotective effect revealed by the aqueous extracts of *Cymbopogon flexuosus*. Due to the similarities between the zebrafish and human neurotransmitter systems and genomes; this model is an effective, efficient, and ethically sound approach for investigating neurotoxicity.

MATERIALS AND METHODS

Chemicals

In this study, acrylamide, donepezil, disodium hydrogen phosphate, potassium dihydrogen ortho- phosphate, H_20_2, SDS, ascorbic acid, acetic acid, thiobarbituric acid, pyridine, EDTA, GSSG, NADPH, DTNB, acetylthiocholine iodide, were used.

Plant material collection and identification

Cymbopogon flexuosus leaves were employed in this study. They were collected from the CSIR-Central Institute of Medicinal & Aromatic Plants in Lucknow. The authors gathered and verified the samples at Maharana Pratap College of Pharmacy's Plant Systematics Laboratory, Department of Pharmacology, Kanpur, Uttar Pradesh. They placed a voucher specimen in the Plant Systematics Laboratory. (No. CIMAP-HA-1000420251).

Extraction

After being allowed to air dry, *Cymbopogon flexus* leaves were ground into a powder (20 mesh size). 52 grams of powdered seeds were then extracted using Soxhlet equipment and 500 milliliters of ethanol and water as solvents. After that, evaporation in a water bath was used to concentrate the filtrate. Weighing the drained extracts, we used the computation to determine the yield of the soluble constituents **(Dubey *et al.*, 2023a)**.

$$Yield\ \% = \frac{\text{Weight of dry extract}}{\text{Weight of dry seeds powder}} \times 100$$

Fig. 1. Aqueous extract of *Cymbopogon flexuosus*

***In vitro* antioxidant activity**

DPPH scavenging assay

In a 96-well plate, 0.2ml of 0.1mM DPPH solution (SRL Chem, Cat no. SR-29128) in methanol (SD fine, Cat no. 10930lC250) was mixed with 10µl of a separate stock of the test substance (as stated in the excel sheet). The standard was ascorbic acid (SRL, Cat no. 23006). The reaction was set up in quadruplicate, and duplicate blanks were made using 10µl of standard/sample at various concentrations and 0.2ml of DPPH. Wells without reagent (DPPH) were regarded as red blank, and wells without treatment were regarded as controlled. The plate was left in the dark for half an hour. Using a micro plate reader (iMark, BioRad), the de-colorization was measured at 517nm at the conclusion of the incubation. The control was a reaction mixture with 20µl of deionized water. In comparison to the control, the scavenging activity was displayed as a percentage of inhibition. Software Graph Pad Prism 6 was used to determine the IC_{50}. A graph was created with the X (sample concentration) and Y (inhibition percentage relative to control) axes.

Calculations:

% RSA= (ABS control-Abs Sample)/Abs control) x100

RSA=Radical Scavenging Activity
Abs Control=Absorbance of control
Abs Sample= Absorbance of Sample

GC-MS analysis

The aqueous extract was analyzed using GS-MS. The GC system (PerkinElmer Clarus 600) accepts the Rtx-5MS capillary column with ease. Using 99.99 percent pure helium, a steady flow rate of 1.0 mL/min was attained and sustained. With an ionization energy of 70 eV (electron volt), a scan time of 0.2 seconds, a fragment spanning from 40 to 650 m/z, an injection quantity of 1 L (split ratio 10:1), and a temperature of 260 °C, the GC-MS spectral lines were found utilizing an ionization energy approach. In contrast, the column oven ran for three minutes at 500 degrees Celsius. To get to 3000°C, the temperature was increased by 100°C every minute. Retention times, peak areas, peak heights, and spectral line patterns of the plant samples were compared to those of recognized chemicals in databases from the Wiley-8 library and the National Institute of Standards and Technology (NIST) library in order to identify the components **(Dubey *et al.*, 2023a, b)**.

Animals

Adult male and female wild zebrafish (*Danio rerio*), aged 60–90 days, were collected from local ponds. The specimens were of similar size and weight, averaging 3.5 ± 0.5 cm in length and 0.4 ± 0.1 g in weight, respectively. Upon collection, fish were quarantined in 10-liter tanks and fed commercially available feed once every 24 hours. The fish were acclimated to laboratory conditions for one week while maintained on a regular feeding regimen to allow adaptation to the new environment.

Experimental phase

Fresh acrylamide exposure solution (0.75mM) was prepared in distilled water. The fish were divided into five g (n=5) different groups as:

Group (I)- Control group (Fed by water)

Group (II)- ACR (induced by acrylamide)

Standard Group (III)- Standard acrylamide (0.75mM) + Donepezil 10Mg/ liter

Test Group (IV)- Acrylamide (0.75mM) +Aqueous leaves extract of *Cymbopogon flexuosus* 200Mg/ liter

Test Group (V)- Acrylamide (0.75mM) +Aqueous leaves extract of *Cymbopogon flexuosus*440Mg/ liter

Five-liter tanks were labeled according to the five experimental groups previously described. Fish in the control group tank were fed daily following the same regimen as the main tank. Four separate one-liter tanks, designated as ACR exposure tanks, were prepared by dissolving 0.7 mM acrylamide (ACR) in one liter of water. Fish from the corresponding five-liter tanks of the four experimental groups (excluding the control group) were transferred into their respective ACR exposure tanks and exposed to ACR for 30 minutes. Subsequently, the fish were returned to their original five-liter tanks and fed once daily. This exposure protocol was repeated for three consecutive days. For treatment of ACR-induced fish, three additional one-liter tanks were prepared and labeled according to the treatment groups. Appropriate concentrations of the treatment extracts and the standard drug were dissolved in one liter of water and placed in these tanks. Fish from the five-liter tanks of the three treatment groups (excluding the control and ACR-only groups) were transferred to their respective treatment tanks and exposed to the treatment solutions for 60 minutes. Following treatment, the fish were returned to their original five-liter tanks and fed once daily. This treatment procedure was also conducted for three consecutive days **(Dubey *et al.*, 2023a, b)**.

Biochemical estimations

Zebrafish were euthanized by hypothermic shock using ice water maintained at 2–4°C. Brain tissues were dissected using a mortar and pestle and homogenized in 5mL of 0.1M phosphate buffer (pH 7.2). The homogenate was centrifuged at 5000 rpm for 15 minutes at 4°C, and the resulting supernatant was used for the quantification of glutathione reductase, acetylcholinesterase, total protein, lipid peroxidation, and nitrite levels **(Prasad *et al.*, 2016; Batista *et al.*, 2018)**.

Estimation of glutathione reductase activity

Glutathione reductase activity was determined following the method described by **Stahl *et al.* (1963)**. The assay involved addition of brain homogenate to a reaction mixture containing phosphate buffer, EDTA, oxidized glutathione (GSSG), NADPH, and distilled water. The reduction of NADPH was monitored spectrophotometrically by measuring the decrease in absorbance at 340 nm at 30-second intervals over 2 minutes. Enzyme activity was expressed as micromoles per minute per milliliter ($\mu mol \cdot min^{-1} \cdot mL^{-1}$) **(McEntee *et al.*, 1993; Ensafi *et al.*, 2008)**.

Estimation of lipid peroxidation

Lipid peroxidation levels were assessed by quantifying thiobarbituric acid reactive substances (TBARS) as described by **Ohkawa *et al.* (1979)**. The absorbance of the malondialdehyde (MDA)-TBA adduct was measured at 532nm using a UV-visible spectrophotometer. TBARS were extracted into a 15:1 butanol:pyridine mixture prior to measurement **(Ellman *et al.*, 1961; Ohkawa *et al.*, 1979)**.

Acetylcholinesterase assay

Acetylcholinesterase activity was measured using the **Ellman**'s **(1960)** method. Brain homogenate was suspended in 0.1 M potassium phosphate buffer (pH 7.2), followed by addition of 3.3 mM DTNB [5,5'-dithiobis-(2-nitrobenzoic acid)]. The mixture was incubated at 25°C for 20 minutes. Subsequently, acetylcholine iodide was added, and absorbance at 412 nm was recorded every minute.

Enzyme activity was calculated based on the molar extinction coefficient of acetylcholine **(Jayanth *et al.*, 2014; Satpathy *et al.*, 2021)**.

Estimation of nitrite

Nitrite concentration in brain supernatant was determined using the Griess reagent according to **Green *et al.* (1982)**. A 100µL aliquot of sample or standard (100µg/ mL) was diluted with 400µL distilled water, followed by addition of 500µL Griess working reagent (0.1% N-(1-naphthyl) ethylenediamine dihydrochloride, 1% sulphanilamide, and 5% phosphoric acid). After incubation at room temperature for 5 minutes, absorbance was measured. Nitrite levels were quantified using a sodium nitrite standard curve and expressed as micromoles per milligram of protein **(Green *et al.*, 1982)**.

Estimation of protein

Total protein content was determined by the Biuret method **(Gornall *et al.*, 1949)**. To 0.1mL of tissue homogenate supernatant, 2.9mL of NaCl and 3mL of Biuret reagent were added and incubated at room temperature for 10 minutes. Absorbance was measured at 540nm using a UV spectrophotometer **(Gornall *et al.*, 1949)**.

Statistical analysis

Data were presented as mean ± standard deviation (SD). Statistical significance was evaluated using the student's t-test with Prism Pad 5 software. Differences were considered significant at $P< 0.05$.

RESULTS

Identification of components

Identification was done using the calculated fragments, molecular mass, and molecular structure. The NIST database and the Willy8-library, which has more than 62,000 pattern entries, were used to interpret the GC-MS spectra. Together with their molecular weights and chemical structures, the components of the test materials were determined. The percentage amounts discovered in the sample were contrasted with component spectra from the Willy8 collection and the NIST library. This is done in order to determine whether this plant species has any chemicals or a combination of them that could support its traditional and commercial therapeutic purposes. It also helps determine the most effective methods for removing harmful compounds. The test materials' constituent parts were determined (Table 1 & Fig. 2).

Table 1. Compound of CFW (*Cymbopogon flexus*-Water)

Peak	R. Time	Area	Area%	Name
1.	9.671	2289371	2.27	β-Myrcene
2.	11.669	529208	0.53	γ-terpinene
3.	11.785	420151	0.42	Linalool oxide
4.	13.413	4504197	4.47	Camphor
5.	13.474	6514918	6.47	Spirojatamol
6.	14.180	960517	0.95	2-Bornanol, 2-Methyl-
7.	15.074	3482967	3.46	2,6 Bis (1,1-dimethylethyl)-4- [(4-chloro-6-(3,5, bis (1,1-dimethylethyl)-4- hydroxy anilino)-1,3,5 triazin-2-yl) amino] phenol -
8.	15.450	7028372	6.98	2,2,7,7-Tetramethyl-Tricyclo [6.2.1.0 1,6] Undec-4-En-3-One
9.	15.536	1744090	1.73	2-Propenal, 3-(2,4,5,6,7,7a-Hexahydro-3,7-Dimethyl-1h-Inden-4-Yl)-2-Methyl-, (4s-(4alpha(E),7beta,7aalph A))-
10.	15.828	12189437	12.10	1,3a-Ethano(1H) Inden-4-Ol, Octahydro-2,2,4,7a-Tetramethyl
11.	15.983	6275373	6.23	Valerianic Acid, Methyl Ester
12.	16.220	1175032	1.17	4a,5-Dimethyl-3-(Prop-1-En-2-Yl)-1,2,3,4,4a,5,6,7-Octahydronaphthalen-1-Ol
13.	17.061	782712	0.78	Hexadecenoic Acid, Methyl Ester
14.	18.702	1113918	1.11	9,12-Octadecadienoic Acid (Z, Z)-, Methyl Ester
15.	18.764	1457587	1.45	9-Octadecenoic Acid, Methyl Ester, (E)-
16.	23.692	2195767	2.18	Oleoyl Chloride
17.	24.043	1673673	1.66	1,4-Benzenedicarboxylic Acid, Bis(2-Ethylhexyl) Ester
		100709079	100.0	

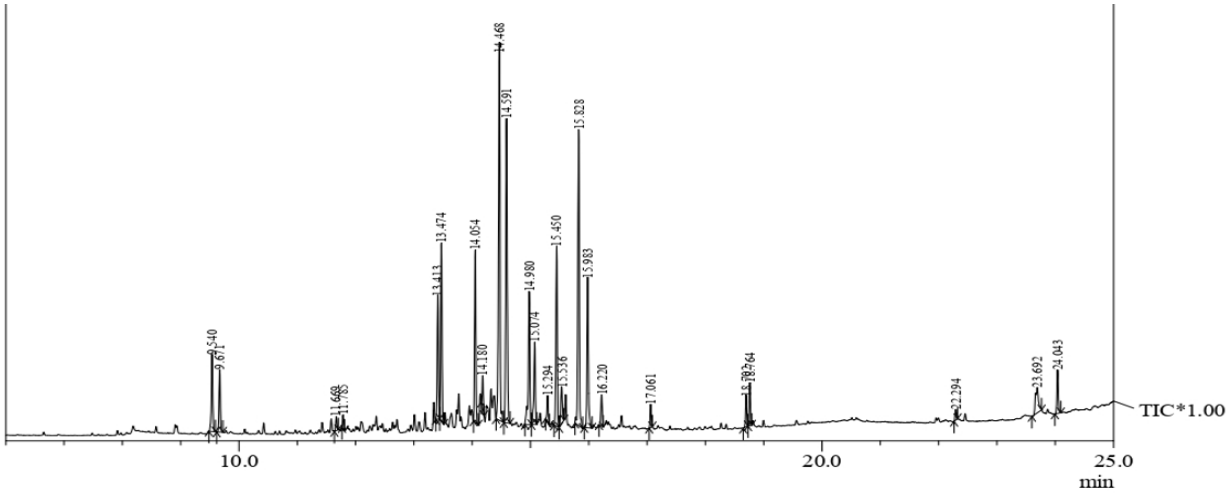

Fig. 2. Gas chromatography- mass spectrometry (GC-MS) *Cymbopogon flexuous*-water

Activities of antioxidants

Oxidative stress has been linked to age-related neurodegenerative diseases in a number of studies. Numerous studies have also examined the protective effects of antioxidants in preventing or reducing neuronal death that occurs in the pathophysiology of this disorder. Indicators of a compound's antioxidant potential also include its total antioxidant activity and ability to scavenge radicals. To determine the plant extracts' ability to act as antioxidants, activities—DPPH were evaluated. The 1, 2-diphenyl-2-picryl hydroxyl radical (DPPH) was used to examine the antioxidant activity of extracts. The results are presented in Table (2).

Table 2. DPPH scavenging assay

Sample code	**IC_{50} value (µg/ml) (Mean ± SEM)**
Ascorbic Acid	11.95 ± 0.02
CFW	575.2 ± 0.06

Different doses of the aqueous leaf extract of *Cymbopogon flexuosus* produced a significant increase in glutathione reductase activity, lipid peroxidation, and protein levels, along with a significant decrease in acetylcholinesterase activity and nitrite content in the brain homogenate of zebrafish, compared to the acrylamide-treated control group. The results are presented in Table (3).

Table 3. Effects of aqueous leaves extract of *Cymbopogon flexuosus* on biochemical parameters

Group	**Glutathione reductase e (Microg ram /Milligram m of protein)**	**MDA (nmol/ Milligram m of protein)**	**Nitrite (µmol/ milligra m of protein)**	**ACh E (Micr ogra m/ millig ram of protei n)**	**Total protein (milligram of tissue**
Group (I)- Control group	24.83 ± 0.24	0.53 ± 0.06	24.23± 1.04	0.15 ±0.05	4.45 ± 0.46
Group (II)- ACR 0.75mM	8.24 ± 0.31@	1.79 ± 0.09@	40.04± 1.04@	0.58 ± 0.07 @	3.09 ± 0.27@
Standard group (III)- Donepezil (10 mg/L)	22.15 ± 0.20** *	0.52 ± 0.04**	27.2±1.29 ***	0.18± 0.05*** *	4.21 ± 0.41***
Test group (IV)- *Cymbopogon flexuosus* (200mg/l)	21.15 ± 0.46** *	1.0 ± 0.08***	33.8±1.38 ***	0.31 ± 0.07***	3.59 ± 0.22** *

Test group (V)- *Cymbopogon flexuosus* (440mg/l)	22.15 ± 0.51***	0.56 ± 0.04**	28.3±1.64***	0.22± 0.05***	4.13 ± 0.23**

Values are expressed as mean ± SEM' n=5 at *P*< 0.001 as compared to normal control group, and ****P*< 0.001;***P*< 0.01; **P*< 0.05 as compared to ACR control group.

DISCUSSION

Oxidative stress is a hallmark of various neurodegenerative disorders, including Alzheimer's disease. One of the primary mechanisms involves the generation of free radicals through the peroxidation of polyunsaturated lipids, which subsequently produce unsaturated carbonyl compounds. These compounds not only mediate synaptotoxicity but also exert direct neurotoxic effects. Acrylamide (ACR)-induced neurotoxicity is associated with elevated levels of malondialdehyde (MDA) and a significant reduction in the activities of endogenous antioxidant enzymes such as catalase and glutathione reductase, highlighting the critical role of oxidative stress in ACR-mediated neuronal damage **(Erkekoglu *et al.*, 2014)**. In the present study, the findings support previous reports suggesting that *Cymbopogon flexuosus* possesses various pharmacological properties, including neuroprotective activity. Phytochemical analysis of the aqueous leaf extract of *C. flexuosus* revealed the presence of flavonoids, phenolics, and tannins, all of which are well-known for their potent antioxidant effects. Notably, treatment with the extract enhanced antioxidant enzyme activity in zebrafish brains, indicating a protective effect against oxidative damage. Following ACR exposure, zebrafish exhibited significant neurotoxicity, characterized by a threefold decrease in glutathione reductase levels, a 3.4-fold increase in lipid peroxidation, a 1.7-fold rise in nitrite levels, a 3.9-fold increase in acetylcholinesterase activity, and a 1.4-fold reduction in total protein content compared to the wild-type control group. Administration of Donepezil, a standard therapeutic agent, significantly ameliorated ACR-induced neurotoxicity. In Donepezil-treated zebrafish, glutathione reductase activity increased by 2.7-fold, lipid peroxidation decreased by 3.4-fold, nitrite levels declined by 1.5-fold, acetylcholinesterase activity was reduced by 3.2-fold, and total protein content increased by 1.4-fold—values comparable to those of the wild-type group. Treatment with various aqueous extracts of *C. flexuosus* after ACR exposure led to restoration of glutathione reductase levels, reduction in lipid peroxidation and nitrite concentrations, normalization of acetylcholinesterase activity, and an increase in total protein levels, all approaching the levels observed in untreated control zebrafish. Among the tested solvent extracts, the ethanolic extract demonstrated superior neuroprotective efficacy in ACR-induced zebrafish. Specifically, treatment with 440 mg/L of the aqueous extract of *C. flexuosus* resulted in a 2.7-fold increase in glutathione reductase activity, a 3.1-fold reduction in lipid peroxidation, a 1.4-fold decrease in nitrite levels, a 2.7-fold reduction in acetylcholinesterase activity, and a 1.3-fold increase in total protein levels, again comparable to wild-type controls.

CONCLUSION

The present study hypothesizes that the aqueous leaf extract of *Cymbopogon flexuosus* possesses neuroprotective properties capable of mitigating oxidative stress induced by acrylamide (ACR) toxicity. This hypothesis is strongly supported by previous findings highlighting the plant's antioxidant and free radical scavenging activities. The current results further underscore the therapeutic potential of *C. flexuosus*, suggesting its prospective application in the development of novel pharmacological agents for the management and treatment of dementia-related neurodegenerative disorders.

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