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Role of Aqueous and Ethanolic Seed Extract of *Asparagus racemosus* on Acr-Induced Neurotoxicity in Adult Zebrafish: Emergence of Neuroprotective Results

Anubhav Dubey[1], Niladry S. Ghosh[1, 2]*, Ranjit Singh[1], Chiranjib Bhattacharjee[3]

[1]Adarsh Vijendra Institute of Pharmaceutical Sciences, Shobhit University Gangoh, Saharanpur, Uttar Pradesh, India
[2]Faculty of Pharmaceutical Science, Assam down town University, Guwahati, Assam, India
[3]Professor, Bir Bikram College of Pharmacy, Khayerpur Old Agarlata, India

***Corresponding Author: niladry_chem@yahoo.co.in**

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ABSTRACT

The primary objective of this work was to examine the potential neuroprotective properties of a seed extract derived from *Asparagus racemosus* for neurotoxicity in an acrylamide-induced zebrafish model. After ACR treatment, fish went through neurotoxic effects where glutathione reductase levels decreased by 3 times; lipid peroxidation activity increased by 3.4 times; nitrite levels increased by 1.7 times; acetylcholinesterase levels increased by 3.9 times, and total protein levels decreased by 1.4 times, compared to the wild-type zebrafish. Treatment with the standard drug vinpocetine showed a significant level of neuroprotective activity in ACR-induced zebrafish, where glutathione reductase level was increased by 2.7 times; lipid peroxidation activity decreased by 3.4 times; nitrite level decreased by 1.5 times; acetylcholinesterase decreased by 3.2 times, and total protein increased by 1.4 times. The results were comparable to those of a wild type of zebrafish. Therapy using different solvent seed extracts of *Asparagus racemosus* after ACR exposure restored glutathione reductase, lipid peroxides, nitrite, protein, and acetylcholinesterase activity, which was comparable to control group levels. Among the two solvent extracts, the ethanolic extract showed much better results for neuroprotective activity in ACR-induced zebrafish. After treatment with 440mg/ l ethanolic seed extract, glutathione reductase level increased by 2.7 times; lipid peroxidation activity decreased by 3.1 times; nitrite level decreased by 1.4 times, and acetylcholinesterase decreased by 2.7 times. While, total protein increased by 1.3 times in ACR-induced zebrafish. The results were comparable to those of the wild type of zebrafish.

INTRODUCTION

Numerous oxidative stress-related illnesses are the consequence of interactions between people and animals and the numerous chemicals released into the environment as a byproduct of human and industrial activity **(Grajek *et al.*, 2015)**. One example is acrylamide, an alkene that is white and crystalline and dissolves in water. Industrial exposure, acrylamide-contaminated drinking water, and thermally processed starchy

foods like potato chips and cereal goods expose humans to acrylamide because, at high temperatures (over 120°C), acrylamide is formed when free amino groups combine with a carbonyl source **(Erkekoglu *et al.*, 2014; Albalawi *et al.*, 2018)**. Exposure to monomeric ACR causes carcinogenicity, developmental toxicity, neurotoxicity, and genotoxicity, according to extensive investigations in rats and other experimental animals. Second, acrylamide binds to sulfhydryl groups on cysteine residues of membrane fusion-related proteins, leading to dysfunctional neurotransmitter release. Lethargy, ataxia, skeletal muscle weakness, and numbness of the extremities are among the indications of central nervous system dysfunction that may be caused by ACR poisoning **(Kim *et al.*, 2015; Singh *et al.*,2020)**.

Asparagus racemosus, often known as shatavari, is a key plant in Ayurveda since it has the potential to cure or prevent hundreds of different diseases. It reigns supreme among herbs and earns the title "herb's queen." Steroidal glycosides, saponins (most notably Shatavarins I, II, III, and IV), polyphenols, flavonoids, alkaloids (racemosol), and vitamins are some of its bioactive components. Sapogenin, found in shatavari, is a precursor to numerous pharmacologically active steroids, making this plant widely used in folk and Ayurvedic medicine. Roots, stems, and leaves are the most important parts although the plant as a whole has therapeutic properties. The "Rasayanas" made from shatavari are excellent for warding off illness. Many ailments are treated with it because of the phytochemicals it contains. The phytochemicals in it are used to treat a wide range of conditions. Antispasmodic, antioxidant, anti-diabetic, anti-allergic, anti-malarial, protective, anti-neoplastic, immune response enhancing, anti-arthritic, anti-inflammatory, anti-periodic, anti-ulcerogenic action, immune modulatory, antistress, anti-diarrheal, antidepressant, infections, tuberculosis, and so on are just some of the many medicinal properties of shatavari. Medications made from Shatavari extract on the market have been shown to have beneficial effects against leprosy, abortion, infection, fever, and pain. Dyspepsia, mental disorders, cough, bronchitis, throat, and female reproductive system problems may all be alleviated by shatavari root, leaf, flower, and stem extracts **(Dubey *et al.*, 2023)**.

Vinpocetine is a synthetic ethyl ester of the alkaloid apovincamine, which is extracted by the leaves of Vinca minor, commonly known as the lesser periwinkle and plant has relation with Apocynaceae family. It is authorized by the European and British Pharmacopoeias. It was first found out, improved, and marketed, authorizing the trade name Cavinton (Chemical performs of Gedeon Richter Ltd., Budapest Hungary). Considering the establishment of vinpocetine drug in the market in 1978; this drug has been extensively used in many countries.

It has been utilized in the protection of different cerebrovascular disorders (CVD), including stroke, carotid stenosis, vertebral stenosis, thrombosis, hemorrhage, etc. Vinpocetine easily passes the blood-brain barrier. In the pharmaceutical market of America, vinpocetine drug is present as a dietary supplement. It can dilate blood vessels,

raise circulation in the blood, increase oxygen utilization, and constrain red blood cells leaving them more flexible or restrain the aggregation of platelets, as experiments on the periwinkle plant extract recorded. Researchers investigated the various pharmacological and biochemical activity of vinpocetine. It has antioxidant activity, anti-epileptic activity, cardioprotective activity, and it improves neuronal plasticity, Alzheimer's and related dementias, the aging process, analgesic anti-inflammatory activity, tinnitus, and Parkinson's disease. Importantly, no significant vinpocetine drug adverse effects have been mentioned. Studies on human individuals showed that vinpocetine drug is eventually absorbed from the upper part of the gastrointestinal tract, and its efficient metabolite apovincaminic acids are absorbed from the abdomen. It is further dissolved in gastric pH (PH1.2) rather than intestinal pH (pH 6.8). The half-life of the drug is 1-2 hours, while after 8 hours, it is perfectly excreted from the body **(Dubey *et al.*, 2020; Dubey A *et al.*, 2020)**. 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 and ethanolic seed extracts of *Asparagus racemosus*. 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

Acrylamide, vinpocetine, disodium hydrogen phosphate, potassium dihydrogen ortho-phosphate, hydrogen peroxide, SDS, acetic acid, thiobarbituric acid, n – butanol, pyridine, EDTA, GSSG, NADPH, DTNB, acetylthiocholine iodide etc.

Preparation of plant extract

The *A. racemosus* seeds were purchased from Vatika Agro Shop in Jaipur, 302020, in the Indian state of Rajasthan. A well-known botanist confirmed that the plant was real. Janta Postgraduate College, A.P.S. University, Rewa (486001), M.P. India, is where the specimen was deposited at the university's herbarium house. J/Bot/2022APS-019, a voucher specimen of the plant's seeds was removed and dried in a separate oven at 45 degrees Celsius. The mechanical grinder turned the dry seeds into a powdery substance. Solvent ethanol and water (500mL) were used to extract the powder of the seeds (52g) using a Soxhlet device. The extracted filtrate was concentrated using normal-pressure evaporation in a water bath **(Roy *et al.*,2014; Dubey *et al.*, 2023)**.

Fig. 1. Preparation and extraction of *Asparagus racemosus* seeds

Animals

Male and female wild zebrafish (Danio rerio) adults, aged 60– 90 days, were collected from local ponds. These fish were of comparable size and weight (3.5 0.5 cm and 0.4 0.1 g, respectively) for their ages. The fish samples were given commercially available feed once every 24 hours upon being in quarantine in a 10-liter tank. The fish were maintained in the lab for a week on a regular diet to get accustomed to the environment. The Hi-Tech Medical College and Hospital's Ethics Committee for Animal Research (ECR/273/Inst/OR/2013/RR-20) authorized all experimental procedures before they were carried out.

Experimental phase

Fresh acrylamide exposure solution (0.75mM) was prepared in distilled water. The fish were divided into seven (n=7) different groups as

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

Group (II)- ACR (induced by acrylamide)

Standard Group (III)- Standard acrylamide (0.75mM) + Vinpocetine 30Mg/ liter

Test Group (IV)- Acrylamide (0.75mM) +Aqueous seed extract of *Asparagus racemosus* 200Mg/ liter

Test Group (V)- Acrylamide (0.75mM) +Aqueous seed extract of *Asparagus racemosus* 440Mg/ liter

Test Group (VI)- Acrylamide (0.75mM) + Ethanolic seed extract of *Asparagus racemosus* 200Mg/ liter

Test Group (VII)- Acrylamide (0.75mM) + Ethanolic seed extract of *Asparagus racemosus* 440Mg/litre

An equal number of fish (n = 7) were taken from the main tank and kept in 7 different 5-liter tanks, labeled with the 7 above-mentioned group names. The fish in the control group tank were daily fed as was done for the main tank. Six different 1-liter tanks were taken and named as ACR exposure tanks." 0.7 mM ACR was dissolved in 1 liter of water and kept in those 6 tanks. Fish from the 5- liter tanks of the remaining 6 groups (except the control group) were transferred into their respective ACR exposure tanks. They were exposed to ACR for 30 minutes. After that, the fish were re-transferred to their respective 5-liter tanks, where they were fed once a day. The same exposure procedure was carried out for three consecutive days. Afterward, for the treatment of ACR-induced fish with two extracts of AR and the standard drug, five different 1-liter tanks were taken and named according to the treatment groups. Suitable concentrations of treatment drugs were dissolved in 1 liter of water and kept in those 5 tanks. Induced fish from the 5 lit tanks of the remaining 5 treatment groups (except the control group and ACR group) were transferred into their respective 1 lit treatment tanks. They were exposed to treatment drugs for 60 minutes. Then, the fish were re-transferred to their respective 5-liter tanks, where they were fed once a day. The same treatment procedure was carried out for three consecutive days **(Erkekoglu *al.*,2014; Singh *et al.*, 2020)**.

Figure-A Aqueous seed extract AS Figure-B Ethanol seed extract AS

Fig. 2. Zebrafish acute toxicology testing using water and ethanol extracts of *Asparagus racemosus* Linn seeds.

Biochemical estimations

All of the zebrafish were put to sleep by a hypothermic shock in ice water between two and four degrees Celsius. We used a mortar and pestle to dissect the fish brains, which we then mixed with 5ml of a 0.1M phosphate buffer with a pH of 7.2. We next centrifuged the homogenate at 5000rpm for 15 minutes at 4°C. Glutathione, acetylcholinesterase, protein, lipid peroxidase, and nitrite concentrations were all determined by analyzing the supernatant **(Prasd *et al.*, 2016; Batista *et al.*, 2018)**.

Estimation of glutathione reductase activity

The approach of **Stahl *et al*. (1963)** was used to measure glutathione reductase activity. The addition of brain homogenate was followed by spectrophotometric measurements evaluating the change in the optical density of the reaction mixture at 340nm at 30-second intervals for 2 minutes. The reaction mixture consisted of phosphate buffer, EDTA, GSSG, NADPH, and distilled water. The enzyme activity was reported in units of molar min-1 mL-1**(Mcentee *et al.*, 1993; Ensafi *et al.*, 2008)**.

Estimation of lipid peroxidation

The generation of thiobarbituric acid reactive substances (TBARS) was used to determine the concentration of lipid peroxides, as reported by **Ohkawa *et al*. (1979)**. The absorbance at 532nm was measured using a UV-visible spectrophotometer, and the results were reported as moles of malondialdehyde (MDA). The TBARS were extracted into a 15:1 butanol:pyridine mixture **(Ellman *et al.*,1961; Ohkawa *et al.*, 1979)**.

Acetylcholinesterase assay

The enzyme activity of acetylcholinesterase was measured using the Ellman technique (1960). After suspending the enzyme in buffer, 3.3 mM DTNB [5,5'-dithiobis (2-nitrobenzoic acid)] was added to a 0.1M pH 7.2 potassium phosphate buffer. The mixture was left to sit at 25 degrees Celsius for 20 minutes. Acetylcholine iodide was then added, and the spectrophotometer's absorbance readings at 412nm were taken every minute. Acetylcholine molar extinction coefficients were used to determine enzyme activity **(Jayanth *et al.*, 2014; Satpathy *et al.*, 2021)** .

Estimation of nitrite

Following the method described by **Green *et al.* (1982)**, the nitrite content in the supernatant of brain tissue was assessed using the Greiss reagent. A volume of 100µl of sample or standard (100g/ ml) was added to 400µl of distilled water. To this solution, we added 500µl of Greiss working reagent [0.1% N-(1-naphthyl) ethylenediamine dihydrochloride + 1% sulphanilamide + 5% phosphoric acid] and let it sit at room temperature for 5 minutes. Nitrite concentrations were calculated using a sodium nitrite standard curve and represented as micromoles per milligram of protein **(Green *et al.*,1982)**.

Estimation of protein

The Biuret technique **(Gornall *et al.*, 1949)** was used to calculate protein content. After letting the supernatant from tissue homogenate sit at room temperature for 10 minutes, 2.9ml of NaCl and 3ml of biuret working reagent were added. Using a UV spectrophotometer, the absorbance was determined to be 540nm. For 10 minutes at room temperature, 0.1 ml of homogenate, 2.9 ml of NACL, and 3 ml of working biuret reagent

were added. Measurements of absorbance were determined at 540nm **(Gornall *et al.*, 1949)**.

Histopathological analysis by Hematoxylin and Eosin staining (H&E staining)

The quick decapitation of the used animals came after the last behavioral test. After removing the brain, it was placed in a container of formalin (10% v/v). The brain tissues were placed in paraffin blocks and then microsliced to a thickness of 3mm. De-waxed and H&E-stained brain slices (5–10 m) were prepared. Binocular microscopy was used to examine and take pictures of the stained sections **(Satpathy *et al.*, 2021)**.

Statistical analysis

The data were reported as mean ± SD. T-test comparison test utilizing Prism Pad 5 software on research data statistical significance was determined at $P< 0.05$.

RESULTS

The different doses of the aqueous and ethanolic seed extract of *Asparagus racemosus* showed a significant increase in the glutathione reductase, lipid peroxidation, protein, and significant decrease in AChE, nitrite level & content in brain homogenate of zebrafish, as compared to acrylamide control group. Results are tabulated in Table (1).

Table 1. Effects of seed extract of *Asparagus racemosus* on biochemical parameters

Group	**Glutathione reductase (Microgram /milligram of protein)**	**MDA (nmol/ Milligram of protein)**	**Nitrite (µmol/milligram of protein)**	**AChE (Microgram/ milligram of protein)**	**Total protein (milligram of tissue**
Group (I)- Control group	24.83 ± 0.23	0.53 ± 0.05	24.23± 1.03	0.15 ±0.04	4.45 ± 0.45
Group (II)- ACR 0.75mM	8.24 ± 0.33@	1.79 ± 0.08@	40.04± 1.03@	0.58 ± 0.06 @	3.09 ± 0.26@
Standard group (III)-Vinpocetine (30 mg/L)	22.15 ± 0.21***	0.52 ± 0.03**	27.2±1.28***	0.18± 0.04****	4.21 ± 0.40***

Test group (IV)-AEAR (200mg/l)	18.34 ± 0.35*	1.2 ± 0.05*	37.3±1.29*	0.41 ± 0.06*	3.34 ± 0.20*
Test group (V)-AEAR (440mg/l)	20.22 ± 0.40**	1.02 ± 0.08**	33.6±1.79**	0.37 ± 0.09**	3.73 ± 0.44**
Test group (VI)-EEAR (200mg/l)	21.15 ± 0.45***	1.0 ± 0.09***	33.8±1.39***	0.31 ± 0.06***	3.59 ± 0.21***
Test group (VII)-EEAR (440mg/l)	22.15 ± 0.50***	0.56 ± 0.03**	28.3±1.63***	0.22± 0.06***	4.13 ± 0.22***

Values are expressed as mean ± SEM' n=7 @*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.

Histopathological study

Fig. (3) displays the influence of an aqueous and an ethanolic seed extract of *Asparagus racemosus* on the histological examination of zebrafish brain areas (at a magnification of forty times). The brains of those in the normal group showed unharmed neuronal cells. However, treatment with ACR induces a disarrangement of numerous cell layers in addition to a considerable loss of pyramidal neuronal cells, compared to the normal group and the standard group. On the other hand, compared to animals treated with the toxin, treatment with an aqueous and ethanolic seed extract of *Asparagus racemosus* reduced the loss of neuronal cell density dramatically.

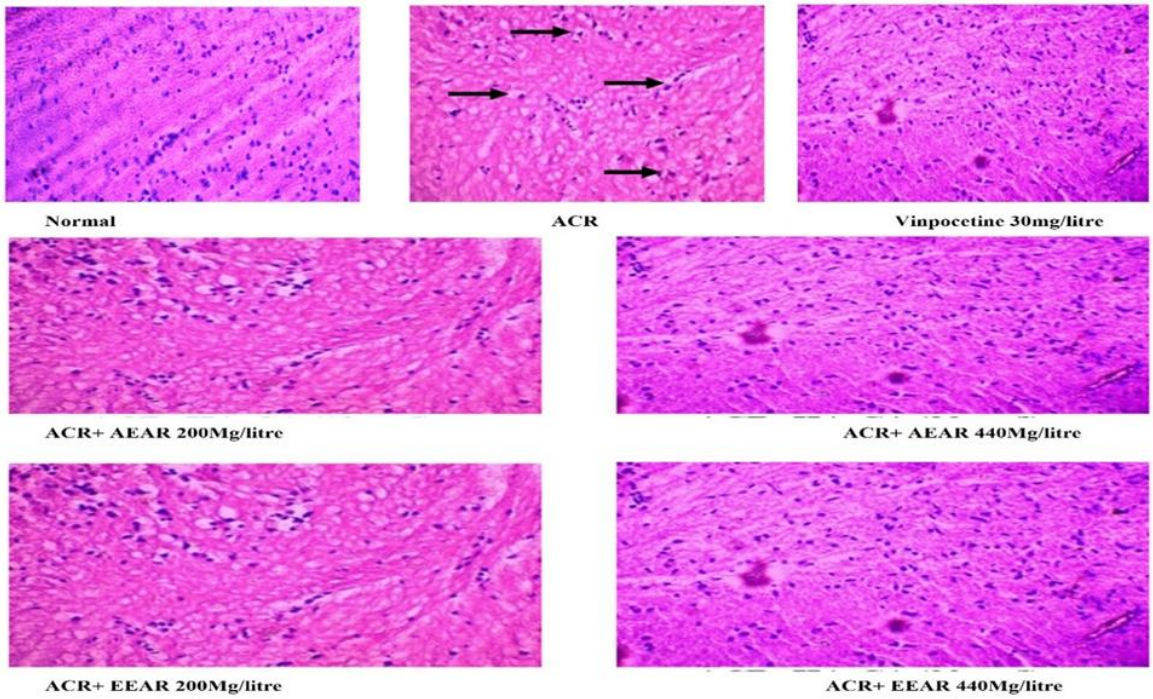

Fig.3. Hematoxylin and Eosin (H and E)-stained zebrafish brain slices and brain area in control and standard experimental groups (40x). Arrows show aberrant neurons.

DISCUSSION

Many neurodegenerative illnesses, such as Alzheimer's disease, are characterized by oxidative damage, and the free radicals generated by polyunsaturated lipid peroxidation are the final mediators of synaptotoxicity. In addition to causing oxidative neuronal damage, the un-saturated carbonyl compounds they produce are also neurotoxic. Increased malondialdehyde levels and decreased activity of the natural antioxidant enzymes catalase and glutathione reductase show that oxidative stress has a role in developing ACR neurotoxicity **(Erkekoglu *et al.*, 2014)**.

The study's findings corroborated previous claims that the *Asparagus racemosus* plant has several medicinal characteristics including neuroprotective ones. Upon applying the phytochemical analysis, flavonoids, phenolics and tannins, which are linked to powerful antioxidant activity, were detected in *Asparagus racemosus* seed extract. Antioxidant enzyme activities were increased, providing further evidence that *Asparagus racemosus* extracts have the potential to reduce oxidative stress in the zebrafish brain.

After ACR treatment, fish samples witnessed a neurotoxic effect, where glutathione reductase level decreased by 3 times; lipid peroxidation activity increased by 3.4 times; nitrite level increased by 1.7 times; acetylcholinesterase increased by 3.9 times, and total

protein level decreased by 1.4 times, compared to the wild type zebrafish. Treatment with vinpocetine standard drug showed a significant level of neuroprotective activity in ACR-induced zebrafish where glutathione reductase level increased by 2.7 times; lipid peroxidation activity decreased by 3.4 times, nitrite level decreased by 1.5 times; acetylcholinesterase decreased by 3.2, and total protein increased by 1.4 times. The results were comparable to the wild type of zebrafish. After ACR exposure, upon using different solvent seed extracts of *Asparagus racemosus*, glutathione reductase, lipid peroxides, nitrite & protein were restored and acetylcholinesterase activity was recorded, all of which were comparable to control group levels. Among the two solvent extracts, ethanolic extract exhibited much better result for neuroprotective activity in ACR-induced zebrafish. After treatment with 440mg/ l ethanolic seed extract, glutathione reductase level increased by 2.7 times; lipid peroxidation activity decreased by 3.1 times; nitrite level decreased by1.4 times, and acetylcholinesterase decreased by 2.7 times, while the total protein increased by 1.3 times, as noticed for ACR- induced zebrafish. The results were comparable to the wild type of zebrafish.

In the study **of Jangir *et al.* (2018)**, histological changes were observed in the ACR-treated rat brain, such as neuronal degeneration, and inflammation. Similarly, the present study detected histopathological alterations in ACR-administered zebrafish brain, such as pyramidal cell degeneration and glial cell degeneration. Treatment with aqueous and ethanolic seed extract of *Asparagus racemosus* significantly attenuated the loss of neuronal cell density.

CONCLUSION

The current study assumes that the aqueous and ethanolic seed extracts of *Asparagus racemosus* may have neuroprotective properties reducing the oxidative stress brought on by ACR toxicity. Previous research on the plant's antioxidant and free radical scavenging properties provides strong support for this claim. The results show the potential health advantages of *Asparagus racemosus* and suggest its use in the development of novel drugs for the treatment of dementia.

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