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A Toxicological Study on Seed Extracts of *Asparagus Racemosus* Linn (Ethanolic and Water) in Experimental Animals

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Abstract

The study investigated the in-vivo oral acute toxicity and in-vitro cytotoxicity (Neutral red assay (NRU) by Aqueous and Ethanol extract of Asparagus racemosus Linn seed. In this in-vivo study, water extract was found to be more toxic to zebrafish. The medium lethal concentration (LD_{50}) values of water and ethanol extract were 1070.8 mg/L and 1822.4 mg/L respectively for 96 hours of exposure. The correlation coefficient of water and ethanol extract were 0.972 and 0.9829 for linear regression curves between extract concentration and death percentage. In the study also 50% to 100 % mortality was observed with 968mg/L and 2129.6 mg/L water extract, whereas only 28.57% and 57.14% mortality were observed for ethanol extract in the same concentration range. The lower concentrations, such as 90.90 mg/L, 200mg/L and 440 mg/L having no mortality, were considered safe for zebrafish. In in vitro study (NRU assay) on the SH-SY5Y cell line, the same trend was observed where water extract was found to be more toxic to the cell line. The results indicate that 43% of cell death was caused by Ethanolic extract at 500 µg/ml concentration. Hence, the IC_{50} value was found to be 562.1 µg/ml, whereas approximately 52% cell inhibition was recorded at only 100 µg/mL of aqueous extract. Hence, the IC_{50} value was found to be 114.7 µg/ml for aqueous extract. Both studies showed that the ethanol extract was less toxic, hence more effective compared to the water extract.

Keywords: ***Neutral red assay, Asparagus racemosus, SH-SY5Y cell line, Aqueous and Ethanol extract.***

1. Introduction

Asparagus racemosus (A. racemosus) is an also-known Vedic rasayana that is utilized for treating a wide variety of conditions, including neurological disorders, dyspepsia, tumors, inflammation, neuropathy, and hepatopathy, as well as to prevent aging or promote lifespan. Antiulcer, anti-

diarrheal, anti-diabetic, anti-inflammatory, and immune-modulating are only some of the pharmacological effects attributed to A. racemosus root extract. The A. racemosus root has been advised for use in situations of impending abortion and as a galactogogue, according to a review of traditional Ayurvedic literature. The root of A. racemosus has been used for many different purposes, including as a diuretic, rejuvenator, carminative, stomachic, antibacterial, tonic, bitter-sweet, emollient, and nervine tonic. Hyperdipsia, neuropathy, hepatopathy, cough, bronchitis, hyperacidity, and maybe even certain infectious conditions have all been linked to the root of A. recemosus having a positive impact[1,9,10].

Fig.1: Morphological Characteristic of A. racemous

Source: Dubey A., et al. (2023)

To determine the safest possible dosage of a medicine, an adverse effect study is required. When it comes to evaluating and controlling potential dangers, fish are the vertebrate of choice. The zebrafish, both as an adult and an embryo, has a unique trait that makes it suitable for use as a model animal in toxicology and medicinal research[9,12].

Danio rerio zebra fish are now invaluable research tools because of their striking similarity to humans. There are numerous similarities between zebrafish and mammals. Most mammalian organs, such as the circulatory, excretory, urinary, and neurological systems, are developed during this time. Because of their small size and low cost, zebrafish are ideal for rapid drug discovery and toxicity studies due to their adaptability and the ability to externally fertilize and rapidly develop embryos, allowing direct observation of developing internal organs and tissues in-vivo. Drug administration is simplified since they readily absorb substances that are solubilized in water[5,2].

Fish have a high reproductive rate, making them a viable alternative to in vitro systems. Compared to other animal models, this one requires less money to keep up. Because of their fast growth, zebrafish are useful for testing the effects of drugs on organ maturation. This article looks at the effects of both ethanolic and water-based extracts of fresh Asparagus racemosus Linn seeds on the development of Zebrafish (Danio rerio).

2. Materials And Methods

The A. racemosus seeds were obtained from the Vatika agro shop in Jaipur-302020 in the Indian state of Rajasthan. The famous botanist verified the plant's legitimacy. Janta Postgraduate College, A.P.S. University, Rewa (486001), M.P. India, is where the specimen was deposited at the university's herbarium house. Additionally, Voucher specimen J/Bot/2022APS-019 is included. The plant's seeds were removed and dried in a separate oven at 45 degrees Celsius. A mechanical grinder was used to reduce the dry seeds to powder. Solvent ethanol and water (500 mL) were used to extract the powder of seeds (52g) using a Soxhlet device. Different concentrations of the test solutions were made by concentrating the filtrate from the extraction process by evaporating it in a water bath at atmospheric pressure.

Oral Acute Toxicity Study of Ethanolic and Water Seed of Asparagus Racemosus Linn

Local bender fish were purchased, and quarantine tanks were used to keep the fish safe. The fish were kept in a 5-liter tank and fed once a day with commercially available food. For one week, the fish were exposed to the laboratory's temperature and humidity while being fed their regular meal (ECR/273/Inst/OR/2013/RR-20). According to OECD recommendations 203. (Fish, Acute Toxicity Test), the ethanolic and water seeds of *Asparagus racemosus* Linn were examined for acute toxicity to determine the lethal dosage (LD_{50}) of the test chemical in an adult zebrafish.

Procedure for *In-vivo* Acute Toxicity Study in Adult Zebra-fish

Adult wild-type zebra fish weighing 0.5 to 1.5 g were bought from a source in the area. The water tank where they were housed has enough ventilation. The standard day/night cycle of 12 hours on, 12 hours off continued. Micro pellets were given to the fish three times a day. 15 days were used to help them adjust to the new environment. A total of 77 fish were split up into 11 groups, each containing 7. Each chemical was made in a geometric sequence with five distinct concentrations, rising by a factor of 2.2. As a baseline, the first group received 1ml of DMSO as a placebo. The second group was given doses of 90.90mg, 200mg, 440mg, 968mg, and 21.296mg. The third group was given 90.90mg, 200mg, 440mg, 968mg, and 21.296mg. Before being added to each aquarium, the medication was diluted with dimethyl sulfoxide (DMSO). The duration of the fishes' contact with the test substances was 96 hours. At 24, 48, 72, and 96 hours, mortalities were reported, and the LD_{50} concentration was calculated[11]. To begin toxicity testing, zebrafish were fasted for less than 24 hours and given commercial dry food once daily. If there is no sign of life and no response when you touch the fish, it is presumed to be dead. Once mortality rates were tallied, we emptied the tank of any dead fish. The lethal dose 50 was determined by observing the amount of water and ethanol in the test sample that resulted in the death of 50% of the organisms tested after 96 hours[6,14].

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

Table 1: An adult zebrafish acute toxicity test data table

Compounds	Group	Dose	Abnormal Changes		Mortality			
			Swimming	Pigmentation	24hrs	48 hrs	72 hrs	96 hrs
Control Compound 1	Group I	-	No changes	No changes	-	-	-	-
Water Extract AS	Group II	90.9mg	No changes	No changes	-	-	-	-
		200 mg	No changes	No changes	-	-	-	-
		440mg	No changes	No changes	-	-	-	-
		968 mg	No changes	No changes	-	1	2	4

Compound 2 Ethanol Extract AS	Group III	2129.6mg	No changes	No changes	1	2	4	7
		90.9mg	No changes	No changes	-	-	-	-
		200mg	No changes	No changes	-	-	-	-
		440mg	No changes	No changes	-	-	-	1
		968mg	No changes	No changes	-	1	2	2
		2129.6mg	No changes	No changes	1	2	3	4

Neutral red assay (NRU) for in-vitro cytotoxicity study of Aqueous and Ethanol extract of Asparagus racemosus Linn seed extract

Analysis with neutral red (NRA) and SH-SY5Y cell line (obtained from NCCS Pune) was used to test the cytotoxicity of the materials given. For 24 hours at 37 degrees Celsius with 5% carbon dioxide, the cells (5000–8000 cells/well) were grown in 96-well plates with DMEM media (AT149-1L) supplemented with 10% fetal bovine serum (HIMEDIA-RM 10432) and 1% antibiotic solution. Each well on the plate was emptied of its previous medium and refilled with new media the next day. Five microliters of treatment dilutions (varying quantities of aqueous and ethanol extracts of Asparagus racemosus Linn seed) were applied to the designated wells, and the plates were incubated for 24 hours. After 1 hour of incubation (Heal Force-Smart cell CO2 Incubator-Hf-90), 100 l of NRU (40 g/ml in PBS) was applied to the specified wells. After removing the medium, 100 l of NRU destain solution was used to dissolve the NRU. Last but not least, an Elisa Plate Reader (iMark BioRad-USA) was used to analyze the plates at 550 and 660 nm. Table 4 and Figure 4 show the IC_{50} values [13,10].

3. Results and Discussion

Table 2: LD_{50} calculation Compound-1 Water Extract AS

SL. No.	Concentration (mg/liter)	% of Mortality	LD 50%
01	90.90	0	**1070.8 mg/L**
02	200	0	
03	440	0	
04	968	57.142	
05	2129.6	100	

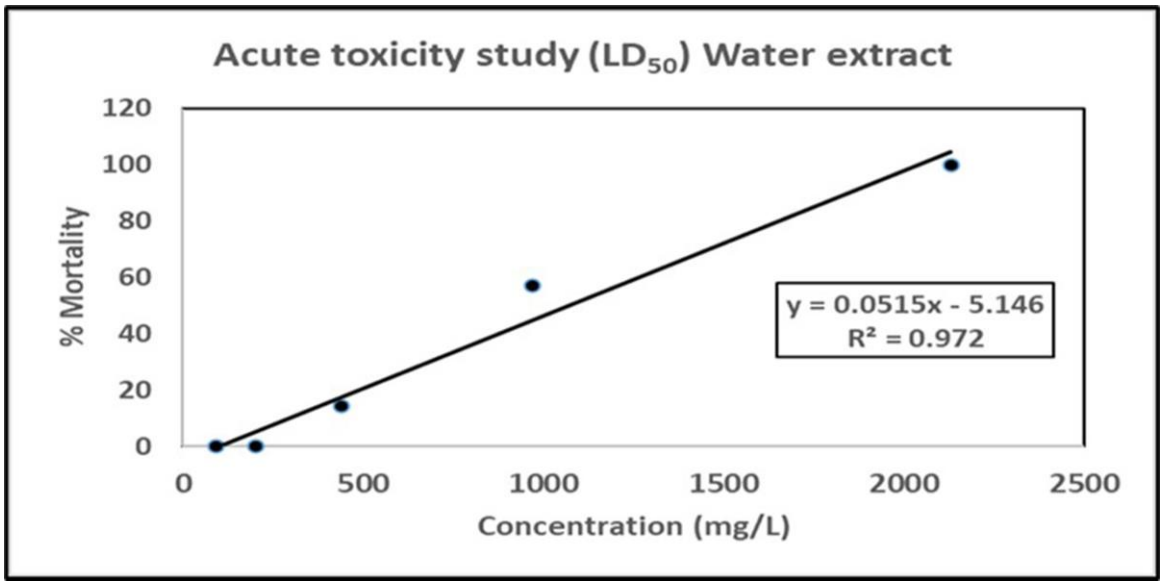

Fig.3: Acute toxicity study Water extract based on the (y=mx+C) equation y = 0.0515x and coefficient is r2 =0.972 from the graph the LD50 was found at 1070.8 mg/L.

Table 3: LD_{50} calculation Compound-2 Ethanol Extract AS

SL. No.	Concentration (mg/litre)	% of Mortality	LD 50%
01.	90.90	0	
02.	200	0	
03.	440	14.28	**1822.4 mg/liter**
04.	968	28.57	
05.	2129.6	57.14	

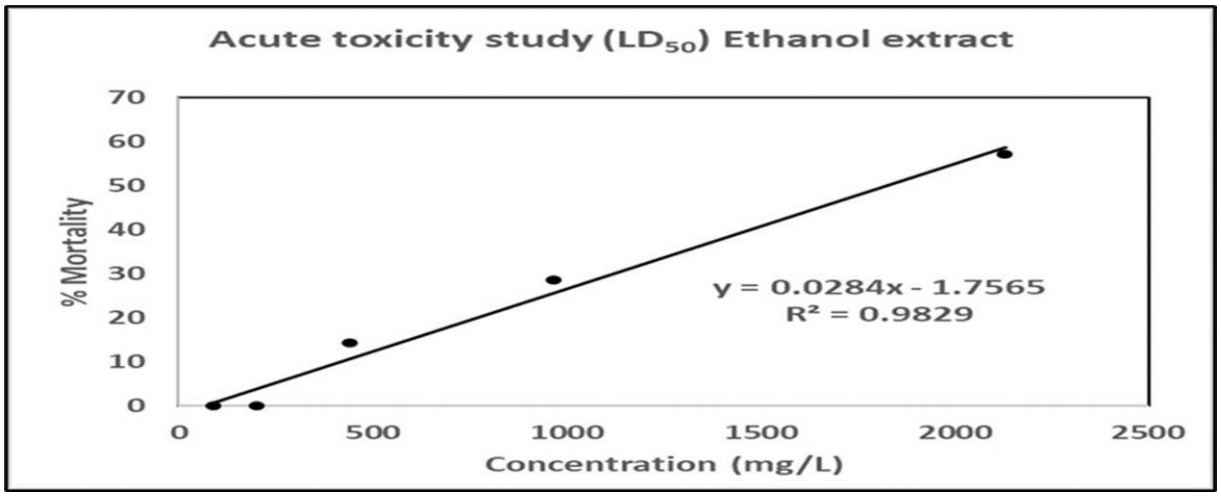

Fig.4: Acute toxicity study Ethanol extract based on the (y=mx+C) equation y = 0.0284x and coefficient is r2 =0.9829 from the graph the LD_{50} was found at 1822.4 mg/L.

No behavioral changes were observed in the control and test fish. The study of acute oral toxicity was conducted with both water and ethanol extracts. Water extract was found to be more toxic to zebrafish. The medium lethal concentration (LD_{50}) value of Water and ethanol was found at 1070.8mg/L and 1822.4mg/L respectively for 96 hours of exposure. The correlation coefficient of water is 0.972 and ethanol is 0.9829. The plan seed extract of Water toxicity was observed at concentrations of 968mg/L and 2129.6 mg/L 50% to 100 % mortality was found and Ethanol seed extract toxicity was observed at 28.57 and 57.14% at concentrations of 968mg/L and 2129.6 mg/L. The lower concentrations that are 90.90 mg/L, and 200mg/L 440mg/L are no mortality and consider safe for zebrafish.

Table 4: IC_{50} value in vitro cytotoxic study on SH-SY5Y cell line in Water and Ethanol Extract AS

Sample code	IC_{50} value (µg/ml)
ARE-01 Water extract	114.7
ARE-02 Ethanol extract	562.1

When tested for cytotoxicity against the SH-SY5Y cell line using the NRU assay, the ethanolic extract was shown to have a greater impact than the aqueous extract. At 500 µg/mL, ethanolic extract was shown to be 43% effective in killing off cells. The IC_{50} value was calculated to be 562.1µg/mL. In addition, the IC_{50} value was determined to be 114.7 µg/mL after recording a cell inhibition of roughly 52% at 100 µg/mL of aqueous extract. Both of the figures (Figures 4 and 5) show a variety of cytotoxic effects.

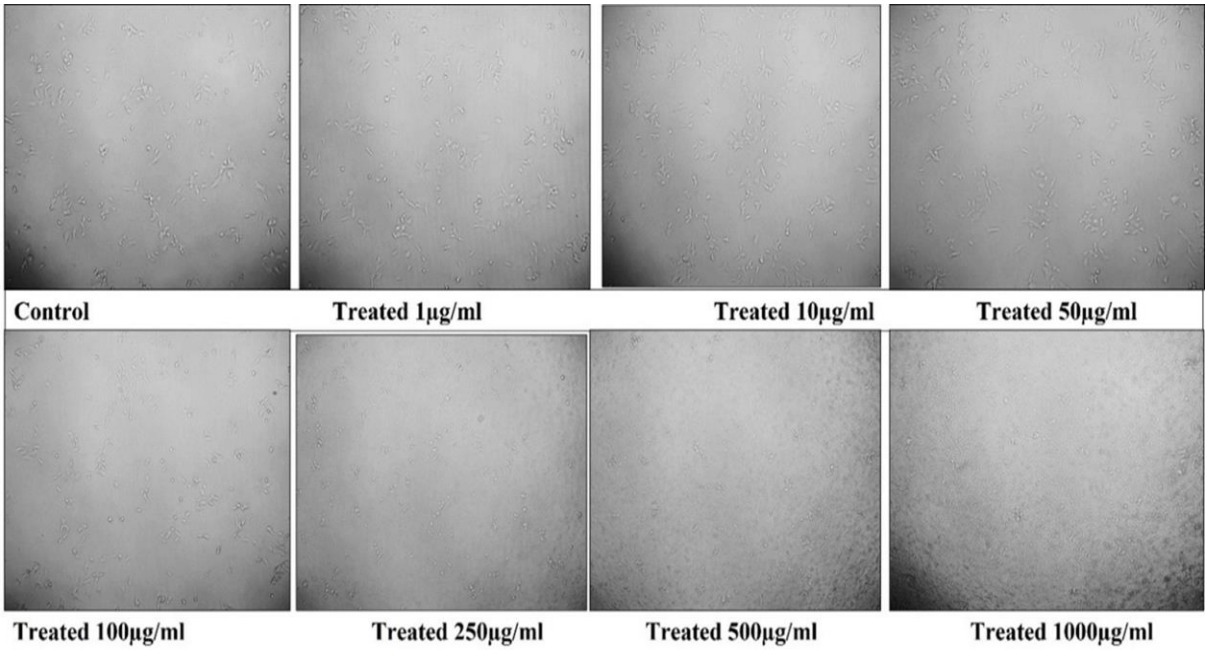

Fig.5: Cytopathic effects on SH-SY5Y treated by water-soluble extracts of ARE-01

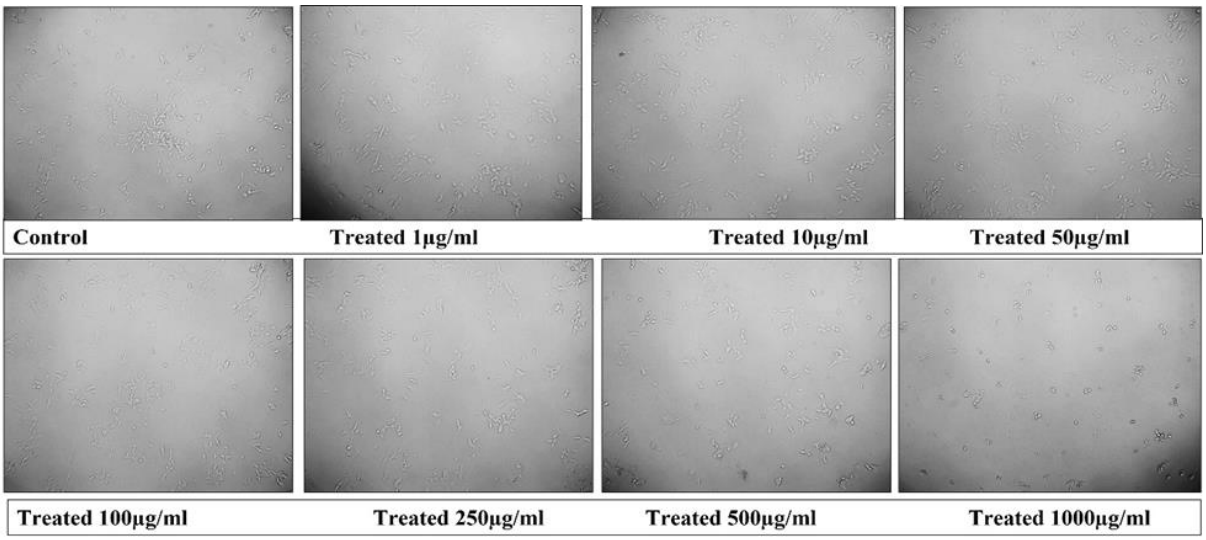

Fig.6: Cytopathic effects on SH-SY5Y treated by ethanol-soluble extracts of ARE-02

This research was conducted to ascertain the LD_{50} value for each test chemical in an in vivo and in vitro situation. To determine the LD_{50} of the test chemical in adult zebrafish, the OECD test guideline 203 (Fish, Acute Toxicity Test) was followed. There were no noticeable differences in the control group fishes' swimming behavior, color, or survival rates. In addition, zebrafish exposed to the test chemical showed no alterations in swimming behavior or coloration. Based on the results presented above, we infer that both extracts are harmful to zebrafish in a dose-dependent manner. Toxic effects might be avoided by ingesting the extract at a low dose. In the current study, it is observed that LD_{50} value of water and ethanol seed extract of AR for oral acute toxicity study on zebrafish were 1070.8 mg/L and 1822.4 mg/liter respectively. According to (Chandakavte *et al*. 2021) The medium lethal concentration (LC_{50}) value of citrus pulp powder and tecoma stans plants extract was founded at 312.5mg/L and 257.5mg/L respectively for 96 hours of exposure. In another oral acute toxicity study (Khedkar *et al*.2018) on zebrafish, LD50 was found to be 2.5 mg, 1.9 mg, 1.25 mg, and 1.47 mg for four different pyrazoline derivatives. In the current study, the LD50 values were much higher than the other studies. Therefore, it can be concluded that AR seed extracts possess some important molecules which are helpful for the survival of Zebrafish. It was also observed that these extracts were non-toxic

to Zebrafish even at very high doses. This research examined whether it was possible to use an extract prepared in water and ethanol in an animal model to conduct additional pharmaceutical screening[9,3].

Again, it was also observed that IC_{50} value of these two extracts for NRU assays on the SHSY cell line were 114.7 (µg/ml) and 562.1(µg/ml) respectively. According to Adewusi *et al*. (2013), IC_{50} value in NRU assay was found 223±6.4µg/ml for the same cell line treated with extract of Boophone distal plant containing cyclocucaenol. Hence, it was seen that the ethanolic extract of *Asparagus racemosus* seeds extract was having high IC_{50} value for the cell line. It can be concluded that ethanolic extract *Asparagus racemosus* seeds were less toxic to neuro cells; rather it can protect the neuro cells[1.]

The limitation associated with in vitro toxicity approach is that most cell systems are representing only one cell type when compared to whole animal experiments, where hundreds of tissues interact with one another physiologically. The main limitation of using zebrafish is that they require a water system to maintain them. They are not mammals and are not as closely related to humans as a mouse is.

4. Conclusion

In the study, it was observed that the LD_{50} value of water and ethanol seed extract of AR for oral acute toxicity study on zebrafish were 1070.8 mg/L and 1822.4 mg/liter respectively. It was also observed that the IC_{50} value of these two extracts for NRU assays on the SHSY cell line were 114.7 (µg/ml) and 562.1(µg/ml) respectively. It can be concluded that the ethanol extract of AR seed was safer than the water extract. The seed extracts of both water and ethanol are highly toxic in higher concentrations. In this study, adult zebrafish was demonstrated as a viable model for pharmacological screening compounds toxicity. Based on the toxicity research on *Asparagus racemosus* Linn, the seed extract was safe to use on an animal model for further pharmacological screening activities.

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Conflict of interest:
The authors declare no conflict of interest.

Authors Contribution
Prof. Ghosh conceptualized and supervised the study. Dubey arranged resources. Dubey performed the experiment and data analysis and Prof. Ranjit reviewed the manuscript. all authors read and approved the final manuscript for publication.

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Data Availability
The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Ethics Statement
This study was approved by the Institutional Ethics Committee for Research at Hi-Tech Medical College and Hospital (ECR/273/Inst/OR/2013/RR-20).

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