

VOLUME 8 ISSUE 1 2022

ISSN 2454-3055



**INTERNATIONAL
JOURNAL OF
ZOOLOGICAL
INVESTIGATIONS**

*Forum for Biological and
Environmental Sciences*

Published by Saran Publications, India



International Journal of Zoological Investigations

Contents available at Journals Home Page: www.ijzi.net
Editor-in-Chief: Prof. Ajai Kumar Srivastav
Published by: Saran Publications, Gorakhpur, India



ISSN: 2454-3055

Acute Toxicity of *Stevia rebaudiana* in Embryo of Zebrafish (*Danio rerio*)

Afrose Banu A.^{1*}, Anuradha V.¹, Nair Aneesh² and Tilton Florida³

¹Department of Zoology, JBAS College for Women, 56 K.B. Dasan Road, Teynampet, Chennai 600018, Tamilnadu, India

²Bio zone Research Technologies Pvt. Ltd., Chennai 600117, India

³Sree Balaji Medical College and Hospitals, Chennai 600044, India

*Corresponding Author

Received: 20th December, 2021; Accepted: 17th January 2022; Published online: 25th January, 2022

<https://doi.org/10.33745/ijzi.2022.v08i01.016>

Abstract: Medicinal plants are found across different corners of the world and have been a boon to mankind. Many people have derived numerous benefits from different medicinal plants for centuries. All plants are not safe there are chances that might cause toxicity and other complications. Therefore, toxicity studies on medicinal plants are to be essential before being used by humans. This study aimed to evaluate the toxicity of ethanolic extract of *Stevia rebaudiana* Bertoni leaves in embryo of zebrafish (*Danio rerio*). The acute toxicity was conducted as per the OECD guidelines 236 July 16. The result provided the evidence that the maximum mortality of 75% was observed after exposure at the higher concentration of 100 µg/ml ethanolic extract of *Stevia rebaudiana* Bertoni leaves 96 hour post fertilization and no mortality was found in negative control group embryo.

Keywords: *Stevia rebaudiana*, Acute toxicity, Fish embryo, Zebrafish, Medicinal plant

Citation: Afrose Banu A., Anuradha V., Nair Aneesh and Tilton Florida: Acute toxicity of *Stevia rebaudiana* in embryo of zebrafish (*Danio rerio*). Intern. J. Zool. Invest. 8(1): 124-128, 2022.

<https://doi.org/10.33745/ijzi.2022.v08i01.016>

Introduction

With the development of herbal industry, people started to use many plant extracts in the preparation of herbal products and food. Commercially people are in assumption that all plants are safe to consume and there is no need to investigate on their safety aspects. There exists only few reports regarding the toxicity of the herbal plants. The leaves of *Stevia rebaudiana* Bertoni (compositae) have been reported as an abundant source of sweetener (Gosling, 1901; Bertoni, 1905; Lewis, 1992; Zhnag *et al.*, 2017; Ruiz-Ruiz *et al.*, 2017). *Stevia rebaudiana* leaves

contain naturally high level of steviol glycosides (Zhang *et al.*, 2017). A number of earlier reviews on *Stevia* and its glycosides have been published (Kinghorn and Soejarto, 1991; Bakal and O'Brien Nabors, 1986).

Zebrafish (*Danio rerio*) is an ideal model for studying toxicity (Jusuf and Haris, 2009; Abdelkader *et al.*, 2012; Caloudova *et al.*, 2018; Sehonova *et al.*, 2019; Blahova *et al.*, 2021; Perumal *et al.*, 2021; Denisa *et al.*, 2022). The embryos are strong enough to manipulate in scientific experiments such as transplanting and

micro-injection experiments. Thus, zebrafish has now gained an influential model organism for studying environmental toxicology (Yang *et al.*, 2009).

The importance of toxicity testing is to provide the dose-dependent changes against the toxicity effect. Acute toxicity studies can provide important information regarding the biological activities of the test substance as well as valuable information about the dangers that are associated with the use of new chemical substances in humans. Toxicity studies play a vital role in drug discovery and development. Performing toxicity studies of herbal plants in an animal model are considered to be safe in human use. The present study was designed to investigate the toxicity of the extract of leaves of *Stevia rebaudiana* for the embryo of zebrafish (*Danio rerio*).

Materials and Methods

Extract preparation:

The *Stevia rebaudiana* plant was collected from Alandur nursery garden. The plant was authenticated by Institute of Botanical Science Plant Anatomy Research Centre. The leaves of collected plant samples were allowed to dry. After drying the leaves were powdered and extracted with absolute ethanol (99.9%) through coal percolation method. The crude extract so obtained was purified through column chromatography and further analysis was done through HR - LCMS. Successful optimization of this technique will pave simple and bulk organic extraction of stevioside and rebaudioside from *Stevia rebaudiana* which is used as a test sample for the assessment of acute toxicity in embryo of a zebrafish (*Danio rerio*).

Test concentration:

As per OECD guidelines 236, five test concentration viz., 100 µg/ml, 50 µg/ml, 25 µg/ml, 12.5 µg/ml, and 6.25 µg/ml were selected with the spacing factor 2.0 (Not exceeding 2.0).

Collection of fish embryo and experimental design:

Wild-type adult zebrafish with well- documented fertilization rate of eggs and free from infection

and disease were selected and allowed for breeding in the ratio of 2:1 (Male : Female). After spawning, eggs were collected and rinsed with water viable and fertilized embryos were separated from unfertilized egg and transferred to test plates (24 well plates) which were filled with 2 ml of exposure medium of respective concentration.

Fish were divided into nine groups in the following manner and treated with leaf extract of *Stevia rebaudiana* for 96 hour post fertilization (hpf):

Groups	Total number of embryos	Concentration (µg/ml) (Leaf extract of <i>Stevia rebaudiana</i>)	Volume of test item added per well (µl)
Group I	20	100	2000
Group II	20	50	2000
Group III	20	25	2000
Group IV	20	12.5	2000
Group V	20	6.25	2000
Group VI (control)	20	No treatment, only dilution water	2000

Physico-chemical parameters of exposure medium:

Exposure medium were analyzed and maintained at optimum hardness, pH, Dissolved oxygen (DO), conductivity and temperature at the beginning and at the end of the experiment in controls and all the test groups.

All embryos were observed for coagulation, lack of somite formation, non- detachment of the tail, and lack of heart beat. Observations were taken at 24, 48, 72 and 96 hpf. Additionally, hatching rate was recorded in treatment and control groups on a daily basis starting from 48 hpf.

Results

Tables 1 and 2 illustrate the acute toxicity assessments (OECD guide lines 236 July 16) in embryo of zebrafish (*Danio rerio*). The maximum mortality of 75% was observed at the higher

Table 1: Apical observations of acute toxicity in Zebrafish embryos after exposure to extract of leaves of *Stevia rebaudiana* at 96 hpf

Observation/ Groups	Group I	Group II	Group III	Group IV	Group V	Group VI
Coagulated embryos	50%	10%	0%	0%	0%	0%
Lack of somite formation	0%	0%	0%	0%	0%	0%
Non-detachment of the tail	0%	0%	0%	0%	0%	0%
Lack of heartbeat	0%	0%	0%	0%	0%	0%
Hatching rate (%)	5%	5%	10%	NA	NA	NA
Other phenotypic abnormalities	Nil	Nil	Nil	Nil	Nil	Nil
Mortality percentage	50%	10%	0%	0%	0%	0%

Table 2: Cumulative mortality after exposure to extract of leaves of *Stevia rebaudiana* at 96 hpf

Test groups	Mortality (%) at the end of 96 hpf
Group I	75%
Group II	50%
Group III	25%
Group IV	20%
Group V	10%
Group VI	0%

concentration (100 µg/ml) after 96 hpf. The exposure of embryos to *Stevia* leaf extract caused mortality within 96 hpf.

Discussion

The leaves of *Stevia rebaudiana* Bertoni is a low-calorie natural sweetener called steviol glycosides which reduce harmful effect of sugar and improving the nutrient properties (Sharma *et al.*, 2016). The extract has anti-hyperglycaemic, anti-hypertensive, anti-hypolipidemic, anti-tumor, anti-diarrheal, anti-oxidant, anti-viral, anti-diuretic, immunomodulatory properties and has positive

effect on haematological problems (Uswa Ahmad *et al.*, 2020).

According to the OECD guidelines 236, in toxicity assay, fish embryos are considered as dead when they show coagulated, lack of somite formation, non-detachment of the tail and lack of heart beat. Additionally hatching rate was recorded in treatment and control group on a daily basis in all the six groups. In zebrafish embryo apical and cumulative mortality were observed. The present study revealed the mortality in zebrafish which were exposed to 100

µg/ml leaf extract of *Stevia rebaudiana* (75% mortality at 96 hpf). No mortality was observed in control. Toxic substances may lead to physical, mental and behavioural changes. The motility of zebra fish embryo can be affected by toxins, as exhibited by coagulation, heart failure, and non-development of yolk embryo's tail (Wibowo *et al.*, 2018). Thus, it is suggested that further experimental studies should be conducted on zebrafish to identify the chronic toxicity of the plant *Stevia rebaudiana*.

Zhang *et al.* (2017) reported that oral administration of ethanolic extract of *Stevia rebaudiana* leaf extract at dietary levels of 1.04%, 2.08% and 3.12% for 90 days did not induce significant behavioral, hematological, clinical, or histopathological changes in rats, however, significant reduction of cholesterol, total protein and albumin was observed in female animals only at high dose level. On the basis of their results these authors have suggested that *Stevia rebaudiana* Bertoni leaves may potentially be used in functional foods as well as nutritional supplements beyond sweetener.

There is an emerging trend in the world with the sophisticated lifestyles of human towards the artificial sweeteners which contain lot of additives (Al-Qudsi and Al-Jahadali, 2012). The high concentration of food additives can cause adverse effects to the human health, especially in early developmental stages. Hence this should be investigated using animal models. Because of exposure to nutritional and environmental challenges during critical stages of early development of life, the metabolism in later life can be affected (Jackson *et al.*, 2010). Embryo of zebrafish can provide an ideal model for the toxicity studies due to easiness as well as more similarities with human genome.

Acknowledgements

The authors are thankful to the management, JBAS College for Women, Chennai, India and Biozone

Research Technologies Pvt. Ltd., Chennai 600117, India for providing the lab facilities for this work.

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