



"ASSESSMENT AND EVALUATION OF ACUTE TOXICITY; LETHALITY ASSAY & PHYTOTOXICITY OF ROSARIN ON DIFFERENT EXPERIMENTAL MODELS"

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ABSTRACT:

Toxicology testing is crucial for establishing the characteristics of a test chemical and its hazardous effects (Arome D, 2013). As a result, the OECD test guidelines recommend advice on how to employ techniques for characterization and identifying chemical substances' hazards. Rosarin, a cinnamyl alcohol glycoside derived from *Rhodiola rosea* plant, was chosen as test substance for toxicity studies. It is found that the drug Rosarin has anti-inflammatory, neuroprotective, and other therapeutic properties. Different experimental models were subjected to various concentrations of the drug Rosarin for determining the toxicity. The experimental models chosen are zebra fish for determining acute toxicity and to evaluate its histopathological studies, brine shrimp for lethality assay and fenugreek seeds for phytotoxicity. The zebra fish were exposed to different concentrations of drug for 96 hours, and the toxicity levels were determined by analyzing histopathological studies of the drug-treated fish. While brine shrimp eggs were taken and hatched for a period of 24 hours, then the larvae were isolated and percentage of live shrimps was calculated within 24 hours. The fenugreek seeds were sown in soil after being treated in various concentrations of the drug for few hours to test for phytotoxicity whose observations need to be recorded once every 24 hours.

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KEYWORDS: Rosarin, Acute toxicity, Histopathology, Lethality assay, Phytotoxicity

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I. INTRODUCTION

In order to characterise the test material and determine its harmful effect, toxicity testing is essential. Prior to being used on humans, newly created medications must undergo extensive toxicity testing. The majority of toxicity testing is done on experimental animals, and it identifies any potential risks that a test chemical is anticipated to cause as well as how it acts. Because of the advantages they provide in analysing a whole functional

organism, the use of animals in toxicity testing is most likely to continue for the foreseeable future. Acute oral toxicity assessment is typically the first stage in assessing and evaluating the hazardous properties of all substances. Acute toxicity gives recommendations for the dose to be used in longer-term studies and also serves as the foundation for further testing programmes. Rosarin is a cinnamyl alcohol glycoside derived from the *Rhodiola rosea* plant.



Rosarin possesses anti-inflammatory as well as neuroprotective properties.

Zebrafish are vertebrate species that are gaining popularity for use in preclinical drug discovery (Rubinstein AL, 2006). Zebrafish is an intriguing animal model for studying the pathophysiology of metabolic illnesses in humans and exploring possible therapy alternatives. When compared to other vertebrate models used to represent human diseases, zebrafish have significant advantages, particularly for developmental toxicity testing, as well as other biomedical research applications (Choi TY, Choi TI, 2021).

The Brine Shrimp Assay has been widely used to test the toxicity of a wide range of plant products for a long time now (Hamidi MR, 2014). Moreover, the Artemia-based lethality assay (brine shrimp lethality assay, BSLA) is quick, simple, and inexpensive. It has been employed as a preliminary toxicity assay in the study of natural products to screen a large number of extracts and chemicals for drug discovery in medicinal plants (Ntungwe N E, 2020).

Phytotoxicity is referred to as a delay in seed germination, restriction of plant growth, or any detrimental effect on plants produced by certain substances or growing conditions (Paget GE, 1964). To assess the toxicity, the Germination index (GI) was utilised, which includes measures of relative seed germination (G %) and relative root elongation (L %). According to certain studies, fenugreek is high in active chemicals and hence, fenugreek seeds were used for carrying out the phytotoxicity tests.

II. AIM AND OBJECTIVES

The aim of the study is to assess and evaluate the acute toxicity tests of rosarin in zebrafish, as well as histopathological studies, brine shrimp lethality assay, and phytotoxicity studies of fenugreek seeds.

The main objectives of the study are as follows:

- Acute toxicity tests on zebrafish using the drug – Rosarin
- Parameters of acute toxicity tests on zebrafish

- Evaluation of histopathological studies on zebrafish
- Brine shrimp lethality assay
- Phytotoxicity on fenugreek seeds

III. MATERIALS AND METHODS

3.1 Zebrafish

Requirements include fish tank, zebra fishes (30), aerators, distilled water, fish feed, external light source, pH meter and weighing balance. Fishes were purchased from Jasmine Aquarists, Secunderabad. The fish are subjected to the test chemical (drug- Rosarin) for 96 hours. Mortalities and obvious abnormalities in appearance and behaviour are noted.

Selection and holding of fish

To maintain homogeneity, fish should be juveniles from the same source and population. The fish should all be of the same age and have normal appearance. Before being used for testing, all the fish should be kept in the lab for at least 9 days. The first 48 hours are spent settling in. The fish should then be acclimatised for at least 7 days.

Number and handling of fish

For the control and each test concentration, a minimum of 5 fish must be employed. The fish should be divided up into the various treatments randomly.

The following four concentrations were used as test concentrations: 25 mg/L, 50 mg/L, 75 mg/L, and 100 mg/L. For control, only low-toxicity solvents should be used (i.e. acetone, ethanol, methanol) as advised in Guidance Document No. 23 (OECD, 2019), and solvents with uncertain toxicity shouldn't be.

Conditions of exposure

- Duration: 96 hours
- Light: should fall between 12 - 16 hours in the photoperiod range.
- Temperature: should be between a range of 21-25°C. The chosen temperature, which may be 24°C, shouldn't vary by more than 1°C.
- Oxygen concentration: not less than 60% of the saturation value of the air. Aeration can be utilised as long as analytical measurements of test concentrations



show that it does not result in a significant loss of the test chemical.

- Feeding: two times a day
- Disturbance: Disturbing factors, such as excessive vibration or noise, should be avoided or decreased to the greatest extent practicable.

Observations and recording

Within the first 24 hours of the study, at least 2 observations should be made, ideally with at least 3 hours in between. Mortalities and apparent anomalies that affect equilibrium are recorded and additional clinical symptoms may be noted if possible.

Parameters to be observed

- Mortality
- Response to light: Active/ Inactive
- Visible abnormalities: loss of balance/ head up or down/ floating at surface or sinking
- Appearance: weak/ dark pigmentation/ exophthalmia
- Swimming behaviour: hyper activity/ hypo activity/ immobility/ convulsions
- Food intake: low/ high/ normal

Humane killing of fish

At the end of the exposure, any remaining fish in the groups are euthanized. After 96h study, the fishes are euthanized, dissected and various organs such as heart, brain, intestine, eyes are collected and submitted for histopathological studies at NIPER, Balanagar, Hyderabad.

3.2 Brine shrimp

The requirements include brine shrimp eggs, air pump, analytical balance, rectangular glass jar, measuring cylinder, table salt, spatula, Pasteur pipette, light source, test tubes, magnifying glass, and test sample.

Serial dilution of extract

A 200 mg test sample was weighed using an analytical balance. After that, a stock solution was made by dissolving 100 mg of test sample (soluble in water [99ml] and methanol [1ml]). From the stock solution, concentrations of 0.1

Transfer of seeds into disposable cups for germination

ml/L, 0.3 ml/L, 0.5 ml/L, and 0.7 ml/L were prepared along with one control solution. Then, 10 nauplii and 1 ml of seawater were added to the appropriate test tubes with 1 ml of the produced solution. After 24 hours, the dead nauplii were counted.

Hatching brine shrimp

3 liters of water was poured into the rectangular jar using the measuring cylinder. 27g of table salt was weighed and added to the jar containing water and mixed properly using spatula. By maintaining proper aeration, the airline's tip was inserted from an air pump into the container's bottom. 15g of brine shrimp eggs were roughly added at the top of water level in jar and mixed with water. The jar is positioned at a short distance from the light. The nauplii will hatch after 20 to 24 hours. The hatched nauplii were separated from empty eggs by turning off aeration and light. Utilizing a Pasteur pipette, 10 nauplii were transferred to a test tube. The nauplii were exposed to various plant extract concentrations. After 24 hours, number of survivors was counted and the mortality rate was determined as a percentage.

$$\% \text{ Live shrimps} = \frac{\text{Live shrimps in sample}}{\text{Live shrimps in control}} \times 100$$

3.3 Phytotoxicity

The requirements include fenugreek seeds, soil, five transparent beakers, pipette, water, disposable cups, ph meter, weighing balance.

Preparation of test and control solution

4 different concentrations of test sample in 4 different petri dishes (concentrations are 0.2mg/ml, 0.4mg/ml, 0.8mg/ml, and 1.6mg/ml) were taken and labeled.

Soaking of fenugreek seeds

10 fenugreek seeds were placed in each petri dish with a different concentration, and they were allowed to soak for 6 to 8 hours in each concentration's test solution along with the control solution.



5 disposable cups (with small hole at the bottom of the cup for free flow of water) were taken for 4 different test concentrations and one control. The fenugreek seeds were transferred into the cups marked with their corresponding concentrations.

Seed germination

The soil in each cup is ensured of the same height and quality for uniformity. The cups were placed in proper sunlight. Every eight hours, the soil was sprinkled with the appropriate drug concentrations as specified on the label. The germination of fenugreek seeds was observed for 96hrs.

Seed germination % = $\frac{\text{No. of seed germination in compost extract}}{\text{No. of seed germinated in control}} \times 100$

No. of seed germinated in control _____

Root elongation % = $\frac{\text{Mean root length in compost extract}}{\text{Mean root length in control}} \times 100$

Mean root length in control _____

Germination index = Seed germination % × Root elongation %

	Day 1 (3:00 pm) 2.5h	Day 2 (10:45 am) 5.5h	Day 2 (3:30 pm) 24h	Day 3 (11:00 am) 30h	Day 3 (3:45 pm) 48h	Day 4 (11:15 am) 54h	Day 4 (3:30 pm) 72h	Day 5 (11:00 am) 78h	Day 5 (3:00 pm) 96h
1.Mortality	-	None	None	None	None	1 DEAD	None	None	None
2.Response of light	-	Active	Active	Active	Active	Active	Active	Active	Active
3.Visible abnormalities	-	None	None	None	None	None	None	None	None
4.Appearance	-	No changes	No changes	No changes	No changes	No changes	No changes	No changes	No changes
5.Swimming behavior	-	Normal	Normal	Hyper	Hyper	Hyper	Hyper	Hyper	Hyper
6.Food intake	-	Normal	Normal	High	High	High	High	High	High

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100

Table 1: Observation table for fishes in 25mg/L concentration

IV. RESULTS AND DISCUSSION

4.1 Zebrafish

Various parameters and observations for different concentrations were recorded in different tables as follows:

FISHES IN 25mg/L:

	Day 1 (3:00)	Day 2 (10:45)	Day 2 (3:30)	Day 3 (11:00)	Day 3 (3:45)	Day 4 (11:15)	Day 4 (3:30)	Day 5 (11:00)	Day 5 (3:00)
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	pm) 2.5h	am) 5.5h	pm) 24h	am) 30h	pm) 48h	am) 54h	pm) 72h	am) 78h	pm) 96h
1.Mortality	-	None	None	None	None	None	None	None	None
2.Response of light	-	Active	Active	Active	Active	Active	Active	Active	Active
3.Visible abnormalities	-	None	None	None	None	None	None	None	None
4.Appearance	-	No changes	No changes	No changes	No changes	No changes	No changes	No changes	No changes
5.Swimming behavior	-	Normal	Normal	Normal	Normal	Hyper	Hyper	Hyper	Hyper
6.Food intake	-	Normal	Normal	High	High	High	High	High	High

Table 2: Observation table for fishes in 50mg/L concentration

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- No mortalities were seen in this concentration.
- No abnormalities or behavioural changes were observed.

FISHES IN 50mg/L:

- One fish was found dead on day 4.
- No other abnormalities or behavioural changes were observed.

FISHES IN 75 mg/L:**Table 3: Observation table for fishes in 75mg/L concentration**

- 2 fishes were found dead on day 3 and day 4 respectively.
- No other abnormalities or behavioural changes were observed.

- **FISHES IN 100 mg/L:**

Table 4: Observation table for fishes in 100mg/L concentration

	Day 1 (3:00p m) 2.5h	Day 2 (10:45 am) 5.5h	Day 2 (3:30 pm) 24h	Day 3 (11:00 am) 30h	Day 3 (3:45 pm) 48h	Day 4 (11:15 am) 54h	Day 4 (3:30 pm) 72h	Day 5 (11:00 am) 78h	Day 5 (3:00 pm) 96h
1.Mortality	-	None	None	None	1DEAD	1DEAD	None	None	None
2.Response of light	-	Active	Active	Active	Active	Active	Active	Active	Active
3.Visible abnormalities	-	None	None	None	None	None	None	None	None
4.Appearance	-	No changes	No changes	No changes	No changes	No changes	No changes	No changes	No changes
5.Swimming behavior	-	Normal	Normal	Hyper	Hyper	Hyper	Hyper	Hyper	Hyper
6.Food intake	-	Normal	Normal	High	High	High	High	High	High



- Highest mortality is seen in this concentration.
- Skin color was observed to be changed into pale orange in dead fishes.
- Exophthalmia was observed in one of the dead fishes.
- Exophthalmia is the abnormal protrusion of eyeball or eyeballs.



Fig-1: Exophthalmic fish

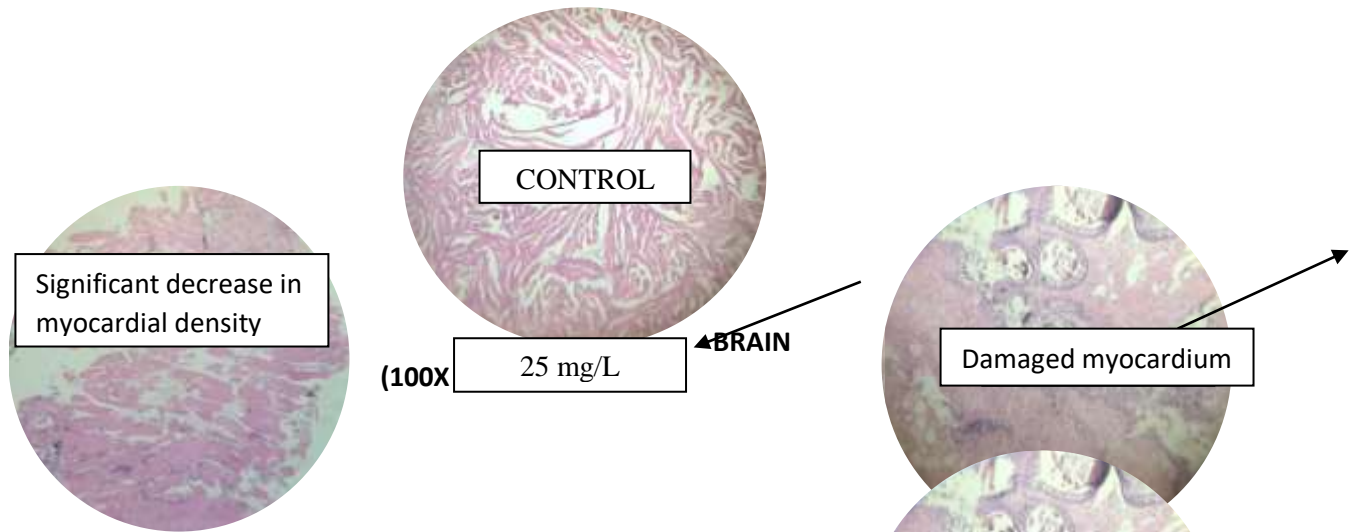
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	Day 1 (3:00 pm) 2.5h	Day 2 (10:45 am) 5.5h	Day 2 (3:30 pm) 24h	Day 3 (11:00 am) 30h	Day 3 (3:45 pm) 48h	Day 4 (11:15 am) 54h	Day 4 (3:30 pm) 72h	Day 5 (11:00 am) 78h	Day 5 (3:00 pm) 96h
1.Mortality	-	None	None	1 DEAD	None	None	1 DEAD	1 DEAD	None
2.Response of light	-	Active	Active	Active	Active	Active	Active	Active	Active
3.Visible abnormalities	-	None	None	None	None	None	None	Exophthalmia seen in dead fish	None
4.Appearance	-	No changes	No changes	No changes	No changes	No changes	Skin colour changed in dead fish	Skin colour changed in dead fish	No changes
5.Swimming behavior	-	Normal	Normal	Hyper	Hyper	Hyper	Hyper	Hyper	Hyper
6. Food intake	-	High	High	High	High	High	High	High	High

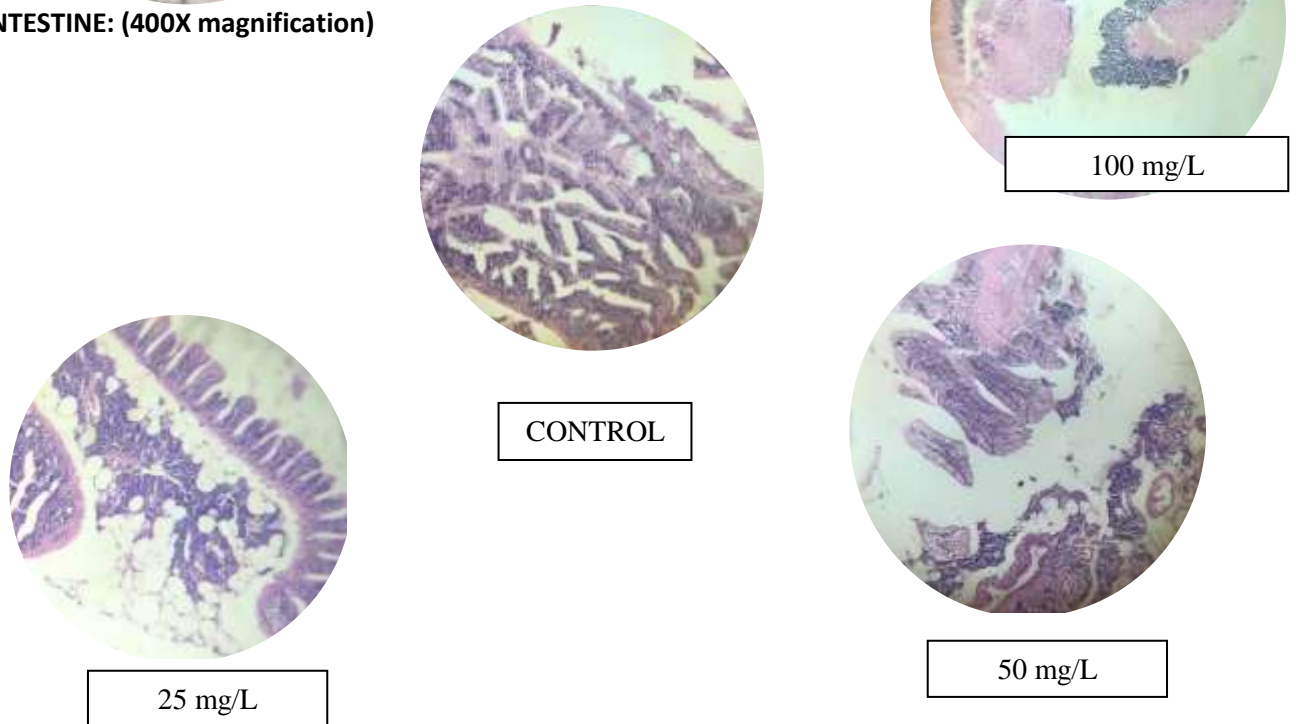


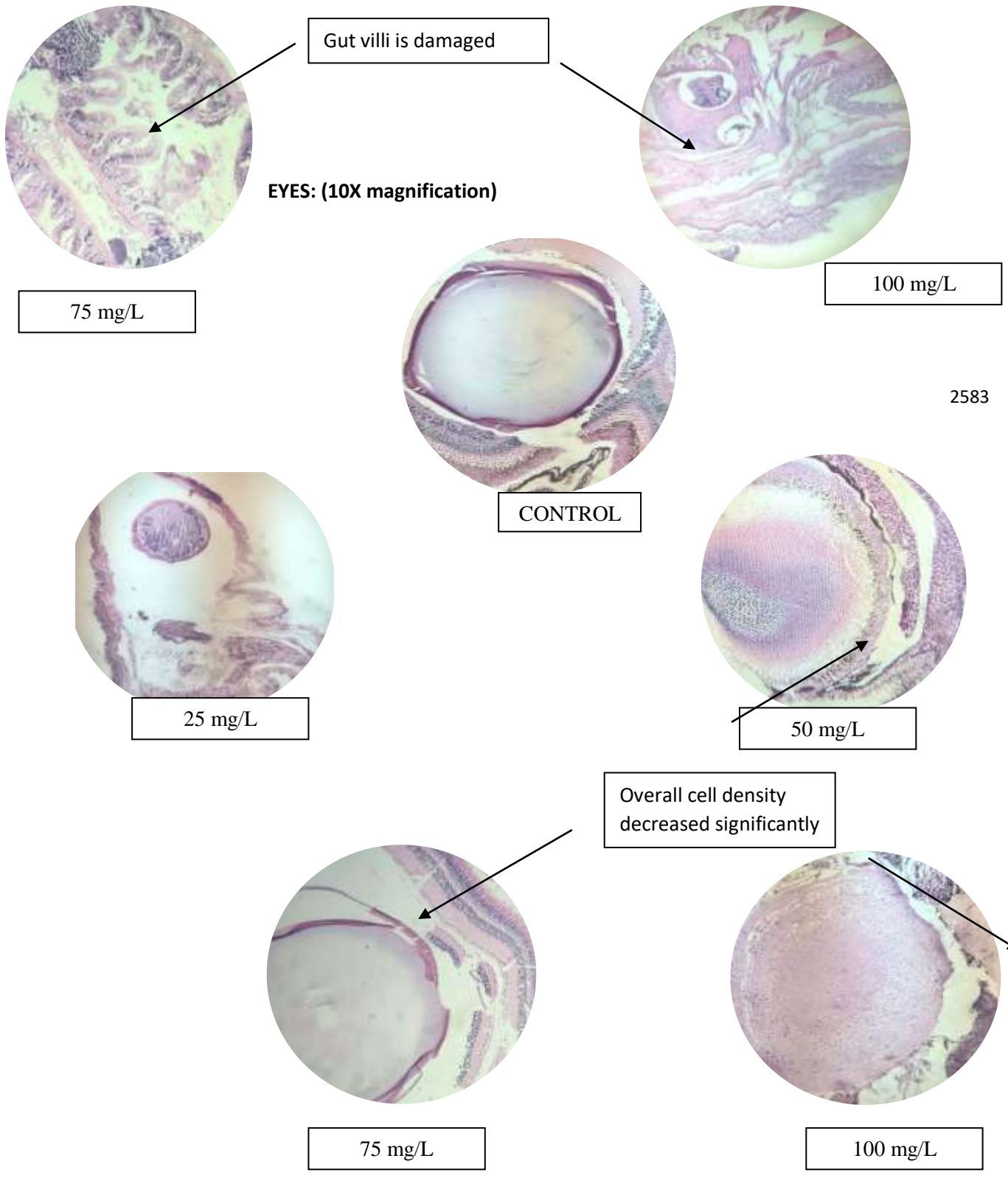
HISTOPATHOLOGICAL STUDIES

HEART (100X magnification):



INTESTINE: (400X magnification)





4.2 Brine shrimp

Table 5: Observation table for brine shrimp lethality assay

PETRI PLATES NUMBER	SAMPLE DETAILS	DAY2 t=2hrs	DAY3 t=0	DAY3 t=6hrs	DAY3 t=12hrs	DAY3 t=18hrs	DAY3 t=24hrs
1.	0.1	15	13	12	8	6	3
2.	0.3	15	14	9	6	4	1
3.	0.5	15	11	8	7	5	2
4.	0.7	15	10	6	5	3	1
AVERAGE	SAMPLES	15	12	8.75	6.5	4.5	1.75
5.	CONTROL	15	15	14	14	14	13
%LIVE SHRIMPS		100%	80%	62.5%	46.42%	32.14%	13.46%

The %live shrimp by the end of the experiment was found to be 13.46%

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4.3 Phytotoxicity

	NO.OF SEED GERMINATION	MEAN SHOOT ELONGATION	MEAN ROOT ELONGATION	GERMINATION INDEX%
AFTER 24 Hrs				
0.2mg/ml	0.4	0.2	0.87	0.003
0.4mg/ml	0.25	0	0.69	0.001
0.8mg/ml	0.21	0	0.6	0
1.6mg/ml	0	0	0.4	0
CONTROL	100	100	100	100
AFTER 48Hrs				
0.2mg/ml	0.65	0.62	0.89	0.006
0.4mg/ml	0.43	0.1	0.69	0.003
0.8mg/ml	0.21	0	0.6	0.001
1.6mg/ml	0.9	0	0.4	0
CONTROL	100	100	100	100



AFTER 96Hrs	0.81	0.86	0.98	0.007
0.2mg/ml	0.62	0.03	0.82	0.005
0.4mg/ml	0.3	0.02	0.79	0.002
0.8mg/ml	0.25	0	0.65	0.001
1.6mg/ml	100	100	100	100
CONTROL				

Table 6: Observation table for germination index of fenugreek seeds



Fig-2: Germination after 48hrs



Fig-3: Germination after 96hrs

V. CONCLUSION

5.1 Zebrafish

- Mortality was observed as the dose concentration was increased.
- More number of deaths was seen in the highest dose concentration i.e. 100mg/L out of 5 fishes, 3fishes were observed dead in the highest concentration.
- The skin color was changed [bright orange to pale] in the dead fishes and a clear case of exophthalmia [abnormal protrusion of the eye balls or eyeball] was observed in one dead fish.
- Therefore, we can conclude that highest dose concentration of 100mg/L was lethal to the fishes.



Histopathological studies

- Histopathological changes in the heart, brain, intestine, eyes of zebra fish exposed to different concentrations of drug rosarin are visualized and the changes are observed. Haematoxylin and Eosin are the staining agents used for histopathological studies.
- Heart: significant decrease of myocardial density of ventricles as the concentration increases.
- Brain: fractional decrease of neuronal cells is observed at higher concentrations and also appearance of clear areas.
- Intestine: the morphology of gut villi is mutated upon increase of the concentration.
- Eyes: overall cell density is decreased as the concentration increases.
- Therefore, we can conclude that increase in the concentrations results in the morphological changes.

5.2 Brine shrimp

- From the above conducted brine shrimp lethality assay, it is observed that the percentage of live shrimps has decreased with an increase in the dose concentration along with the duration of exposure.
- The results were compared with the control solution and the conclusion was drawn.

5.3 Phytotoxicity

- It is clearly observed that as the concentration increases, the germination/growth of the seeds are retarded.
- The germination index % is much better in the initial concentrations when compared to higher dose concentrations which are very negligible.
- Hence, we can conclude that higher dose concentrations of Rosarin can affect the germination/growth of Fenugreek seeds.

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REFERENCES

- Arome, D. and Chinedu, E., 2013. The importance of toxicity testing. *Journal of Pharmaceutical and BioSciences*, 4, pp.146-148.
- Akhila, J.S., Shyamjith, D. and Alwar, M.C., 2007. Acute toxicity studies and determination of median lethal dose. *Current science*, pp.917-920.
- Paget, G.E. and Barnes, J.M., 1964. Toxicity tests. *Evaluation of drug activities: pharmacometrics*, 1, pp.135-65.
- Choi, T.Y., Choi, T.I., Lee, Y.R., Choe, S.K. and Kim, C.H., 2021. Zebrafish as an animal model for biomedical research. *Experimental & Molecular Medicine*, 53(3), pp.310-317.
- Rubinstein, A.L., 2006. Zebrafish assays for drug toxicity screening. *Expert opinion on drug metabolism & toxicology*, 2(2), pp.231-240.
- Hamidi, M.R., Jovanova, B. and Panovska, T.K., 2014. Toxicological evaluation of the plant products using Brine Shrimp (*Artemia salina* L.) model. *Macedpharm bull*, 60(1), pp.9-18.
- Ntungwe N, E., Dominguez-Martin, E.M., Roberto, A., Tavares, J., Isca, V., Pereira, P., Cebola, M.J. and Rijo, P., 2020. Artemia species: An important tool to screen general toxicity samples. *Current Pharmaceutical Design*, 26(24), pp.2892-2908.
- Gandhare, B., Kavimani, S. and Raj Kapoor, B., 2013. Acute and subacute toxicity study of methanolic extract of *Ceibapentandra* (Linn.) Gaertn. on rats. *Journal of Scientific Research*, 5(2), pp.315-324.
- Arts, J.H., Muijser, H., Jonker, D., Van De Sandt, J.J.M., Bos, P.M.J. and Feron, V.J., 2008. Inhalation toxicity studies: OECD guidelines in



relation to REACH and scientific developments. *Experimental and Toxicologic Pathology*, 60(2-3), pp.125-133.

Walum, E., 1998. Acute oral toxicity. *Environmental health perspectives*, 106(suppl 2), pp.497-503.

Patton, E.E., Zon, L.I. and Langenau, D.M., 2021. Zebrafish disease models in drug discovery: from preclinical modelling to clinical trials. *Nature Reviews Drug Discovery*, 20(8), pp.611-628.

Chan, W., Shaughnessy, A.E., van den Berg, C.P., Garson, M.J. and Cheney, K.L., 2021. The validity of brine shrimp (*Artemia* sp.) toxicity assays to assess the ecological function of marine natural products. *Journal of Chemical Ecology*, 47(10), pp.834-846.

Montanher, A.B.P., Pizzolatti, M.G. and Brighente, I.M.C., 2002. An application of the brine shrimp bioassay for general screening of Brazilian medicinal plants. *Acta Farm.Bonaerense*, 21(3), pp.175-178.

Banti, C.N. and Hadjikakou, S.K., 2021. Evaluation of toxicity with brine shrimp assay. *Bio-protocol*, 11(2), pp.e3895-e3895.

Nishimura, Y., Inoue, A., Sasagawa, S., Koiwa, J., Kawaguchi, K., Kawase, R., Maruyama, T., Kim, S. and Tanaka, T., 2016. Using zebrafish in systems toxicology for developmental toxicity testing. *Congenital Anomalies*, 56(1), pp.18-27.

Suneka, S. and Manoranjan, T., 2021. Brine shrimp lethality assay with selected medicinal plants extracts. *Vingnanam Journal of Science*, 16(2).

Kopittke, P.M., Blamey, F.P.C., Asher, C.J. and Menzies, N.W., 2010. Trace metal phytotoxicity in solution culture: a review. *Journal of experimental botany*, 61(4), pp.945-954.

Durazzo, A., Lucarini, M., Nazhand, A., Coêlho, A.G., Souto, E.B., Arcanjo, D.D. and Santini, A., 2022. *Rhodiola rosea*: main features and its beneficial properties. *RendicontiLincei. ScienzeFisiche e Naturali*, 33(1), pp.71-82.

