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Embryotoxicity and teratogenic effects of *Parmotrema tinctorum* (Nyl) Hale on Zebrafish (Danio rerio) and cytotoxic activity of lichen extract on HEK293T cell line

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Abstract

Parmotrema tinctorum (Nyl) Hale an edible lichen, used as a spice and flavouring agent for meat and vegetable preparations by ethnic groups in India and Nepal. In Zebrafish embryos and larva, the therapeutic applications of *P.tinctorum* widely reported. However the teratogenic effects not reported. This study was aimed to examine the toxicity of *P.tinctorum* on the promising model for toxicity research, the Zebrafish, because their genetic structure is more similar to human beings. Additionally, we have demonstrated a toxic effect of *P.tinctorum* extract on the human normal cell line (HEK293T) by MTT assay. The methanol extract of *P.tinctorum* was extracted by Soxhlet and for embryotoxicity and teratogenicity activities, we use different concentrations (50µg/ml – 200µg/ml) of lichen extracts on twelve selected fertilized Zebrafish embryos. The extract did not exhibit any effect on Zebrafish Survival rate, hatching rate, heartbeat and teratogenicity which was similar to control groups. To conclude that, methanol extract of *P.tinctorum* is found to have a nontoxic property to zebrafish embryo and human normal cell line (HEK293T) and suggests that this might be safe for consumption.

Keywords- *Danio rerio, HEK293T, MTT assay, Edible Lichen, Toxicity.*

Introduction

Herbals plants contain many natural chemical compounds which have pharmacological and therapeutic characteristics properties. Herbal plants are generally utilized for their culinary purpose also for nutritional supplements to improve health [1]. Nearly 80 per cent of the society uses medicinal plants because they are safer and do not induce any adverse impacts than synthetic drugs [2-3]. So, over the past decade, remedies produced from medicinal plants are used by mass population. But, the idea of safe herbal medicine is wrong [4]. Herbal medicines also have a toxic side, which produces effects on animals and humans. Previous studies of different herb and herbal product have reported that, herbal medicine cause numerous types of toxicity, including development toxicity, genotoxicity, reproductive toxicity and hepatoxicity [5-10]. Moreover, the intake of herbal medicines causes poisoning. Hereafter consumption of herbal plants is done after the in-depth toxicological evaluation which leads to reduce the public health risk. [11]

Traditional preclinical knowledge in *in-vitro* and *in vivo* models are the main compound in current safety and toxicology testing methods. In drug discovery and toxicological screening, the Zebrafish is an emerging model which offers many advantages on Biological, physiological and molecular alteration [12]. Several other advantages of Zebrafish are, they have a genome that is similar to the human genome, high reproductive ability, structural resemblances with vertebrates, embryo's optical transparency, high in external embryonic development, good characterized behaviour and small in size. Mainly it is not expensive so it is a unique animal model for assessing the new drug's toxicity [13].

Lichens are the symbiotic associated organism, which consists of both algae, the photobiont and Fungi, the mycobiont. Lichen produces various chemical constituents which are so unique to them and not found in plants. In several countries, Lichens are commonly used as food ingredients and folklore medicine. Many research has proved that secondary metabolites were isolated from lichen have anti-oxidant, anti-inflammatory, antiarthritic activities and potent larvicidal activity [14-15]. *Parmotrema tinctorum* (Nyl) Hale (Parmeliaceae) is prominent foliose which are mostly corticolous or saxicolous. This foliose lichen is widely used as a spice in food and add as a flavouring agent in a food product [16]. *Parmotrema tinctorum* have many bioactive compounds such as lecanoric acid, isolichenin, lichenin and vitamins such as B and C. the biological activity of *P. tinctorum* is not studied fully and this effort is made to investigate the toxicity of *P.tinctorum* in the Zebrafish model.

2.Materials and Methods

2.1 Collection and identification of the lichen sample

Fresh thalli of Corticolous *Parmotrema tinctorum*, were collected from Kodaikanal hills of Western Ghats, Southern India, during January 2019. Samples were collected at an altitude of 2130 meters. Identification of lichen was carried out at Biomedical Research Lab, Bharathiar University, Coimbatore, Tamil Nadu, India. Voucher specimens were deposited at lichen herbarium Centre, Bharathiar University, Coimbatore, India and NBRI, Lucknow, India. The lichen specimens were identified with the help of identification keys documented on Awasthi's identification key manual [17]. Spot test (K, C, I, KC) were done for the Identification of representative specimen employed in the present study [18].

2.2 Extraction of *P.tinctorum*

The extraction of the lichen sample was done using the Soxhlet apparatus. The powdered lichen samples were loaded in pockets of thimbles and introduced in the extractor. Then, extraction was done separately using 250 mL methanol and heated up to 80°C for 8 hours. Then, the methanol extract was concentrated using a rotary vacuum evaporator. (Superfit make, India)

2.3 Invitro Cytotoxic Activity of Lichen extract by MTT assay.

Using MTT assay, the cytotoxicity activity of lichen extract was done against the HEK-293T Cell line [19]. In this method, HEK293T cells were seeded into 96 well plates (2000-4000 cells/well) with a total volume of 100 µl medium per well and allowed to attach for 24 hours and 48 hours. After incubation, the cells were treated with lichen extract without FBS. Once the incubation period was over, the cells were washed with 1x PBS and different concentration of the extract (50µl, 100µl, 150 µl, 200µl and 250µl) was poured into each well. The experimental setup was left under UV light for 24 hrs. After incubation 10ul MTT/ well was added and incubated for 3 hrs in a CO2 incubator. After 3 hrs the media along with MTT was removed and 100 µl DMSO was added and the setup was left in dark condition for 15 min. Reading were taken at 570 nm in a UV spectrophotometer.

2.4 Zebrafish maintenance and egg collection

According to OECD guidelines, the embryotoxicity test was performed. Totally 6 to 8 months aged, 30 females and 10 males *Danio rerio* wild type, commonly as Zebrafish were used to obtain embryos. The animals were adapted for two weeks at the laboratory in a 20 L glass aquarium with a photoperiod 14: 10 (light: dark). The parameters of the aquarium water were 27 ℃ ± 1℃ temperature should be maintained, 7.1 ± 0.5 pH and more than 95%Saturation dissolved oxygen. The Zebrafishes were fed with commercial feed five times per day. After selection, at 24-hour post-fertilization, using a Pasteur pipette the embryos were transferred into each of the 96 well plates [20].

2.5 Experiment design and treatment

The different series of concentration of the sample was pipetted into each well in the 96 well plates. Subsequently 24 to 72 hours of treatment, the embryo development was observed. Using an inverted microscope, the zebrafish embryo was viewed by focusing on parameters like survival rate, heartbeat hatching rate also Morphological observation. The tests were done in accurate triplicate.

2.6 Evaluation of Zebrafish Embryo Hatch Rate:

Determination of the hatch rate of Zebrafish was done by treating them with different concentration of *P.tinctorum* (50 µl – 200 µl) for three days. Using an inverted microscope, the embryo hatching was observed.

2.7 Evaluation of Zebrafish Embryos Survival Rate:

The rate of survival was monitored from 0hrs to 72 hours. If the survival rate is > 75%, it is considered as non-toxic [21].

2.8 Evaluation of Zebrafish Larvae Heart Beat:

After three days of treatment with *P.tinctorum* extract (50 µl – 200 µl), the heartbeat of larvae was examined. The heartbeat counting of larvae was done by using an microscope connected with a camera device for visual observation. The heart rate was counted and monitored per minute with a stopwatch.

2.9 Teratogenicity assay

To check the teratogenic effect of extract, the embryos exposed to different concentration (50 µl – 200 µl) of the *P. tinctorum* extract. On everyday basis, the medium was changed and the development of the embryo was monitored. The embryos malformation was analysed at every 24 hpf to 72 hpf (hours of post-fertilization). The parameters used were listed in Table 1. The embryos were assessed for the heartbeat, blood circulation, detachment of tail, somites, skeletal deformities and motility. If some abnormalities are found or appeared low development, then score was given as 1 and if appeared any sort of normal development, then score was indicated as 0. By the cumulative score of malformation, the teratogenicity effects of the different concentration of the extracts were analysed [22].

Table .1 Parameters for teratogenic scoring for the different time interval

Parameters / Time	Somites	Tail detachment	Otolith	Eyes	Heart beat	Blood circulation	Hatch rate	Skeletal deformities	Motility
24hrs	*	*	-	-	-	-	-	-	-
48hrs	*	*	*	*	*	*	*	*	*
72hrs`	*	*	*	*	*	*	*	*	*

* normal development

- no observation / development

3. Resul**t**

3.1 Cytotoxicity activity of lichen extract by MTT assay

To examine the impact of *P.tinctorum* on cell growth was done by MTT assay. HEK293T cells were used in the present study. These cells were treated with different concentrations (50µl, 100µl, 150 µl, 200µl and 250µl) of *P.tinctorum and* observed for 24 hours.

The survival percentage were similar in both control and treated HEK293T cells. All the tested concentrations of lichen extracts were showed that a survival percentage of > 95%.

The results are shown in Fig 5. This was additionally confirmed by light and fluorescence microscopy. The test results have also shown that the cell retained their originality. This was compared with the the control and treated groups.

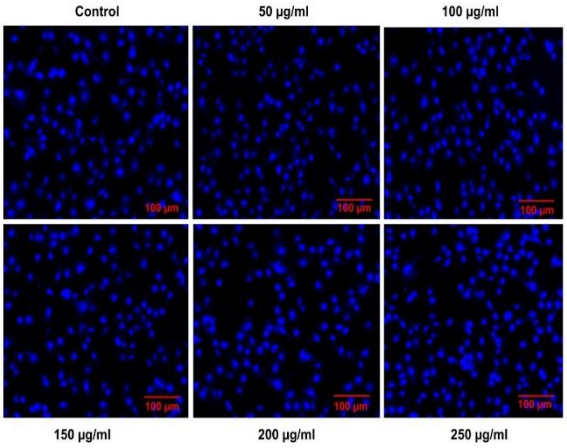

Figure. 5 Fluroescence microscopy images of the morphologies of HEK293T cells treated with a series of concentrations of *P. tinctorum.*

3.2 Embryotoxicity and Teratogenicity

Toxicity of *P.tinctorum* was examined by treating embryos with various concentration (50ul, 100ul, 150 ul,200ul and 250ul) of lichen extract for 72-period post-fertilization. The heartbeat, phenotypes, survival rate, hatching rate of the embryos were monitored for 72 hours. At all concentrations the hatching rate was found to be similar. The survival rate was also observed from the low concentrations of 50 µg/mL and to the maximum concentration of 200 µg/mL (Fig.1-3).

There was no significant difference in the percentage hatching rate, survival and heartbeat rate when the larvae were exposed to various concentration of *P. tinctorum* extract. The result was evident that there were no noticeable morphological abnormalities such as stunted growth, kink and bend tail observed in Zebrafish.

In all concentration, the larvae were active and healthy. The results are shown in Fig. 4. These results suggest that even at 200 µg/mL concentration, zebra fish embryos was safe.

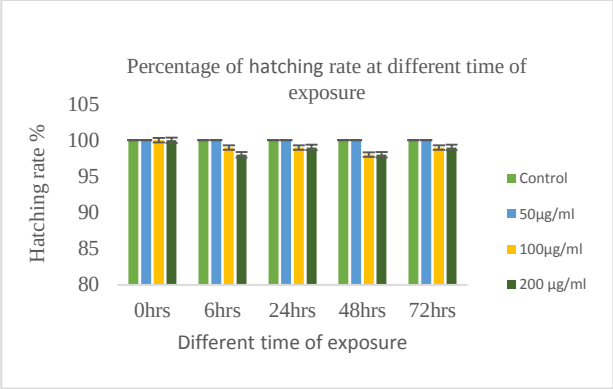

Figure 1. Percentage of hatching rate at different time of exposure to *P.tinctorum* extract

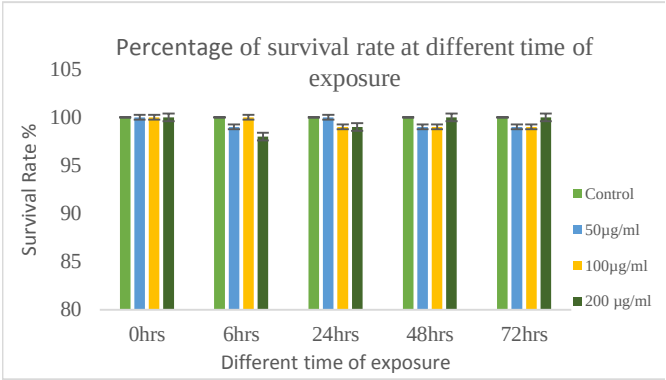

Figure 2. Percentage of hatching rate at different time of exposure to *P.tinctorum* extract

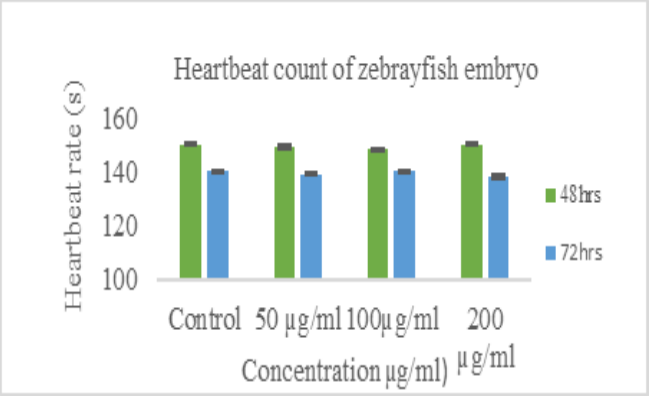

Figure 3. Effects of *P.tinctorum* extract on zebrafish larvae heartbeat

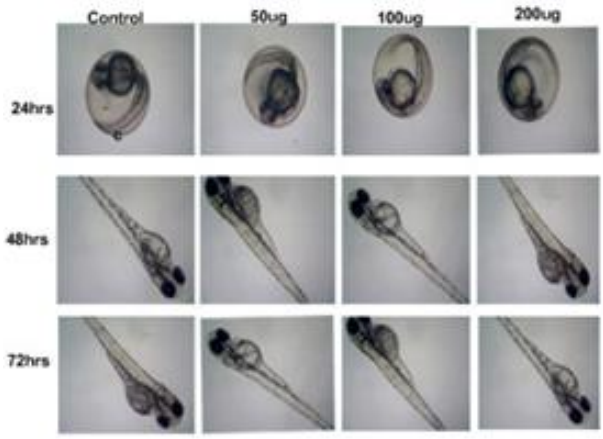

Figure. 4 Development toxicity of *P.tinctorum* extract on zebrafish embryo

4. Discussion

Indian Siddha is a well-known traditional method of treatment, has been used since ancient historical times to cure various diseases. Various lichen extracts are employed in ayurvedic Indian medicine as an antidote for many diseases. In recent years, various lichen species are investigated for their biological activities. The non-toxic properties of many lichen species are still unknown due to the lack of extensive research. The lichen *Parmotrema tinctorum* is one such genus. To examine the biosafety observation of the methanol extract of *P.tinctorum* on Zebrafish (*Danio rerio*) embryo this investigation was carried. These species are habituated to grow at higher altitudes and found distributed in the Western Ghats. In Siddha water has been used conventionally as the extraction solvent. As lichen extracts are partially soluble in water, the present study used methanol for the complete elution of active biological agents from *P.tinctorum*. The non-toxic nature of such extracts was documented and the data generated in the current study are similar to the work carried out by other researchers. The embryotoxicity assay shows the cytotoxic property of the lichen extracts and this study is one of the first attempts with the *P.tinctorum* to assay the embryotoxicity of methanolic extracts against the embryo of zebrafish. The *P.tinctorum* did not exhibit a toxic effect even at the highest concentration of 200µg/mL. In support of the presently obtained results, the highest concentration of

ethanolic extract of corticolous lichen *G. reticulatum* was tested non-toxic to the Zebrafish embryo. The embryos death, deformities and teratogenic effects of *P.tinctorum* extract have not been illustrated in previous studies. The results of this present investigation suggest that the extract of *P.tinctorum* might be totally safe. The results are in line with the findings of Saikowska. [23]

In the present study, we tested the toxicity of *P.tinctorum* extract on HEK293T normal cells *in vitro.* Using fluorescence microscopy and MTT assay it was observed that the lichen extract did not show any significant toxicity in the cell line. The present study results are in accordance with the work done by Nael [24]. in fruit extract of *C.fistula* on normal human lung cell BEAS-2B *in vitro*. Even though the *in vitro* results cannot necessarily be extrapolated to the human body, using other models the actual health risk should be evaluated.

Conclusion.

These overall findings suggest that baseline information nourishes the potential of the *P.tinctorum* extracts explored non-toxic effects on zebrafish embryos and HEK293T human normal cell line at different concentrations. For a deeper understanding of the nontoxic effect of *P.tinctorum* for human use, we did further molecular analysis and preclinical studies with mice.

Conflicts of interest

The authors declare that they do not have any conflicts of interest.

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