Phytochemical screening and evaluation of genotoxicity and acute toxicity of aqueous extract of *Croton tiglium* L.

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Abstract- Extensive and indiscriminate use of synthetic compounds and natural compounds obtained from plant sources have resulted into serious threats to not only the aquatic ecosystem but also to human health. Aqueous seed extract of the plant *Croton tiglium* L. is used in many traditional medicines to treat various ailments in many developing countries. The extract is also used for killing fishes for consumption in Manipur, India. However, the side effects and safety measures are not well studied and evaluated. The present study aims to investigate major phytochemical constituents, acute toxicity and genotoxicity of the aqueous extract. The phytochemical screening was carried out using chemical methods; acute toxicity test was performed using zebrafish as a model organism and genotoxicity potential was evaluated by *in vitro* plasmid DNA fragmentation analysis. Our results show that the exposure of aqueous extract of *Croton tiglium* cause increase plasmid DNA strand breakage in a dose dependent manner. The aqueous extract of *Croton tiglium* also showed pisicidal activity. So, the plant extract need to be evaluated for its long term human health hazards and safety thoroughly before it could be used for therapeutic medicinal interventions.

Index Terms- Phytochemical screening, Genotoxicity, Acute toxicity, Zebrafish, *Croton tiglium*.

I. INTRODUCTION

Treatment of various ailments using medicinal plants has been practiced from prehistoric times. Their use has been considerably increased among the populations of developing countries because of their beneficial and having few significant side effects [1, 2]. However, there is limited report of the proper evaluations of the toxicity of these medicinal plants. Thus, proper phytochemical screening of the plant is necessary because plants can synthesize toxic substances to protect themselves against infections, insects and other organisms which feed on them. Various groups of compounds are responsible for the toxic effects of these plants. Major bioactive compounds responsible for these toxic effects include alkaloids, cardiac glycosides, phorbol esters, lectins and cynogenic glycosides. Previous studies had reported the cases of acute poisoning of patients admitted to hospitals and resulted into death mainly due to ingestion of toxic medicinal plants [3, 4, 5]. Recent investigations have also revealed the presence of genotoxic, mutagenic and carcinogenic compounds in many plants used as traditional medicine or food both *in vitro* and *in vivo* assays [2, 6, 7]. However, some toxic plants are used by doctors for the treatment of diseases [8]. Assessment of the potential genotoxicity of the traditional medicines is very important as damage to the genetic material may lead to mutagenesis and carcinogenesis as well as other toxic effects [9, 10, 11].

Croton tiglium L. is a shrub native to South East Asia and belongs to the family Euphorbiaceae. It is indigenous to India and widely distributed in North-Eastern part of India. In the state of Manipur, it is used as folk medicine for treating gastrointestinal disorders. The seeds and young leaves of this plant are extremely toxic to fishes, as a result it has been extensively used as a source for killing fishes for consumption. The oil obtained from the seeds of *Croton tiglium* has been shown to act as a potent tumour-promoting agent in mouse skin and a potent mitogenic agent in contact inhibited tissue cultures [12, 13]. Phorbol esters extracted from the seeds of *Croton tiglium* showed inhibitory effect on HIV-induced cytopathic effect (CPE) on MT-4 cells and a potent inhibitory effect on the proliferiation of HIV-1 [14]. In Ayurvedic system of medicine, the croton seed and oil has been used in minute doses in dropsy, constipation, cold, cough, asthma and fevers. From ancient times, Chinese people have been using this plant to treat gastrointestinal disorders, intestinal inflammation, rheumatism, headache, peptic ulcer and visceral pain [15, 16, 17, 18]. Previous reports also have shown that the oil of *Croton tiglium* has purgative, analgesic, antimicrobial and anti-inflammatory properties [15, 18]. Moreover, this plant is used as a pisicidal plant in North Eastern part of India. The pisicidal and molluscidal effects of this plant had also been reported [19, 20, 21].

Despite the popular use of this plant as folk medicine and as a fish poison, there is no scientific data available for phytochemical screening, acute toxicity and genotoxicity of the aqueous extract of *Croton tiglium* L. The present study aims to investigate the genotoxicity of this plant extract by *in vitro* DNA strand breakage analysis using plasmid DNA and acute toxicity test using zebrafish as model organism. Moreover, the major phytochemical constituents of the aqueous extract of *Croton tiglium* are being reported for the first time.

II. MATERIALS AND METHODS

Plant extract preparation

The seeds of *Croton tiglium* were collected from Nambol, Manipur state, India in September 2011. The seeds were washed with tap water and cut into small pieces and subjected to shade dry at 28°C. Then the seed-pieces were crushed into fine powder with mortar and pestle. 40gm of the powder was mixed with 80mL of distilled water and then after proper maceration, the

suspensions were kept for 24hours at 28°C. The resultant suspension was filtered with nylon cloth. The filtrate so obtained was centrifuged at 6000 rpm for 10 minutes. The clear supernatant was subjected to lyophilization, and then the dried aqueous extract of *Croton tiglium* (AECT) were kept at -20°C until further use.

Phytochemical screening

Phytochemical screening was performed for the detection of various bioactive compounds from the aqueous extract of *Croton tiglium* using chemical methods. The following major classes of bioactive compounds were screened: phenolic compounds (ferric chloride test and lead acetate test); alkaloids (Mayer's test, Wagner's test, Hager's test and Dragendorff's test); saponins (Foam test); terpenoids (Libermann-Burchard test); cardiac glycosides (Keller-Killani test); anthraquinone glycosides (Borntrager test); flavonoids (Alkaline reagent test, Shinoda's test) and carbohydrates (Molisch's test, Fehling's test, Brafoed's test and Benedict's test). Among the phenolic compounds the presence of tannin was screened using the gelatin test and the presence of flavonoids using the NH_4OH-test. These entire tests were carried out using the protocol given elsewhere [22, 23, 24].

Experimental animal

Wild type Zebrafish (*Danio rerio*) were obtained from local vendors, India. The fish were acclimatised for one month in a glass aquarium of 50 litre capacity and kept in continuously well aerated water containing 2mg/L Instant ocean salt at approximately 28°C under a 14h:10h light-dark photoperiod. Zebrafish were fed with commercial food twice a day with flakes in the morning and live artemia in the evening. The pH, dissolved oxygen content and total hardness of the aquarium water were analysed by standard methods [25].

Acute toxicity testing of AECT

A 48 hour acute toxicity (LC_{50}) test of the AECT was conducted using adult zebrafish (length 2.6-2.8cm and weighed 0.2-0.3 g) in a static water renewal experiment, according to the Organisation for Economic Cooperation and Development (OCED) guideline for testing of chemicals [26]. Ten randomly selected zebrafish of both sexes were exposed to a particular dose in a 5 litre capacity rectangular glass tank containing different concentrations of the AECT. Five different concentration of AECT (4, 8, 12, 16, 20, 24mg/L), each with two replicates along with one control were used for this test. The selections of the test concentration of AECT were based from the result obtained from the range finding experiment. Mortality was monitored continuously and the fish were considered dead when operculum movement was no longer detected and the fishes could not response when contacted with a glass rod. The dead fish were immediately removed from the tank. After 24 hours the fish were transferred to new tanks containing their respective concentrations of the AECT. The fish were not fed prior to or during the experimental period. During the experiment, the behaviour of the experimental fish was monitored regularly.

Plasmid DNA fragmentation analysis

pTZ57R/T plasmids were isolated from the transformed DH5-Alpha *E. coli* cultures following the protocol given in GeneJET Plasmid Miniprep Kit (Thermoscientific). Agarose gel electrophoresis (0.8%) was performed in order to evaluate plasmid DNA treated with different concentrations of AECT. Here the electrophoresis assay was performed to separate different conformational form of plasmid DNA in order to analyse strand breaks in the plasmid DNA treated with different concentrations of AECT. A comparison between the plant extracts treated DNA bands and the positive and negative control DNA bands was used to access any possible genotoxic effects of the plant extracts. In this experiment pTZ57R/T plasmid DNA aliquots (1µg) were incubated with different concentrations of the AECT (300µg/mL, 225µg/mL, 150µg/mL and 75µg/mL), at 37°C for 40 min. Negative control was performed using ultrapure Milli-Q water and positive control with $SnCl_2$ solution (200µg/mL) with the same incubation temperature and time period. After incubation, each sample was mixed with loading buffer (0.2% xylene cyanol FF; bromophenol blue; 30% glycerol in water), loaded in a horizontal 0.8% agarose gel electrophoresis chamber in Tris acetate-EDTA buffer at pH 8 and run at 6V/cm. Then the DNA bands were visualised in the agarose gel coupled with ethidium bromide staining (O.5µg/mL) by fluorescence using an ultraviolet trans-illuminator system [6]. The assay was repeated three times and the best image was documented using the gel documentation system, JH Bio AlphaDigiDocRT2.

III. Results and Discussions

In the present study, we investigate the acute toxicity and genotoxicity of the AECT. Moreover, we also perform the phytochemical investigation of the AECT for the first time. Phytochemical analysis of AECT using chemical methods showed the presence of saponins, alkaloids, phenolic compounds, tannins, triterpenoids and carbohydrates (Table 1). We have used the crude aqueous extract for all our experiments. We presume that studying the crude aqueous extract form is the most appropriate as people have been found using the crude aqueous extract only for folk medicine and/or for killing fishes. Working with crude extracts, means working with complex mixtures of biologically active compounds, some of the compounds in such a mixture can be genotoxic or antigenotoxic. So, screening of the genotoxic and antigenotoxic properties is important to predict the potential health hazards of using the plant for medicinal purposes.

In order to fully understand the genotoxic potential of the AECT, we performed *in vitro* plasmid DNA fragmentation analysis. From the *in vitro* DNA fragmentation analysis, we observed that exposure of AECT caused increased plasmid DNA breakage in a concentration dependent manner. The untreated control pTZ57R/T plasmid consists of the supercoil form (SC) and open circular form (OC). After treatment with different concentrations of AECT, we observed that some part of the super coiled form (SC) changed to linear form (L) and open circular form (OC) which is not observed in the control plasmid. Same effect is also observed in case of the positive control experiment also. This showed that AECT contain some compouds which can damage the plasmid DNA. The open circular form results from single strand breaks and the linear form results from double strand breaks. In addition to this observation, we showed DNA smear formation and this effect was more pronounced in the

plasmid DNA treated with higher concentration of the AECT. This also showed that AECT also contains other compounds which act on the plasmid DNA but with different mechanisms. Our *in vitro* plasmid DNA fragmentation analysis assay clearly showed that AECT has the ability to mediate DNA strand breaks and damage in the plasmid pTZ57R/T DNA, in a concentration dependent manner. The plasmid strand breakage after treating with AECT is shown in Fig. 1. Previous study had also reported the genotoxicity of the plant extracts in plasmid DNA [6, 27]. But no single test is enough to predict the genotoxic potential of a compound. So, we performed the *in vivo* assay using the micronucleus test and comet assay in zebrafish. We observed the genotoxic effects after treating the zebrafish with AECT (Data unpublished). The results obtained in our study are in agreement with the reports of Lopes et al., [28]. It is difficult to speculate on the compounds responsible for the genotoxic response detected with this extract but we speculate that certain saponin mixture and alkaloids might be responsible for the genotoxic effects of AECT to plasmid DNA. Previous study had also reported that certain group of bioactive compound like alkaloids and saponin mixtures are associated with DNA damage [29]. But further studies with other test system are required for confirming the genotoxic potential of AECT.

In order to evaluate the acute toxicity test of the AECT, we used zebrafish as a test model organism. The fundamental similarities in cell structure and biochemistry between animals and humans facilitate the use of this model animal for the early prediction of the likely effects of chemicals and complex mixtures on human populations. From acute toxicity test, we observed that AECT has pisicidal activity and also observed that fish mortality rate increased with increasing concentration of AECT. But we observed a negative correlation between LC values and exposure period. The LC_{50} value exhibited a decrease from 11.883mg/L for 24h to 8.159mg/L for 48h. The LC_{50} values of the AECT for two different time periods are shown in Table 2. Previous studies had reported the compounds mainly responsible for most of the ichthyotoxic properties of plants; these include rotenoids, phorbol esters and saponin etc. [30, 19]. As the AECT contains a mixture of compounds, we can't predict which bioactive compounds are responsible for fish death but as per our expectations it may be mainly due to the presence of a saponin mixture and phorbol esters. After exposure of the extracts, the fish behaviour was also assessed. After some minutes of the exposure of AECT to the zebrafish, the zebrafish showed the signs of stress. The behaviour observed after exposure include increased respiratory rate, loss of equilibrium, jerky movements and circular swimming just before they lose equilibrium. After long exposure they cannot move and finally sank to the bottom. No such behaviour was observed in the control fishes. There are reports that genotoxicity can be correlated with gametic loss, embryonic mortality and heritable mutation, thereby affecting survival at the individual and population level [31, 32, 33]. As the AECT contains genotoxic compounds and also have pisicidal activity, the extensive and indiscriminate use of this pisicidal plant for killing fish might cause loss of aquatic bio resources. It had also reported that used of higher quantities of pisicidal plants to catch fish resulted in the loss of biodiversity in natural aquatic ecosystem [34].

IV. Conclusion

The present study clearly illustrates that aqueous seed extract of *Croton tiglium* have the potential to cause pisicidal activity as well as genotoxic activity. However, there is evidence that specific genotoxins can induce different responses in prokaryotic and eukaryotic organism. So, further *in vivo* study is required to identify the active compounds responsible for causing genotoxicity to ensure the safe use of using *Croton tiglium* for medicinal purposes. The present finding may help to predict the ecotoxicity assessment and potential health risk of using *Croton tiglium* as fish poison and traditional medicine.

Bioactive compounds	**Aqueous extract of *C. tiglium***
Phenolic compounds	+
Alkaloids	+
Saponins	+
Terpenoids	+
Cardiac glycosides	-
Anthraquinone	-
Flavonoids	-
Carbohydrates	+
Tannins	+

+: Present; - : Absent.

Table1. Qualitative analysis of phytochemicals present in aqueous extract of *Croton tiglium*

Exposure period	**Estimates (mg/L)**	**Limits (mg/L)**	
		LCL	**UCL**
24h	LC_{10}=5.210	3.127	6.852
24h	LC_{50}=11.883	9.807	14.049
24h	LC_{90}=27.099	21.421	41.362
48h	LC_{10}=3.062	1.501	4.429
48h	LC_{50}=8.159	6.169	9.978
48h	LC_{90}=21.742	16.923	33.683

Table2. Acute toxicity (LC_{50}) for the aqueous extract of *Croton tiglium* at different intervals against zebrafish. LCL: Lower confidence limit; **UCL**: Upper confidence limit.

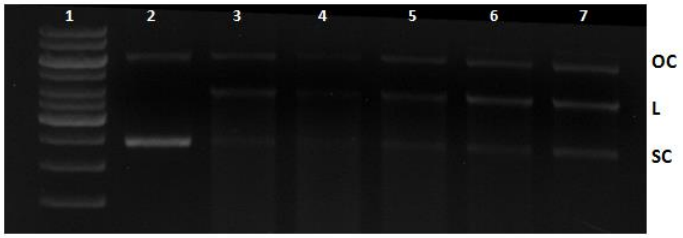

Fig.1. Agarose gel electrophoresis of pTZ57R/T plasmid exposed to aqueous extract of Croton tiglium or with $SnCl_2$. Lanes: 1: 500bp ladder; 2: pTZ57R/T; 3: $SnCl_2$ (200µg/mL); 4: 300µg/mL; 5: 225µg/mL; 6: 150µg/mL and 7: 75µg/mL; OC: Open circular; L: Linear; SC: Super coil.

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