

TOXICOLOGICAL EVALUATION OF *SPHAERANTHUS INDICUS* ON NON-TARGET FISH SPECIES.

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ABSTRACT

Sphaeranthus indicus has been proved effective larvicidal against mosquito larvae. Before application in the field its effect on non-target species must be evaluated, so in the present study toxicological evaluation of *Sphaeranthus indicus* extract was carried on *Gambusia affinis* as a model organism of an aquatic system. The fish were exposed to ethanol based *Sphaeranthus indicus* extract of various concentrations ranging from 20µl/L - 140µl/L. The LC₅₀ value of ethanol extract of *Sphaeranthus indicus* against *Gambusia affinis* was found to be 59.47µl/L. Treatment of *Gambusia affinis* with varied concentrations of the extracts reported many behavioral changes, like erratic swimming, hypo-pigmentation, gathering at one corner, hyper secretion of mucous, downward and vertical swimming patterns etc. The biochemical changes in the gills of fish were also noted at sublethal concentrations of toxicant. DNA and RNA content both were found to be decreased after treatment by 32.49% and 44.75%. The results suggest the meticulous use of even these botanicals as insecticides.

KEYWORDS: DNA, *Gambusia affinis*, larvicidal, Piscicidal activity, RNA, *Sphaeranthus indicus*.

INTRODUCTION

Mosquitoes have been declared as a “public enemy number one” by WHO because of the numerous diseases associated with it. Over the ages scientists are working to explore various methods to control these and the usage of organophosphates and organochlorines have come up as an easy option. These adulterations of environment with varied pollutants have surged the deadly consequences. An increasing number of well-designed epidemiological and molecular studies offer enough evidences about the association of pesticide usage with the high cancer risk (Engel *et al.*, 2017). Inhibition of Acetyl cholinesterase activity was shown on exposure to several pesticides (Maria Giulia Lionetto *et al.*, 2013). The problems of bio accumulation, resistance, persistence has also been associated with chemical pesticides. Therefore the development of the alternate technology based on neem and other plant products which have been found to be effective against mosquito larvae (Oadiipupo *et al.*, 2019; Dua *et al.*, 2009). Many botanicals has been investigated for their pesticidal and insecticidal properties like pyrethrins (Kraiss and Cullen, 2008), essential oil like those obtained from garlic (Susheela *et al.*, 2016), Quinones (De Sousa *et al.*, 2010, Magdum *et al.*, 2001), neem extract (Oadiipupo *et al.*, 2019) *Sphaeranthus indicus* as larvicidal compound (Kalimuthu, 2012).

Sphaeranthus indicus has been studied extensively as a botanical to be used against mosquito larvae (Arivoli *et al.*, 2016; Kalimuthu 2012; Purnakanti *et al.*, 2016) and cockroaches (Shrinivasan *et al.*, 2006) although; many other such insecticides are in the market for competition. But unfortunately, the random selection and usage of different insecticides causes lots of damage to non-target species. When insecticides enter into the aquatic systems, the environmental costs can be high. Numerous means by which insecticide can seek its way out in aquatic systems could be through agricultural runoff, spraying operations, by leaching through soil and other times we directly add it into the water bodies to deal with mosquito larvae. Fishes are very sensitive to a wide range of toxicants. Many species of fishes have been noted for the uptake and accumulation of various pesticides (Abdul-Khaliq and Al-Jowari, 2011).

Recent studies have proved that even low dosage of pesticides which enter the aquatic environment produce many physiological and biochemical changes (Prashanth *et al.*, 2011; Yilmaz, 2004 and Sarikaya and Yilmaz, 2003). This may be reflected in their behavior which can be studied and analyzed as indicators of pesticide in aquatic system.

Botanicals have an advantage over chemicals pesticides as they have been substantiated comparatively safer for non-target species. So in the present study the botanical *Sphaeranthus indicus* has been taken under investigation to find out its toxicity against *Gambusia affinis*.

MATERIALS AND METHODS

Sphaeranthus indicus is an annual spreading herb, which grows approximately 15–30 cm in height. The plant is distributed throughout the plains and wetlands in India, Sri Lanka and Australia (Gogate, 2000). All the parts of the plant have medicinal uses. In folk medicine, the plant is reportedly used in treating epileptic convulsions, mental illnesses and hemicranias Kritikar and Basu (1987). The whole herb is used in Ayurveda preparations to treat various ailments like epilepsy, diabetes, leprosy hernia, cough etc. (Paranjape *et al.*, 2001). Fresh aerial part of *Sphaeranthus indicus* were washed thoroughly in tap water and shade dried at room temperature ($28 \pm 2^\circ\text{C}$) for 5 to 10 days. The air-dried materials were powdered separately using an electrical blender. From the plant, 50 gm dried powder is extracted with 200 ml of ethanol in a Soxhlet apparatus for a period of 72 h each and filtered. The extracts were concentrated at reduced temperature on a rotary vacuum evaporator and stored at a temperature of 4°C . One gram of the plant residue was dissolved in 100 mL of ethanol (stock-solution) considered as 1 % stock solution. From this stock solution, different concentrations were prepared like 20 $\mu\text{L/L}$, 40 $\mu\text{L/L}$, 60 $\mu\text{L/L}$, 80 $\mu\text{L/L}$, 100 $\mu\text{L/L}$, 120 $\mu\text{L/L}$ and 140 $\mu\text{L/L}$.

The fish used for toxicity testing was *Gambusia affinis*. Fish were made available by a local fish breeder and transported to laboratory in plastic bags partially filled with water and air. The fish of similar size were taken out from the tank and transferred to another aquarium where they were acclimatized in dechlorinated water for 15 days. In the course of this period the fish were fed on commercial pellet food at least once a day. In order to nullify the influence of the excreta and left over food material the water in the aquarium was changed on alternate day. The water was aerated intermittently to avoid the effect of stratification and to maintain dissolved oxygen not less than 4mg/lit. The temperature for the acclimatization & test was maintained as $27^\circ\text{C} \pm 2$. The feeding was stopped 24 hours prior to the start of experiments. The toxicant used in static bio assay was a potential larvicidal compound against Mosquito larvae.

Acute Toxicological Studies

Acute Toxicological Studies (Determination of LC_{50}) was conducted in three replicates of the control and eight treatments with different concentrations of the toxicant ranging from 20 $\mu\text{L/L}$ - 140 $\mu\text{L/L}$. ten fish of equal size were introduced in each jar. The extract concentration of each container were freshly made and replaced after every 24 hours. The mortality of fish in each concentration of the toxicant was observed and recorded daily at the intervals of 24, 48, 56, 72 and 96 hours exposure and the behavioral changes in the fishes were observed and noted. In this study the acute toxicity of ethanol extract of *Sphaeranthus indicus* on *Gambusia affinis* was determined by the use of NCSS statistical software LC_{50} . Fishes treated with sublethal concentrations (20-60 $\mu\text{L/L}$) of *Sphaeranthus indicus* were taken after 96 hours of treatment and dissected to take out gills. Gills were washed in buffered saline and 10% homogenate was prepared in buffered saline (0.15 mol/L NaCl and 0.15 mol /L sodium citrate, pH 7). Homogenate was centrifuged at 8000rpm for 15 mins and obtained supernatant was taken for estimation of DNA and RNA by DPA and orcinol methods respectively (Schneider 1957) and absorbance was measured at 595 and 665nm respectively for DNA and RNA.

RESULTS

Acute toxicity assessment:

Table 1: Mortality of *Gambusia affinis* over the treatment period of 96 hours.

Sr. No.	Concentration of <i>Sphaeranthus indicus</i> extract ($\mu\text{L/L}$)	Percent mortality (%)
1	control	0
2	20	10
3	40	30
4	60	40
5	80	50
6	100	80
7	120	100
8	140	100

Percent Mortality of *Gambusia affinis* during the acute toxicity assessment period of 96 hours was shown in table no. 1. The mean LC_{50} value of ethanol extract of *Sphaeranthus indicus* on *Gambusia affinis* was found to be 59.47 $\mu\text{L/L}$ SE ± 7.25 .

Behavioral changes observed during acute toxicological studies:

In present study, various behavioral anomalies were reported on subjecting *Gambusia affinis* to various concentrations of ethanol extract of *Sphaeranthus indicus* ranging from 20 µl/L- 140 µl/L. Fish in control jar exhibited normal behavior although fishes in treated jars exhibited varied behavioral and morphological abnormalities with the increase in the concentrations of extract of *Sphaeranthus indicus*. Initially with the exposure of toxicant the fish showed vertical and downward swimming patterns. However, within 1-2 hours of exposure they calmed down and swimming speed of the fish decreased with increase in the time of exposure and concentration of *Sphaeranthus indicus*. Some other changes were also observed like; Hypo-pigmentation, decreased level of sensitization, Surfacing phenomenon, mucous secretion, hemorrhage regions, increased distance between gills and operculum and Jerky movements.

Biochemical alteration:

Increasing the concentration of ethanol extract of *Sphaeranthus indicus*, the reduction in the quantity of DNA and RNA (DNA estimation by DPA method and RNA estimation by Orcinol method is shown in table no. 2)

Table 2: Variation in DNA and RNA content in gills of intoxicated *Gambusia affinis*

Sr. No.	Concentration of <i>Sphaeranthus indicus</i> (µl /L)	DNA content of gills (µg/10 mg gills ± SD)	RNA content of gills (µg /10 mg gills ± SD)
1	Control	94.40±1.21	48.00±10.5
2	20	89.53±2.15	40.71±6.39
3	40	67.13±3.7	34.63±9.7
4	60	63.73±4.6	26.52±3.6

DISCUSSION

Mosquito breeding is the major issue involved with stagnant water bodies irrespective of their size. Varied solutions in the form of synthetic insecticides are readily available in the market, but those have their own consequences. Botanical insecticides may serve as suitable alternatives to synthetic insecticides as they are relatively safe, degradable and are readily available in many areas of the world. Several plants from different families have been reported for their activity against mosquito, in spite of that only few botanicals have moved from the laboratory to field use, like neem-based insecticides, which might be due to the light and heat instability of phytochemicals (Green *et al.* 1991). The plants giving out insecticidal substance is massive. The effect of *Sphaeranthus indicus* extracts on *Gambusia affinis* at different concentrations and at different exposures time was substantial. These behavioral anomalies and decline in nucleic acid content indicates that even botanicals have to be used wisely and in a controlled manner.

The mean LC₅₀ value of *Sphaeranthus indicus* on *Gambusia affinis* was observed to be 59.47 µl/L. Concentration less than 20 µl/L was considered as a safe concentration for *Gambusia affinis* where zero mortality was observed without any behavioral anomalies. The toxicity increased with the increase in exposure time and concentration. Behavioral changes are the most sensitive indication of potential toxic effects of compounds. Optomer responses are very useful in evolution of the behavioral changes of fish. In the present study behavior of control fish was normal with well-coordinated movement and alertness. Though, in the sets with *Sphaeranthus indicus* extract, the fish exhibited irregular swimming pattern, erratic and jerky movement. Fish also exhibited decreased level of sensitization which was in accordance with the work of Singh-Gupta *et al.* (2018), Gupta and Jawale (2013), Gul *et al.* (2009), Shambanagouda *et al.* (2009). This desensitization was probably due to the inhibition of AchE activity, leading to accumulation of acetyl choline in the cholinergic synapsis further leading to hyper stimulation (Rao *et al.* 2005). Mucus secretion was also observed here and similar findings were reported by Gupta and Jawale (2013), Prashanth *et al.* (2011) and Sindhe *et al.* (2007). Mucous secretion has been explained as an effort of an organism to reduce its contact with toxicant by creating barrier between the body and toxic media. Beyond LC₅₀ value hemorrhage region was observed in fish which coincides with the results obtained by Saxena *et al.*, (2005). Initially at lower concentrations of *Sphaeranthus indicus*, hypo-pigmentation of the fish was observed whereas at higher concentrations they acquired hyper pigmentation just before dying. A similar result of hypo pigmentation was observed by Shahi and Singh (2011). This change in body color was explained by Rao *et al.* (2005), as impairment of pituitary functions reflected by reduction in number and size of the chromatophores and their pigment content. Increased distance between gills and operculum was observed in accordance

with the results of Singh-Gupta *et al.* 2018 and Khunyakari *et al.* 2001. Surfacing phenomenon or Gathering of fish at the surface of medium was observed in sub-lethal concentrations which were in accordance with the Prashanth *et al.* (2011). This Gathering at the surface may help the fish to avoid contact with the toxic medium, as seen by Katja *et al.* (2005).

Finally fish sank to the bottom with the least opercular movements and died with their mouths open. These behavioral changes observed in the fish can be the effect of biochemical alteration. Nucleic acids are the major components that control all metabolic activities. If there is any degradation of these nucleic acids, it will disturb the complete metabolic processes resulting in physiological and behavioral changes. The alterations in DNA levels could be due to the disturbances in the synthesis and turnover rate of DNA besides degenerative changes. Nucleic acid content is considered as measure of capacity of an organism for protein synthesis. In the present study the effect of *Sphaeranthus indicus* on the DNA and RNA in the gills of *Gambusia affinis* was observed as declining, which could be summed up as an effect of uptake of toxicant across gills resulting in degradation of DNA and RNA. This result was in accordance with the Gautam *et al.* (2002), Durairaj and Selvarajan (1992). In the present study the decrease of DNA is not very prominent as compared to RNA and can be attributed to the increased activity of DNAase as suggested by Tayyaba *et al.* (1981). Hence it can be concluded that the present test compound, ethanolic extract of *Sphaeranthus indicus* which has medicinal usage (Ali *et al.*, 2011), (Nahata *et al.*, 2012) has a potential to be used as an insecticide (Kalimuthu, 2012) also, although its usage as an insecticide should be a meticulous one as its usage in large quantity has been proved harmful for *Gambusia affinis*, a nontarget organism.

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