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Research Article

EFFECT OF *SPIRULINA PLATENSIS* EXTRACTS ON EARTHWORM, *EISENIA FETIDA*

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ABSTRACT

Earthworms are farmer's friend, which play an important role in the soil ecosystem. Hence, application of any agrochemical, biopesticides and any other biological products for crop protection needs to be evaluated for its effectiveness (toxicity) towards Earthworm. In the present experiment, three types of *Spirulina platensis* extracts were tested against earthworm, *Eisenia fetida* in a 14 day acute earthworm toxicity study. The *Spirulina* extracts were prepared using solvents, Acetone (100%), 95% Ethanol and 95% Methanol respectively. The concentrations of the extracts were prepared using in deionised water and mixed with artificial soil and incubated under controlled environmental conditions (at 20±2°C temperature in 400-800 Lux light intensity) for 14 days. Four replicates were maintained per treatment. Concurrent solvent controls and a control with deionised water were maintained for comparison. On day 14, the biomass change and the mortality of the earthworms were determined to assess the LC_{50} (Lethal Concentration) and the NOEC (No Observed Effect Concentration) (related to biomass change and mortality) of the tested concentrations of the extracts. The study results revealed that all the extracts are safer to earthworms in the tested concentration. Hence, it is concluded that the *Spirulina* extracts are non-toxic to earthworms in artificial soil under laboratory conditions.

Keywords: *Spirulina platensis*; *Eisenia fetida*; OECD 207; Acute earthworm toxicity;LC_{50}

1. INTRODUCTION

The extensive use of the agrochemicals influences the soil fertility and ecosystem adversely. Literature is available on frog deformity in agro ecosystem due to high level pesticide usage [1]. Similar observations are made in other species too. Hence, these observations enlighten the necessity of alternative eco-friendly sources of chemical pesticides for crop protection to conserve ecosystem. Certain biological systems like *Blue green algae* (BGA) are studied for its antibacterial and antifungal effects against plant pathogens [2]. These properties of BGA help in developing an eco-friendly biopesticide. The influence of algal extracts including *Spirulina platensis* on plant seed germination and plant growth was also studied and verified [3]. However, they too possess biotoxins which in turn affects the ecosystem. Some of the biotoxins are tested against animal model like mouse and aquatic model (micro algae) [4, 5]. Studies conducted with algal extracts reveal the positive and negative impact of crude extracts against micro algae [6]. In *vitro* studies revealed that acetone and ethanol extract of *Spirulina platensis* are effective against rice fungal pathogens *Magnaporthe grisea* and *Rhizoctonia solani* respectively [7]. However such studies against terrestrial organisms are scarce. Hence the present research helps in understanding the effect of algal extracts on earthworm, one of the crucial terrestrial organisms in ecosystem.

In the current study, three solvent extracts *viz.,* Acetone (100%), 95% Ethanol, 95% Methanol of *Spirulina platensis* are compared for its suitability to use without harming earthworm ecosystem.

The extracts were represented as A+SP, 95% E+SP and 95% M+SP respectively.

2. MATERIAL AND METHODS

The experiment was conducted in the department of Ecotoxicology, International Institute of Biotechnology and Toxicology (IIBAT), Padappai, Tamil Nadu, India.

The test species, *Eisenia fetida* (Savigny, 1826) was procured from a GLP certified laboratory, Germany and bred in the department of Ecotoxicology, IIBAT under standardized conditions. The species were also confirmed by Dr.Sultan Ismail, Ecoscience Research Foundation, Chennai.

The *Spirulina platensis* CCC 477 culture was procured from CCUBGA (Blue green algae division), Indian Agriculture Research Institute (IARI), New Delhi and

mass cultured in IIBAT using modified Zarrouk medium (pH 9.5 to 10.0).

2.1. Preparation of The Extracts

The dried powder of *Spirulina platensis* were (25 grams each) mixed with 100 ml of solvents, Acetone, 95% Ethanol and 95% Methanol respectively and allowed to stand for 24 hours with intermittent manual shaking in dark condition. After 24 hours, the extracts were filtered using Whatman No.1 filter paper. The crude residue was soaked again in fresh respective solvents for another 48 hours. The filtrate from the first soaking and second soaking was then mixed together. The combined filtrates were concentrated using rotary evaporator until the material becomes gummy. The gummy material was collected using 10ml of respective solvents [8, 9] (**Fig.2**). The phytochemical analysis was done for the extracts and solvents [10-12] (See **Table1)**.

Fig. 1: Microscopic view (40X) of 14 days old *Spirulina platensis* culture in modified Zarrouk medium

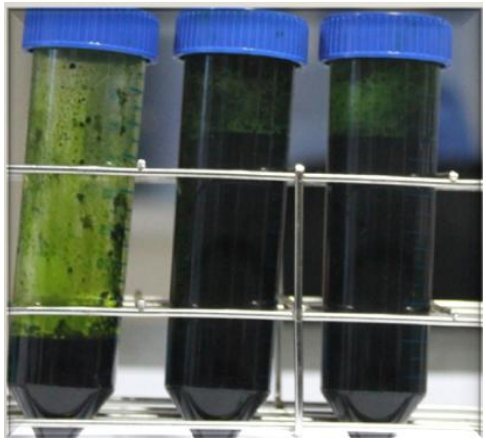

Fig. 2: Acetone, Methanol and Ethanol extracts

2.2. Preparation of Artificial Soil

The artificial soil was prepared according to OECD 207("Earthworm, Acute Toxicity Tests"), (1984), ISO 11268-I, (2012) and KCR, (2014) test guidelines [13-15]. The soil ingredients, sphagnum peat, kaolin clay and fine sand were mixed in proportion of 10:20:70 using a soil homogenizer. The Sphagnum peat blocks were imported from Gramoflor GmbH, Germany, ground and sieved using sieves with pore size less than 2 mm. The kaolin clay was purchased from ROMAC India Industrial minerals, Chennai, and fine sand was procured from Bhuvaneswari Hardwares, Chennai and sieved using 50 micron and 200 micron sieves to achieve the required particle size. After the preparation, the pH of the artificial soil was confirmed as 6.4.

2.3. Determination of Water for Moistening the Soil

The water for moistening the soil is calculated based on Maximum Water Holding Capacity (MWHC) of the soil ISO No. 11268 -1(2012) and OECD 222(2016) [15, 16]. Water corresponds to 40 - 60% of the Water Holding Capacity (WHC) is used for moistening artificial soil which is ideal for earthworm survival.

The method of WHC determination is described below:

A double end open glass tube (10.0 × 5.0 cm) with filter paper covering one of its ends was weighed (T) and artificial soil (approximately 100 g) was filled compactly through the open end to a depth of 5 - 7 cm of the tube. The tube was then gradually immersed in water taken in a wide mouth bowl, until the water level was just above the top of the soil. This experimental setup was kept undisturbed for 3hours. After 3 h the excess water from the soil samples was drained under saturated conditions. For this, the tubes were placed on the sand saturated with water for 2h by covering with another bowl. The sample was weighed (S) after 2 h and dried at 105°C for 3hours in a hot air oven.

The sample was weighed after drying (D) and the maximum water holding capacity of the artificial soil was calculated as follows:

$$\text{WHC (\% dry mass)} = \frac{S\text{-}T\text{-}D}{D} \times 100 \quad (1)$$

where, S = water saturated soil + mass of the tube + mass of filter paper (g)

T = Tare (mass of tube + mass of filter paper) in g

D = Dry mass of the soil (g)

The WHC calculated for the artificial soil was 43.65%

2.4. Evaluation of Earthworm Acute Toxicity

The Glass beakers of 1L capacity with a cross sectional area of 113 cm^2 was used for the experiment. About 500g dry artificial soil was filled into the beaker. The water for moistening the artificial soil was calculated as 25ml/100g soil (corresponds to 57.27% of WHC).Hence the water required per replicate is calculated as 125 ml. Half of this amount was added with the soil one day prior to the experiment to limit the dust emission during the extracts application. The concentrations selected for the extracts were 62.5, 125, 250, 500 and 1000 mg/Kg with a geometric factor 2 for conducting the experiment. To compare the effect of the *Spirulina* extracts with that of its respective solvents, corresponding solvents were tested at 1000 mg/Kg concentration (the maximum tested concentration of an earthworm study). Apart from this an untreated control (soil moistened with deionised water) was also maintained for over all comparison. The extracts and solvents were weighed for 1Kg dry soil and applied to the soil with the remaining water after premoistening. The soil was homogenized with a soil blender to mix the extract uniformly in the soil. Healthy earthworms, 4-5 months old with well-developed clitellum with wet weights of approximately300-600mg/worm (with gut content) were selected. The worms were acclimatized for one day in the artificial soil, washed with tap water, blotted carefully with filter paper, weighed and released on the surface of both treated and untreated artificial soil. Four replications for each test concentrations and control were maintained with ten earthworms each. The test containers were incubated in a controlled room with a temperature of 20±2°C and 400-800 LUX continuous light for 14 days.

After 7 and 14 days exposure, the artificial soils from the containers were emptied and observed for live / dead earthworms. Due to rapid decomposition in the soil, the lost earthworms were considered as dead. After the mortality check on day 7, the live earthworms and the artificial soil were returned to the respective test containers. The total and the mean body weights of all live earthworms in each test container were determined at the test start (day 0) and on the final day (day 14). Based on the weight difference between initial and final weight, the biomass change was calculated. At the test start (day 0) and on the final day (day 14), moisture content and pH of the artificial soils were assessed [17, 18].

2.5. Statistical Analysis

Since no mortality was observed in any of the concentrations, analysis of LC_{50} and NOEC related to mortality was not applicable for this experiment. The NOEC based on biomass change was analysed using Dunnett test ($\alpha = 0.05$). All the statistical analysis was done using ECOSTATS statistical software in SAS environment (SAS version 9.3). One-way ANOVA followed by LSD (least significant difference) for post hoc comparison, was performed to do the overall comparison of data [16].

3. RESULTS AND DISCUSSION

3.1. Phytochemical Analysis of Extracts

Qualitative phyto chemical analysis of extracts was done to understand the nature of the extracts (Table 1). All the extracts possessed the phytochemicals like Alkaloids, Tannins, Flavanoids and Saponins.

3.2. pH and moisture content of treated artificial soil

The pH and moisture content of treated artificial soil on day 0 (at start) and on day 14 (final day) is assessed (Table 2). The moisture content was maintained within 10% deviation from day 0 - day 14.

3.3. Effect on Mortality

No mortality was observed in any of the concentration tested indicating that the extracts have no lethal effects on Earthworm, *Eisenia fetida*.

Table 1: Phytochemical Analysis of Extracts

Parameter	Extracts and solvents		
	Acetone	Ethanolic (95%)	Methanolic (95%)
Alkaloids (Mayer's Test)	Present	Present	Present
Tannins (Ferric Chloride test)	Present	Present	Present
Flavanoids (Sodium Hydroxide test)	Present	Present	Present
Terpenoids/Steroids (Salkowski Reaction)	Present	Present	Present
Saponins (Foam test)	Present	Present	Present

[1] *Representative sample from 4 replication;* [2] *Mean of 4 replications*

Table 2: pH &Moisture Content of Treated Artificial Soil of Earthworm, *Eisenia fetida* Treated with the *Spirulina* Extracts

Extracts	Concentrations (mg/Kg) dry soil	pH[1]		Moisture content(%)[1]	
		Start (day0)	End (day14)	Start (day0)	End (day14)
Control	Deionized water	7.17	7.53	25.10	24.98
A+SP Extract	Acetone (Control)	7.70	7.76	25.34	25.10
	62.5	7.88	7.89	25.12	25.17
	125	7.85	7.90	25.03	25.11
	250	7.86	7.72	24.99	2.52
	500	7.84	8.00	25.07	25.09
	1000	7.76	7.96	25.04	25.08
95% E+SP Extract	95% Ethanol (Control)	7.87	7.92	25.22	25.14
	62.5	7.91	7.85	25.32	25.22
	125	7.77	7.79	25.14	24.99
	250	7.91	7.86	25.08	25.19
	500	7.88	7.92	25.06	25.14
	1000	7.86	7.90	25.09	25.16
95% M+SP Extract	95% Methanol (Control)	7.79	7.82	25.04	25.11
	62.5	7.56	7.77	25.06	25.14
	125	7.76	7.86	25.07	25.09
	250	7.76	7.84	25.09	25.13
	500	7.79	7.92	25.11	25.21
	1000	7.74	7.81	25.24	25.01

[1] *Representative sample from 4 replications*

3.4. Effect on Biomass

The biomass change was calculated using the following formula:

$$\text{Moisture content\%(dry weight basis)} = (I - F)/F \times 100 \quad (2)$$

I- Initial weight

F- Final weight

The decrease in biomass change from the pre weight is indicated by '-' symbol.

The biomass changes (weight changes after exposure) in the controls were-12.16% (Deionised water), -7.17% (Acetone), -3.55% (95% Ethanol) and -5.44% (95% Methanol). Among the controls, 95% Ethanol exhibited less biomass change (Table 3 and Fig. 3).

Since deionized water and solvent controls used for comparing the data within the same extract group, significant difference between the deionized water control group and the corresponding solvent control group was analysed in SAS. The NOEC related to biomass change of the each extract was analysed by Dunnet test.

In the acetone based *Spirulina* extract group (A+SP), no significant difference in the biomass change was observed between the water and Acetone control. Hence the average of the controls was taken for comparing the different concentrations of the A+SP extract in order to find out the NOEC related to biomass change.

In 95% E+SP extracts, significant difference was observed between the water control and 95% Ethanol control. Hence to do the analysis in the worst case scenario, the control group which exhibited maximum biomass change was taken for comparison. Here the water control showed maximum biomass change and the same was compared with the concentrations of 95%E+SP extract (Table 3) for adjudging NOEC. Here, even though the concentrations 62.5 (-2.40%),125 (-2.99%),250 (-1.50%) and 500 (-0.44%) mg/Kg exhibited significant difference in biomass change compared to the water control, it was considered as positive effect, that is gain in biomass compared to the water control(-12.16%).

Table 3: Biomass Change of Earthworm *Eisenia fetida* Exposed to *Spirulina* Extracts in 14 days Artificial Soil Test

Extracts	Concentrations (mg/Kg) dry soil	Biomass change (%)					Biomass change NOEC (mg/kg dry soil)
		RI	RII	RIII	RIV	Mean	
Control	Deionized water	-7.21	-11.54	-19.01	-10.87	-12.16d	
A+SP Extract	Acetone (Control)	-9.79	-6.45	-4.58	-7.87	-7.17bcd	1000[1]
	Average (Deionised water + Acetone) controls	-8.51	-9.01	-11.72	-9.37	-9.65	
	62.5	-4.33	-2.58	-6.81	-8.08	-5.45abc	
	125	-12.02	-6.30	0.16	-2.96	-5.28abc	
	250	-5.25	-6.18	-7.75	-5.25	-6.11abc	
	500	-7.47	-10.67	-4.31	-4.56	-6.75bcd	
	1000	-19.36	-10.71	-4.05	-6.10	-10.05cd	
95% E+SP Extract	95% Ethanol (Control)	-3.59	-7.25	-1.18	-2.16	-3.55ab	1000[2]
	62.5	-4.78	0.22	-4.25	-0.77	-2.40ab*	
	125	0.29	-5.84	-3.93	-2.47	-2.99ab*	
	250	-4.00	-0.36	1.06	-2.70	-1.50ab*	
	500	3.27	-0.48	-2.15	-2.38	-0.44a*	
	1000	-3.85	-0.05	-15.43	-6.36	-6.42bcd	
95% M+SP Extract	95% Methanol (Control)	-6.50	-6.54	-6.21	-2.49	-5.44abc	1000[3]
	62.5	-4.58	-12.54	2.75	-8.93	-5.83abc	
	125	-2.68	-7.25	-6.92	-3.92	-5.19abc	
	250	-4.74	-15.75	0.69	0.27	-4.88abc	
	500	-5.67	-5.00	-5.07	-4.26	-5.00abc	
	1000	-8.74	-1.71	-4.46	-1.26	-4.04ab	

*The overall comparison done by One-way ANOVA followed by LSD (least significant difference) for post hoc comparison (Means with the same letter are not significantly different);[1]-For NOEC of A+SP extract, the data was compared with the mean of Deionized water and Acetone controls; [2]-For NOEC of 95% E+SP extract, the data was compared with the Deionized water control. Even though significant difference observed in all the concentrations except 1000 mg/Kg, it was considered as positive difference, that is significant increase in biomass compared to the control and so the NOEC is considered as 1000 mg/Kg; [3]-For NOEC of 95% M+SP extract, the data was compared with the Demonized water control; *- Values are statistically different from control by Dennett's test; $P < 0.05$.*

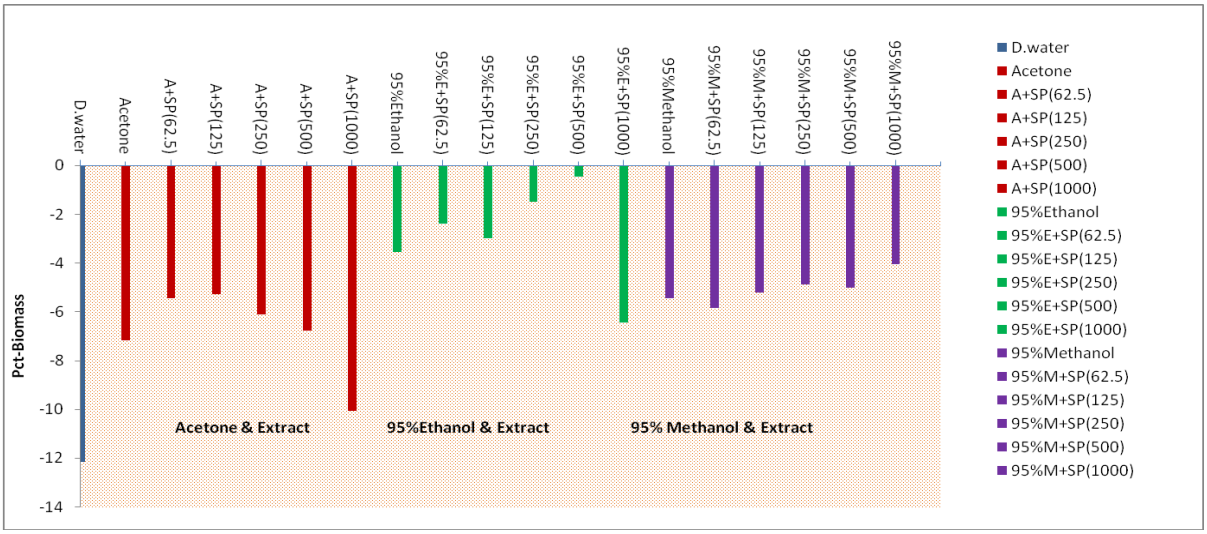

Fig. 3: Effect of *Spirulina* Extracts (Overall Comparison) on Earthworm Biomass Change in the Artificial Soil after 14 days Exposure

Similarly in 95% M + SP extracts, significant difference observed between the water and 95% Methanol controls. The water control was compared with the 95% M + SP extract concentrations.

The NOEC in all the extract group was observed as 1000 mg (extract)/Kg dry soil which indicates that none of the extract has negative influence on earthworm biomass.

Over all comparison using ANOVA was done among all the controls and extracts and found that all the extracts were comparable with the controls.

Here, the adverse toxicity effect towards the earthworms are reduced by *Spirulina* extracts which was in comparable with the findings of Mohmed Abdel-Daim et al.,(2016) who observed a reduced toxicity in mice due to *Spirulina* supplementation [20]. The non-toxic effect of the biologic compound against earthworms was in contrary with the findings of Altaf Hussain *et al*., (2016), where the toxicity of neem leaf extract was studied against earthworms [21]. But the toxicity of *Spirulina platensis* was observed in *Biomphalaria alexandrina* snails due to the presence of phytochemicals like total phenolic compounds, alkaloids and saponins [22]. However the earthworms have the capability of reducing the plant poly phenol toxicity by a class of unique surface-active metabolites termed 'Drilodefensins' present in their gut [23]. Similar mechanism is suspected in the reduction of toxic effect of *Spirulina* polyphenols against earthworms in this experiment.

4. CONCLUSIONS

The following conclusions could be drawn based on the results of the study:

- *Spirulina* extracts are not causing mortality effects in earthworms.
- The biomass change is lesser than the water control, indicating the enhancement of growth in the extracts compared to that of water control.
- Based on the overall results, it is concluded that under the laboratory experimental conditions, *Spirulina platensis* extracts are safer to earthworms despite of the solvents used for its preparation.

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