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Cytotoxicity Analysis of Partial Purified Extract of *Schizophyllum commune* (Schizophyllaceae) against *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae) †

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Abstract: In search for ecofriendly substitute to chemical pesticides, the present study was conducted to find the toxicity of a partially purified extract of an endophytic fungus *Schizophyllum commune* on *Spodoptera litura* (Fabricius). Toxicity was assessed by cytotoxic analysis using fluorescence microscopy on third instar larvae of *S. litura*. In addition, various morphological deformities in different life stages of *S. litura* (larvae, pupae and adults) were also recorded, after feeding second instar larvae to a diet having partial purified *S. commune* extract up to a prepupal stage. The results showed various morphological deformities in different life stages (larvae, pupae and adults) of the insect. In cytotoxic analysis, living cells percentage was highly reduced however apoptotic and necrotic cells percentage significantly increased in larvae fed with partial purified fungal extract for different time intervals (24hr, 48hr, 72hr and 96hr). The recovery of cytotoxicity induced by the fungal extract was almost negligible indicating its long term effect. Overall the study highlights the significance of pesticides which are biologically originated, eco-friendly and safe.

Keywords: Endophytic fungus; Polyphagous insect pests; Biocontrol agents; Ecofriendly.

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Introduction

Agriculture plays an important role to support global environmental, economical and social system. As the world's population is increasing at an alarming rate, in order to meet the need of food, the global challenge is to increase the yield, improve the quality of agriculture production as well as make it environmentally compatible. For this reason, the utilization of chemical pesticides as plants protective agents occupies the leading place. Despite long time effective control by synthetic insecticides, there are many issues which threaten the continued use of these agents. These include development of resistance in insects, their negative effect on human health and environment due to non-biodegradable nature [1]. Therefore, the present situation demands an eco-friendly alternative.

Biological control using fungi is a most promising technique as these microorganisms possess unique mechanisms of action while infection [2]. Among all microorganisms endophytic fungi have been known for their insect pests resistance nature. They grow inside the plant tissues without damaging to host plant [3]. They produce different secondary metabolites which secure their host plants from infectious agents and from various unfavorable conditions [4]. Number of studies has been reported the negative impact of fungal endophytes on development of insects [5, 6]. They were reported to induce morphological deformities in different stages of insects, reduce adult emergence and ultimately cause mortality [7-13].

Although fungal endophytes have been extensively used against various insect pests, but very less reports are available on the cytotoxicity of endophytic fungi against *Spodoptera litura*. (Fabricius) (Lepidoptera: Noctuidae) [13-15]. *S. litura* is a noctuid moth also called as Cluster caterpillar, Tobacco caterpillar, Oriental leafworm moth, Cotton leafworm, and Tropical armyworm. It has wide range of distribution throughout tropical and temperate Asia, Australia and Pacific basin [16]. *S. litura* is an economically important and a major polyphagous pest which seriously damage different vegetables as well as cash crops. Previous studies reported that more than 112 different plants species has been damaged by this insect [17]. Since it cause serious economic crops damage, the pest has been exposed to excessive high doses of insecticides. As consequences of using heavy dose of insecticides against this pest, it developed resistance against different insecticides [18]. Moreover, it is challenging to control this pest because of its cryptic habit and a high rate of infestation [17]. The use of fungal endophytes instead of other microbial agents is more beneficial due to their capability of attacking all the life stages of insects [19]. The biocontrol studies using endophytic fungi on *S. litura* has mainly focused on the ascomycetes fungi viz. *Beauveria bassiana*, *Metarhizium anisopliae* and *Nomuraea rileyi* [20-23]. Very fewer reports focus on other fungal groups [15].

On the basis of above discussion, the present study was conducted to analyze the effect of a basidiomycetes, an endophytic fungus *Schizophyllum commune* on *S. litura* by analyzing its effect on different development stages as well as on cytotoxicity parameters. Recovery of the cytotoxic effect was also studied to analyze the longevity of damage caused by the extract.

Materials and methods:

Rearing of Spodoptera litura (Fabricius):

The eggs of Spodoptera litura (Lepidoptera) were collected from the different cauliflower fields around Amritsar (India) and kept in BOD incubator for hatching. Larvae were reared on castor leaves. All stages of the culture were maintained in laboratory at proper temperature (25±2ºC), relative humidity (65±5%) and photoperiod12:12 (D: L) conditions.

2.2. Fungal culture isolation, production and identification

For the isolation of an endophytic fungus the leaves of *Aloe vera* were collected from Amritsar (India) and washed with distilled water. The collected leaves were cut into small pieces after sterilization. Small pieces were positioned on water agar plates containing ampicillin (200mg/ml). The plates were incubated (30ºC) till the emergence of hyphae. The hyphae were point inoculated on PDA (potato dextrose agar) plates. The culture was purified and used for further studies [13].

The production was performed in 50ml malt extract broth in 250ml Erlenmeyer flask for 10 days and extraction was done using different solvents hexane, chloroform and finally with ethyl acetate at 120rpm and 40ºC. In this way different impurities were washed with hexane and chloroform and final ethyl acetate extract we got was a partial purified instead of crude extract. The result of crude extract in which extraction was done only with ethyl acetate extract has been published in our previous reports [13]. The ethyl acetate extract was concentrated using rotavapor and dissolved in 1ml DMSO and stored at 4ºC [13]. The identification of fungus was done by morphological and molecular studies using ITS1 and ITS4 primer which amplifies ITS1-5.8S- rDNA- ITS2 region. Amplified ITS region was sequenced at first base sequencing (Malaysia). The sequence similarity was matched with other available databases retrieved from NCBI using BLAST [13].The identification results showed that the fungus was *Schizophyllum commune* as indicated in our previous study [13].

Bioassay studies:

To check the effect of partial purified *S. commune* extract on development parameters of *S. litura* different concentrations (125, 250, 500, 1000 and 2000µg/ml) of the extract were formed. The Second instar larvae (6 days old) were fed with fungal extract as well as with control diet (0.5% DMSO) under controlled temperature 25 ± 2^{0}C and relative humidity 70±5% conditions. Each experiment has six replicates and each replicate has five larvae. Each larva was reared in separate container (4×6cm). The diet was changed daily till pupation. The observations like larval mortality and deformities in different life stages were recorded.

Cytotoxicity measurements:

To analyze the effect of fungal extract on cytotoxicity in S. litura, Cell viability assay was carried out by using acridine orange/ethidium bromide (AO/EB) staining. At first the artificial diet was formed with fungal extract having concentration 763.629µg/ml (LC50 value) in 0.5% DMSO. The third instar larvae (12 days old) were treated with fungal extract diets and control diet (0.5% DMSO) at controlled temperature 25±2^{0}C and relative humidity 70±5% conditions. Each experiment has three replicates and each replicate has ten larvae. Each larva was kept in separate container (4×6cm) and the diet was changed daily. Cytotoxicity was assessed by taking haemolymph from 12 days old larvae. For this haemolymph was taken from 10 larvae treated with same concentration, pooled and stained with Acridine orange (100µg/ml) and Ethidium bromide (100µg/ml) dye mixer. The stained cells were observed under fluorescent microscope at 400X magnification. Viable cells emit green fluorescence and necrotic cells emit red fluorescence [12]. The effect of fungal extract has been noticed after 24hr, 48hr, 72hr and 96hr.

Recovery:

After giving fungal extract treatment to larvae at each time interval recovery of cytotoxic effect was recorded after 1day and 2days by giving control diet to treated larvae. Each exposure time (ET) has two recovery groups. i.e R1=Recovery after 1day, R2=Recovery after 2days. Each experiment was replicated three times with 20 larvae per replication, out of these 10 were used to check recovery after 1day and 10 for 2day. Each larva was reared in separate container (4×6cm) and the diet was changed daily.

Statistical analysis:

To compare the different concentrations in bioassay studies, to check the effect of duration and in recovery studies one way analysis of variance (ANOVA) with Tukey's test was applied and to analyze the effect of treatment, student's t-test was performed.

Results:

Effect of partial purified ethyl acetate extract of S.commune on survival of S. litura:

The partial purified extract of *S. commune* imparts negative impact on survival of *S. litura* larvae. Larvae fed with diet having partial purified extract of *S. commune* resulted 26.66-66.66% mortality in comparison to 10% in control. With increase in concentration larval mortality has been increased but significant effect was observed at highest concentrations 1000µg/ml and 2000µg/ml, which caused 53.33% and 66.66% mortality, respectively (F=16.240, p≤0.01) (Table. 1). The LC50 value of a partial purified extract of *S. commune* was 763.629µg/ml (Table. 2).

Table 1. Mortality of *S.litura* larvae fed on diet supplemented with different concentrations of partial purified ethyl acetate extract of *S.commune.*

Concentrations (µg/ml)	Larval Mortality (%) (Mean±S.E)
Control	13.33±4.22^{a}
125	26.66±4.21^{ab}
250	33.33±4.21^{ab}
500	40.00±10.32^{bc}
1000	53.33±6.66^{bc}
2000	66.66±8.43^{c}
F value	7.903**

The values are mean ± standard error. Significance ascribed as * p<0.05 and **p<0.01(One way ANOVA). Different letters a, b, c within the column are significantly different (Tukey's test, p≤0.05) and signify the effect of concentration.

Table 2. Determination of LC50 value of *S.commune* partial purified extract on *S.litura*:.

Chemical	Insect	Upper limit	Lower limit	LC50
Ethyl acetate extract of fungus *S.commune*	*Spodoptera litura*	1117.638	566.190	763.629

Effect of partial purified ethyl acetate extract of S.commune on different development stages of S. litura:

As evident from the table.3, partial purified extract of *S. commune* has adversely affected the different development stages. It has induced various deformities in larvae, pupae and adults. More deformities were observed in pupae and adults as compared to larvae which were shown in table. 3. It caused 66.66% abnormalities in pupae and 16.66% in adults at highest concentration (2000µg/ml), however the effect was non significant in case of adult deformity while significant effect was found in case of pupal deformity.

Table 3. Pupal and Adult deformities in *S.litura* under the influence of partial purified ethyl acetate extract of *S.commune*.

Concentration (µg/ml)	Adult deformity (%) (Mean±S.E)	Pupal deformity (%) (Mean±S.E)
Positive control	3.33±3.33^{a}	6.66±4.21^{a}
125	0.00±0.00^{a}	40.26±10.18^{ab}
250	0.00±0.00^{a}	27.76±9.29^{ab}
500	27.76±9.29^{a}	44.43±15.90^{ab}
1000	16.66±10.54^{a}	61.08±9.29^{b}
2000	16.66±16.66^{a}	66.66±16.66^{b}
F value	1.593	3.531*

The values are mean ± standard error. Significance ascribed as * p<0.05 and **p<0.01(One way ANOVA). Different letters a, b, c within the column are significantly different (Tukey's test, p≤0.05) and signify the effect of concentration.

Effect of partial purified ethyl acetate S.commune extract on Cell viability of S. litura:

Partial purified *S. commune* extract has highly influenced the cell viability profile of exposed larvae. Significantly decreased in the living cells percentage and increased in the apoptotic and necrotic cells percentage was observed in all treated groups in comparison to control. In 24hr exposure group, the living cells percentage was decreased from 84.00±1.15% in control to 58.25±1.01% in treated group. The living cells percentage decreased with increase in time interval however decrease was non-significant ($F=5.104$, $p>0.05$) as revealed by ANOVA Tukey's test (Table. 4). Apoptotic cells percentage showed variable trend, maximum apoptotic cells percentage was observed at 24hr exposure group where value increased 3.04 fold than control ($t=9.36, p\leq0.01$). The effect of duration was found to be non-significant ($F=0.036$, $p>0.01$) (Table. 5). The necrotic cells percentage was significantly increased in all treated groups in comparison to controls. Minimum increase was found in 24hr exposure group, from 4.25±7.50 (control) to 7.50±0.57 (exposure group) ($t=4.50, p\leq0.05$) and maximum in 96hr exposure group, from 4.50±0.57 (control) to 17.75±0.43 (exposed group) ($t=18.30, p\leq0.01$). Necrotic cells percentage significantly increased with increase in time interval ($F=54.08$, $p\leq0.01$) revealed by Tukey's test (Table. 6).

Table 4. Living cells percentage in haemolymph of *S.litura* after treatment with partial purified ethyl acetate extract of *S.commune*.

Parameter	Group	Exposure Time (hrs)				
		24	**48**	**72**	**96**	**F value**
Living Cells Percentage	**Control**	84.00±1.15^a	82.75±1.29^a	86.25±0.43^a	83.25±1.01^a	2.261
	EG(LC50)	58.25±1.01^a	57.75±0.72^a	49.75±3.03^a	48.50±3.17^a	5.104*
	t – value	16.783**	16.823**	11.921**	10.428**	

EG=Exposed group. The values represented as mean ± standard error.* ascribes the significant difference between exposed group and control group (t-test, p≤0.05).Different letters a, b, c between the columns are significantly different (Tukey's test, p≤0.05) and signify the effect of duration.

Table 5. Apoptotic cells percentage in haemolymph of *S.litura* after treatment with partial purified ethyl acetate extract of *S.commune*.

Parameter	Group	Exposure Time (hrs)				
		24	**48**	**72**	**96**	**F value**
Apoptotic Cells Percentage	**Control**	11.25±1.88^a	11.02±1.08^a	9.25±0.72^a	12.25±0.43^a	1.154
	EG(LC50)	34.25±1.59^a	33.00±1.15^a	33.25±4.18*^{a}	33.75±3.61*^{a}	0.036
	t – value	9.357**	13.884**	5.650**	5.916**	

EG=Exposed group. The values represented as mean ± standard error.* ascribes the significant difference between exposed group and control group (t-test, p≤0.05).Different letters a, b, c between the columns are significantly different (Tukey's test, p≤0.05) and signify the effect of duration.

Table 6. Necrotic cells percentage in haemolymph of *S.litura* after treatment with partial purified ethyl acetate extract of *S.commune*.

Parameter	Group	Exposure Time (hrs)				
		24	**48**	**72**	**96**	**F value**
Necrotic Cells Percentage	**Control**	4.25±0.43^a	4.00±0.86^a	4.50±0.28^a	4.50±0.57^a	0.169
	EG(LC50)	7.50±0.57^a	9.25±0.43^a	17.00±1.15^b	17.75±0.43^b	54.082**
	t – value	4.503*	5.422*	10.502**	18.360**	

EG=Exposed group. The values represented as mean ± standard error.* ascribes the significant difference between exposed group and control group (t-test, p≤0.05).Different letters a, b, c between the columns are significantly different (Tukey's test, p≤0.05) and signify the effect of duration.

Recovery in cell death after treatment with partial purified ethyl acetate extract of S.commune to S.litura larvae:

Cell viability was also affected by treating larvae to control diet after exposure to partial purified *S. commune* extract. In 24hr recovery groups, living cells percentage was increased in both recovery groups however significant increase was observed only in R2 group, from 58.25±1.01(EG) to 66.50±0.58(R2) (F=8.659,p≤0.05). 48hr, 72hr and 96hr recovery groups showed non-significant decrease (Table. 7). Apoptotic cells percentage showed significant decrease only in 24hr recovery groups, in other groups non-significant decrease was observed. In 24hr recovery groups significant decrease was observed from 34.25±1.59(EG) to 24.50±1.15(R2) (F=7.373,p≤0.05) while in 96hr recovery groups the values decreased non-significantly (Table. 8). Necrotic cells percentage in 24hr and 72hr recovery groups showed non-significant increase. In 96hr recovery group significant increase in necrotic cells percentage was observed from 17.75±0.43(EG) to 25.00±2.31(R2) (F=5.731,p≤0.05) (Table. 9). Overall recovery was not observed in treated larvae even after feeding them to control diet.

Table 7. Recovery in Living cells percentage in haemolymph of *S.litura* after treatment with partial purified ethyl acetate extract of *S.commune*.

Parameter	Group	Exposure Time (hrs)			
		24	48	72	96
Living cells percentage	EG(LC50)	58.25±1.01^{a}	57.75±0.72^{a}	49.75±3.30^{a}	48.50±3.17^{a}
	R1	61.25±2.16^{ab}	53.25±2.74^{a}	51.50±1.44^{a}	51.03±1.71^{a}
	R2	66.50±0.58^{b}	60.50±0.87^{a}	48.25±1.01^{a}	45.25±2.74^{a}
	F value	8.659*	4.571	0.646	1.294

* (p≤0.05), **(p≤0.01). EG=Exposed group, R1= Recovery after 1day, R2= Recovery after 2 days. The values represented as mean ± standard error. Different letters a, b, c between the rows are significantly different (Tukey's test, p≤0.05) and signify the difference between exposed group and recovery group.

Table 8. Recovery in Apoptotic cells percentage in haemolymph of *S.litura* after treatment with partial purified ethyl acetate extract of *S.commune*.

Parameter	Group	Exposure Time (hrs)			
		24	48	72	96
Apoptotic cells percentage	EG(LC50)	34.25±1.59^{b}	33.00±1.15^{a}	33.25±4.18^{a}	33.75±3.61^{a}
	R1	32.00±2.59^{ab}	33.25±2.45^{a}	31.25±2.45^{a}	26.25±0.43^{a}
	R2	24.50±1.15^{a}	30.25±1.59^{a}	31.75±1.29^{a}	29.75±5.05^{a}
	F value	7.373*	0.842	0.129	1.091

* (p≤0.05), **(p≤0.01). EG=Exposed group, R1= Recovery after 1day, R2= Recovery after 2 days. The values represented as mean ± standard error. Different letters a, b, c between the rows are significantly different (Tukey's test, p≤0.05) and signify the difference between exposed group and recovery group.

Table.9: Recovery in Necrotic cells percentage in haemolymph of *S.litura* after treatment with partial purified ethyl acetate extract of *S.commune*.

Parameter	Group	Exposure Time (hrs)			
		24	48	72	96
Necrotic cells percentage	EG(LC50)	7.50±0.58^{a}	9.25±0.43^{a}	17.00±1.15^{a}	17.75±0.43^{a}
	R1	6.75±0.43^{a}	13.50±0.29^{b}	17.25±1.01^{a}	22.75±1.29^{ab}
	R2	9.00±0.58^{a}	9.25±0.72^{a}	20.00±0.29^{a}	25.00±2.31^{b}
	F value	4.610**	22.816*	3.410	5.731*

* (p≤0.05), **(p≤0.01). EG=Exposed group, R1= Recovery after 1day, R2= Recovery after 2 days. The values represented as mean ± standard error. Different letters a, b, c between the rows are significantly different (Tukey's test, p≤0.05) and signify the difference between exposed group and recovery group.

Discussion:

There are various fungal endophytes which are insect pathogenic. They live asymptomatically in healthy plant tissues [24, 25]. Webber [26] probably first time reported the endophytes role, *Phomopsis oblongata* in securing elm trees from beetle *Physocnemum brevilineu* (Say). The insecticidal activity of fungi is due to different secondary metabolites produced by them which have great significance in agriculture for pest management [27]. So, in a present study different metabolites of an endophytic fungus *Schizophyllum commune* were extracted using ethyl acetate. Before ethyl acetate extraction, extraction was also carried out with hexane and chloroform and finally with ethyl acetate extract, so, the ethyl acetate extract was not crude it was partially purified having impurities washed with chloroform and hexane. This partial purified extract of *S. commune* was evaluated for its toxicity on *Spodoptera litura* (Fabricius). Our previous study reported the toxicity of its crude extract on *S. litura* [13]. *S. commune* is an endophytic fungus belongs to basidiomycetes. Bioassay studies of a partial purified extract have showed that fungal extract induced high rate of mortality in *S. litura* which is 66.66% at 2000µg/ml concentration. Previously, the insecticidal activity of endophytes has been reported by various researchers [28-30]. Ingestion of diet having fungal metabolites by the larvae also induced various abnormalities such as larval pupal intermediates and deformities in pupae and adults. The morphological abnormalities recorded in the present study showed similarity to those reported previously in *S. litura* due to fungal endophytes [31-33]. These deformities might be due to imbalance of ecdysteroids as result of secondary plant metabolites [34]. Overall, the fungal extract induced the negative impact on survival and development of *S. litura*. Previous studies have highlighted the adverse effects of endophytes on survival and development parameters of various insect pests [35-37]. Endophytes isolated from mangrove plants reduced the survival of *S. litura* [22]. Metabolic extract of *Cladosporium* sp. [35, 38] and *Aspergillus niger* [31] showed adverse effects on development of *S. litura*. Endophytic fungi *A. flavus, Comothyrium* sp. and *Nigrospora* sp. imparted negative effect on the development of *Aphis gossypii* (Glover) [39].

In addition, the cytotoxic effects of both fungal extracts were also assessed using fluorescent stains, Acridine orange/Ethidium bromide. The method is based on the loss of integrity of plasma membrane as the cell die. The percentage of living cells, apoptotic cells and necrotic cells were used as parameters. Er *et al*. [40] used the methodology to analyze the cytotoxic effect of venom on haemocytes of ***Galleria mellonella*** (Linnaeus). During present investigation fungal extracts was found to cause cytotoxicity in *S. litura* due to which living cells percentage decreased and apoptotic and necrotic cells percentage was increased in treated larvae in comparison to control. This finding showed similarity with the finding of Karthi *et al.* [41] and Kaur et al. [42] which recorded the cytotoxic effects of *Aspergillus flavus* and ***Alternaria alternata*** against *S. litura*, respectively. Reduction in haemocytes was observed after exposure of Oosporein (metabolite of *Beauveria caledonica*) to *Hylobius abietis* (Linnaeus) and *G. mellonella* [43]. Mirhaghparast *et al*. [44] observed the toxicity of *Beauveria bassiana* and *Metarhizium anisopliae* on different haemocytes of *Spodoptera littoralis* (Boisduval). The cytotoxicity of *Aspergillus fumigates* metabolite fumagillin on *G. mellonella* was reported by Fallon *et al*. [45]. In addition to *in vivo* some *in vitro* studies also revealed the cytotoxicity of fungal metabolites. Fornelli *et al*. [46] reported the cytotoxic effect of mycotoxins (nivalenol, fumonisin B_1 and deoxynivalenol) on SF-9 insect cell line. The fungus *Nomuraea rileyi* induced morphological changes in SF21 cell line like membrane blebbing (Tseng *et al*. 2008). Fungal metabolites of *B. bassiana* were reported to cause cytotoxicity in SF-9 and SF-21 insect cell lines [47] and *Fusarium venenatum* found to be cytotoxic for SF-21 insect cell lines [48].

Conclusion

In nut shell the study decipher the cytotoxic potential of partial purified extract of *S. commune* against *S. litura*. After treatment, cell viability was also found to be decreased.

Cytotoxic effects did not get recovered as predicted from repair study. Overall, the study deciphers the potential of the partial purified *S. commune* extract to be exploited in agriculture as biocontrol agents.

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