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Antifungal Activity of *Turbinaria conoides* and Evaluation for the Effective Concentration against the Infection of *Beauveria bassiana* in Silkworm Larvae

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ABSTRACT

Research was aimed at investigation effect of dichloromethane and methanol (1:1) extract of seaweed brown algae, *Turbinaria conoides* for its antifungal activity against *Beauveria bassiana* an entomopathogenic fungus on silkworm based on *in vitro* by agar cups bioassay method and *in viva* application of crude seaweed algal extract to muscardine infected silkworm larvae. The extract of *T. conoides* has shown antifungal activity and inhibitory effect against this fungus through *in vitro* bioassay studies. The inhibitory effect increased with increased concentration of algal curde extract. Further, three different concentrations of this algal extract were tested against *Beauveria bassiana* infected silkworm larvae. The effective concentration was evaluated between 1000 to 1500 μg mL^{-1} of the algal extracts. It was established that with the application of these algal extract about 75 to 85% of larval mortality due to *B. bassiana* infection was controlled with out affecting the other qualitative and quantitative traits. Therefore, present communication sturdily dedicated to find out cheapest and eco-friendly disinfectant for prevent or treat fungal diseases in silkworm could be an alternative treatment process under disease management in sericulture industry.

Key words: Antifungal activity, *Turbinaria conoides, Beauveria bassiana,* fungal disease, *in vitro, in vivo,* silkworm

INTRODUCTION

Silkworm, *Bombyx mori* L. is an important economic Lepidopteron insect and utilized for the commercial production of natural silk fiber Queen of Textile. Its application in all over the world finely contributed to promotion as a powerful laboratory model for the basic and applied research in biology (Tazima, 2001; Ramesha *et al*., 2010). It is very much prone to various diseases caused by fungi, bacteria, viruses and protozoans in its life cycle by which heavy cocoon crop loss has been noticed in all sericulture practicing countries (Su, 1990). Among fungal Muscardine diseases can be caused by over seven fungi viz., *Beaueriana bassiana, Aspergillus flavus, A. oryzae, A. tanei, Paecilomyces farinosus, Sporosporella uvella* and *Metarhizium anisopliae*. The obvious characteristic of this type of infection is mummification of the dead larvae which become hard and powdery white like sticks of chalk. The fungus *Beauveria bassiana* (Bals.) Vuill is the most common and virulent pathogen that caused white muscardine disease in silkworm (Jayaramaiah *et al.,* 1986).

Several attempts have been made to control white muscardine disease by application of chemicals and fungicides. By dusting of ceresin lime on the silkworm body has been effective for control of the white muscardine disease. *In vitro* studies on twenty seven different chemicals in different concentrations were found to be effective for white muscardine disease (Samson and Mummigutti, 1979). Presently lime dusting and application of certain recommended fungicides are being used for the control of *B. bassiana* (Byrareddy *et al.*, 1993; Balavenkatasubbaiah *et al.*, 1994; Bhattacharya *et al.*, 1997).

During last few years of research on seaweed, identification of many more valuable molecules have been initiated (Attaway and Zaborsky, 1993). Several such studies have been carried out on extracts from marine algae on microorganism (Nezha *et al.*, 2004). Almost all these studies were carried from the families of Rhodophyceae, Chlorophyceae and Phaeophyceae (Selvi and Selvaraj, 2000). Extracts from many marine algae show antibacterial (Sastry and Rao, 1994), antifungal (Rao *et al.*, 1986), antimicrobial and antiviral activity (Caccamerse *et al.*, 1981) and antibiotic activity (Lustigman, 1988). Among the marine algae, brown algae show less fungal activity than the red and green algae. Hence, in the present study *Turbinaria conoides* a marine alga extract has been utilized in controlling of *B. bassiana*, a serious fungal pathogen of silkworm as an alternate cheapest and natural eco-friendly antifungal agent under disease management in sericulture industry.

MATERIALS AND METHODS

Chemicals: All chemicals were analytical grade and potato dextrose agar was procured from the Himedia Laboratories, Mumbai, India.

Fungal culture collection: *Beauveria bassiana* (MTCC No. 0984) was obtained from the Institute of Microbial Technology, Chandigarh, India. Culture test of *B. bassiana* was maintained on Potato Dextrose Agar (PDA) slants and were sub-cultured in petridishes prior to testing for *in vitro* and *in vivo*.

Algal sample collection: *Turbinaria conoides*, a marine brown alga was collected from the Mandapam coast on the south east coast of India at a latitude 9°45' N and longitude 79°0' E during the Winter season (1st-2nd week of November). These samples were identified at the Marine Algal Research Station, Central Salt and Marine Chemical Research Institute, Mandapam, Tamil Nadu, India. It belongs to Class-Phaeophyceae; Family-Sargassaceae and Genus: *Turbinaria*; Species-*conoides*.

Preparation of extract from *Turbinaria conoides*: The collected *T. conoides* was washed thoroughly with seawater and allowed to dry in the shade for about 3 to 4 days. The dried samples were then brought to laboratory and again washed thoroughly with distilled water for two to three times for removal of excess salts and debris. Extraction was followed by slightly modified procedure of Sastry and Rao (1994). The 250 g of *T. conoides* were utilized for the extraction. The sample was chopped into fine pieces and packed in soxhlet extractor and extracted with dichloromethane and methanol (1:1) for 36 to 48 h at the temperature of 50-55°C. The extracts were concentrated and dried under reduced pressure in rotary evaporator.

***In vitro* bioassay:** The ready-made potato dextrose agar medium (39 g L^{-1}) was suspended in distilled water (1000 mL) and heated to boiling until it dissolved completely. The medium and petridishes were autoclaved at a pressure of 15 lb inc^{-2} for 20 min.

Further, the agar cups bioassay was employed for testing antifungal activity of the extracts on *B. bassiana* (Linday, 1962). The medium was poured into sterile petridishes under aseptic conditions in laminar flow chamber. When the medium in the plate solidified, 1×10^8 spores mL^{-1} of *B. bassiana* was inoculated and uniformly spread over the agar surface with a sterile L-shaped rod. Solutions were prepared by dissolving the compound in Dimethyl Sulphoxide (DMSO) and different concentrations (100 to 1500 μg mL^{-1}) were made. After incubation, cups were scooped out with 6 mm sterile cork borer and the lids of the dishes were replaced. To each cup different concentrations of seaweed extracts (100 to 1500 μg mL^{-1}) were added. All the samples were kept under 37°C at room temperature. After 24 h inhibition zones were observed, measured and diameter was calculated in mm. The average value of three replications was calculated for the antifungal activity against *B. bassiana*.

Silkworm rearing: Ten dfls (Disease Free Layings) of PM×NB_4D_2, a popular polyvoltine×bivoltine hybrid was used for the study. These layings were incubated at 25±1°C temperature and 70 to 80% Relative Humidity (RH) after surface treatment with 2% formalin solution. The silkworm rearing was conducted under the standard rearing condition as suggested by Krishnaswami *et al.* (1973). The young larvae (1st-3rd instars) reared at 26-28°C with 80-90% RH and late age larvae (4th and 5th instars) maintained at 24-26°C with 70-80% Relative Humidity (RH). The silkworm larvae were fed with freshly chopped good quality of V_1 variety mulberry leaves during the rearing period. The whole processes from silkworm egg incubation to completion of rearing activities were carried out under hygienic conditions in thoroughly disinfected silkworm rearing house with bleaching powder followed by formalin solution.

***In vivo* bioassay of *T. conoides* extract on silkworm:** The most effective concentration of the *T. conoides* extract in *in vitro* was evaluated for bioassay studies on silkworm. Acetone was used for dissolving *T. conoides* extract. The different concentrations were prepared with distilled water before exposed to silkworm because, acetone more than (2%) was found to be toxic on silkworm.

At first day of 5th instar, nine batches with 100 silkworms in two replications were kept separately. One batch with only 0.9% NaCl, one batch with only *B. bassiana* spores (1×10^8 spores mL^{-})^1, one batch with only Acetone (2%), three batches with three different concentrations (1000, 1500 and 2000 μg mL^{-1}) of *T. conoides* and other three batches with *B. bassiana* infected silkworm with these three different concentrations of *T. conoides* were exposed. The silkworms were first inoculated with the *B. bassiana* suspension and after 4 h different concentrations of algal extract were swapped over the silkworm and rearing trays. Data on mortality of silkworm larvae and cocoon yield due to fungal pathogen and with different treatments of algal extracts were recorded everyday and statistically analyzed. The survived silkworm larvae were mounted on plastic collapsible montage after attain ripening stage and allowed for spinning. After 6th to 7th days the silkworm cocoon were harvested and cocoon assessment was carried out with the assistance of following formulae.

Pupation rate: The live and healthy pupa present inside the cocoon while metamorphosis of larva into pupa and expressed in percentage:

$$\frac{\text{No. of good cocoons} + (\text{No. of double cocoons}\times 2)}{\text{Total silkworm larvae retained for the experiment}}\times 100$$

Cocoon weight: The average single cocoon weight in grams will be obtained by 25 male and 25 female cocoons taken randomly after cut open with the sharpen blade and sex separation on 6th or 7th day of spinning:

$$\frac{\text{Weight of 25 male (g) + 25 female cocoons (g)}}{50}$$

Shell weight: The average single cocoon shell weight in grams will be obtained by 25 male and 25 female cocoons shell taken randomly after cut open with the sharpen blade and sex separation. The shell used must be same cocoons used for the cocoon weight:

$$\frac{\text{Weight of 25 male (g) + 25 female cocoon shells (g)}}{50}$$

Shell ratio: To determine the total quantity of silk available from a single cocoon and calculated parameter from the above weighed single cocoon and shell weight and expressed in percentage by following equation:

$$\frac{\text{Single cocoon shell weight (g)}}{\text{Single cocoon weight (g)}} \times 100$$

These investigations were performed repeatedly thrice for its authentication during 2008-2009 at the laboratory of Biology Division, Indian Institute of Chemical Technology, Hyderabad, India.

RESULTS

***In vitro* assay:** Antifungal activity of *T. conoides* crude extract (100 to 1500 μg mL^{-1}) on *B. bassiana* were assessed on the Agar plates. There was no zone of inhibition noticed from 100 to 900 μg mL^{-1} of *T. conoides* extracts. However, from 1000 to 1500 μg mL^{-1} of the algal extracts, the zones of inhibition were increased significantly at every higher concentration (Table 1, Fig. 1).

***In vivo* bioassay on silkworm larvae:** The major aim of the study is to control the muscardine disease in silkworm with the application of crude seaweed algal extract. Three different concentrations (1000, 1500 and 2000 μg mL^{-1}) of *T. conoides* extracts against *B. bassiana* were assessed for its activity on silkworm larvae *in viva*. In negative control batch (0.9% NaCl), the pupation was recorded 98% whereas, in the positive control (*B. bassiana* 1×10^{8} spores mL^{-1}) it was

Table 1: Effect of *T. conoides* extract on *B. bassiana in vitro*

		Concentrations of *T. conoides* (μg mL^{-1})														
Organism	Replication	100	200	300	400	500	600	700	800	900	1000	1100	1200	1300	1400	1500
Beauveria	1	-	-	-	-	-	-	-	-	-	10.00	12.00	15.00	18.00	21.00	23.00
bassiana	2	-	-	-	-	-	-	-	-	-	8.00	11.00	16.00	19.00	23.00	25.00
	3	-	-	-	-	-	-	-	-	-	11.00	12.50	15.00	17.50	19.00	23.00
Average											9.67	11.83	15.33	18.17	21.00	23.67
SD											1.53	0.76	0.58	0.76	2.00	1.15

Zone of inhibition (diameter in mm) values for each replication; -: No zone of inhibition; SD: Standard Deviation

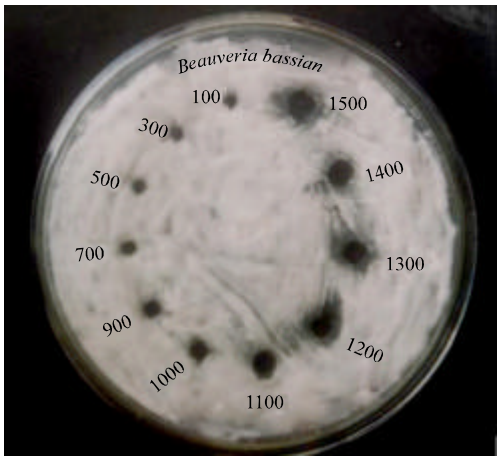

Fig. 1: Effect of different concentrations of *T. conoides* crude extracts (μg mL^{-1}) on *B. bassiana*

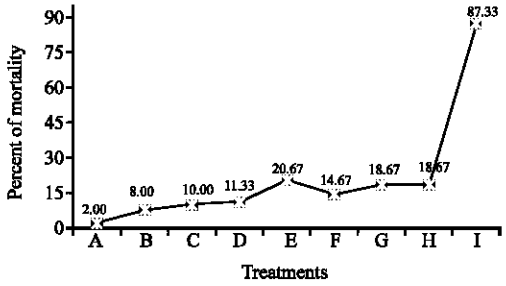

Fig. 2: Percentage of silkworm mortality with different concentration of seaweed crude extracts (*T. conoides*). A = 0.9% NaCl; B = 2% acetone; C = 1000 μg mL^{-1} of Tc; D = 1500 μg mL^{-1} of Tc; E = 2000 μg mL^{-1} of Tc; F = Bb+1000 μg mL^{-1} of Tc; G = Bb+1500 μg mL^{-1} of Tc; H = Bb+2000 μg mL^{-1} of Tc; I = Bb (1×10^8 spores mL^{-1}); Tc = *Turbinania conoides*; Bb = *Beauveria bassiana*

12.67%. The batches those were exposed 2% Acetone, the pupation was found 92%. Three batches those were treated three different concentrations of the *T. conoides* the average pupation were recorded 90.00, 88.67 and 79.33%, respectively. While other three *B. bassiana* pre-inoculated batch with three different concentrations of *T. conoides* extracts, the pupation were recorded 85.33, 81.33 and 81.33%, respectively. The average mortality percentage among all the treatments was shown in Fig. 2.

The maximum good cocoon percentage of (88.67%) was observed with the treatment of 0.9% NaCl followed by treatment of *B. bassiana* with 1500 μg mL^{-1} of *T. conoides* (77.33%), with the treatment of *B. bassiana* with 2000 μg mL^{-1} of *T. conoides* (76.67%) and minimum of 20.67% with the treatment of *B. bassiana* (1×10^8 spores mL^{-1}) (Table 2).

Though pupation rate of silkworm rearing was more in all algal extract treatment groups, but less percentage of good cocoons was observed. Around 30-40% of cocoons were with very low qualitative traits like higher percentage of flimsy and loose cocoons. The average cocoon and shell weight did not show much difference among all the algal treatment alone and also with the *B. bassiana* treatment groups. But there was a significant difference in the shell ratio (%) among

Table 2: Effect of seaweed extract (*T. conoides*) on the rearing parameters of *B. bassiana* infected silkworm larvae ($PM \times NB_4D_2$)

Treatments	Pupation (%)	Good cocoon (%)	Cocoon weight (g)	Shell weight (g)	Shell ratio (%)
0.9% NaCl	98.00	88.667±3.055	1.779±0.087	0.290±0.009	16.297±0.949
2% acetone	92.00	54.667±8.083	1.727±0.018	0.285±0.013	16.519±0.709
1000 μg mL^{-1} of Tc	90.00	66.667±8.083	1.596±0.088	0.273±0.010	17.084±0.788
1500 μg mL^{-1} of Tc	88.67	53.333±2.309	1.663±0.034	0.274±0.013	16.480±0.859
2000 μg mL^{-1} of Tc	79.33	50.333±5.033	1.831±0.028	0.286±0.035	15.623±2.074
Bb + 1000 μg mL^{-1} of Tc	85.33	70.000±2.000	1.765±0.073	0.324±0.005	18.360±1.023
Bb +1500 μg mL^{-1} of Tc	81.33	77.333±6.429	1.815±0.010	0.319±0.001	17.561±0.043
Bb + 2000 μg mL^{-1} of Tc	81.33	76.667±8.083	1.838±0.014	0.329±0.004	17.918±0.145
Bb (1×10^8 spores mL^{-1})	12.67	20.667±3.055	1.527±0.004	0.232±0.018	15.190±0.926

Each data collected for three replications; ±SD; Tc: *Turbinaria conoides*; Bb: *Beauveria bassiana*

all the treatments. Minimum shell ratio of 15.19% in only *B. bassiana* exposed batch while maximum shell ratio of 18.36% was observed in the treatment of *B. bassiana* and *T. conoides* combinations with the effective concentration at 1000 μg mL^{-1}. While, in the other batches shell ratio was ranged from 5.62 to 17.91% (Table 2).

DISCUSSION

The general hygiene in the silkworm rearing house will reduce the incidence of fungal infection. Prevention of fungal diseases in silkworm could also be possible by spraying 1-2% Dithane M 45 in slaked lime or captain in kaolin, 3% formaline and sanjeevini dust. Tolerance to all these agents is another approach to decreasing the incidence of those silkworm fungal diseases. Finding natural eco-friendly plant products that prevent or treat these diseases could be an alternative natural treatment process. The renewable/cultivable nature of marine flora is another advantage for development of potential antifungal products for use in feed or by other means administration to silkworm in sericulture. Economically feasible standard operating procedures can be developed in preparing the extracts/fractions in large scale with reproducible antifungal efficiency.

Molecules derived from natural products have an excellent record of providing novel chemical structure for development as new therapeutic agents. Many of the worlds most valuable and successful medicines have been derived from nature. Marine halophytes are the specialized group of plants adopted for high saline conditions which include mangroves, seaweeds, sea grass and blue green algae. They are also proven to have rich source of structurally diverse bioactive compounds with valuable pharmaceutical potential (Mayer and Hamann, 2002; Kumar *et al.,* 2009). But biological control of silkworm disease pathogens by herbal derived compounds has not much has been attempted so far.

While pursuing research on diseases control in economically important insects, steady efforts have been made to develop cost-effective, eco-friendly, commercially viable mass production technologies of various biocontrol agents and improved formulations for use under the Integrated Pest Management (IPM) throughout the world (Flint and van Der Bosch, 1981). Several works on marine algae have been carried out to determine many active components in the extracts of the algae species (Cuiloli *et al.*, 2000). Extracts from many marine algae show antibacterial (Sastry and Rao, 1994; Choudhury *et al.*, 2005) antifungal (Rao *et al.*, 1986), antimicrobial and antiviral activity (Caccamerse and Azzolina, 1979; Caccamerse *et al.*, 1981) and antibiotic activity (Lustigman, 1988). It was also well emphasized on the antifungal agent from the fungi source itself from the preceding results (Gagloczy and Vagvolgyi, 2009; Cabral *et al.,* 2010).

Hence, in the present study, extracts of *T. conoides* has been tested its antifungal activity against *B. bassiana* a common fungal pathogen on silkworm. A little or no information is available on the antimicrobial activity of *T. conoides* (Sawai *et al*., 1994). Therefore, these *in vitro* studies revealed on observable antifungal activity of *T. conoides* against *B. bassiana* from 1000 to 1500 μg mL^{-1} whereas, there was no effect upto 100 to 900 μg mL^{-1} of *T. conoides* extracts. Further, these extracts were also tested on *B. bassiana* infected silkworm *in vivo* to find out the maximum control of this pathogen on silkworm without affecting on both qualitative and quantitative cocoon traits. It was observed that, with the effect of different concentrations of *T. conoides* extract, the mortality (%) of silkworm was around 10 to 26.67% whereas; in the *B. bassiana* infected silkworm the mortality was 87.33%. The batches those were treated with the *B. bassiana* and three different concentrations viz., 1000 and 1500 μg mL^{-1} and of *T. conoides* extract, the mortality was found little higher and ranged from 20.67 to 18.67%. Among these three concentrations of algal extracts maximum control of *B. bassiana* on silkworm was observed at the highest concentration (1500 and 2000 μg mL^{-1}). While, observing the qualitative cocoon traits there was not much difference between the control and treated batches. However, in the cocoon shell ratio (%) there was significant difference observed between the control and treated batches. It was found almost same in both 0.9% NaCl silkworm batch and the batch those were treated with three different concentrations of *T. conoides* extracts separately. Interestingly, the other three bathes those were pretreated with *B. bassiana* and three different concentrations of *T. conoides* separately, showed with higher cocoon weight, shell weight and shell ratio than the controls (0.9% NaCl) and Acetone and three different concentrations of *T. conoides* extracts. The result obtained was corroborated with earlier reports emphasized on antifungal effect of *S. isoetifolium* revealed by Kumar *et al*. (2009). It also reveals that, this extract has no effect on the qualitative and quantitative cocoon traits on silkworm and could be utilized safely for the control of *B. bassiana* in commercial silkworm rearing. The molecular mechanism of these crude seaweed extract may cause cellular toxicity due to glutathione depletion and DNA damage due to oxidation of nucleobases as suggested in other antifungal agents by Huq (2007). Further, detail studies are required for isolation of the particular active molecules from this algal sample, which is only acting on the *B. bassiana* and need to elucidate the structure and molecular mechanism of action of this seaweed extract by molecular model analysis.

CONCLUSION

It was sturdily concluded from the *in vitro* and *in vivo* study that divulge on the utilization of *T. conoides* algal extract as an alternate natural antifungal agent against the early infection of *B. bassiana* in silkworm crop protection. Hence, the present communication dedicated to find out cheapest and eco-friendly disinfectant for prevent or treat these diseases could be an alternative treatment process at appropriate concentration under disease management in sericulture industry. However, further studies are needed to elucidate the structure and molecular mechanism of action of these seaweed extracts.

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