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# Research Article
# Antifungal Efficacy of Seaweed Extracts Against Fungal Pathogen of Silkworm, *Bombyx mori* L.

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## Abstract

**Background and Objective:** Marine organisms are the rich source of structurally novel and biologically active metabolites. Secondary or primary metabolites produced by these organisms may be potential bioactive compounds of interest in the pharmaceutical industry. This study aimed to investigate the antifungal activity of the methanol extract of three seaweeds *Caulerpa serrulata*, *Gracilaria edulis* and *Ulva fasciata* against silkworm fungal pathogen, *Nomuraea rileyi*. **Materials and Methods:** The crude extracts of all the seaweeds were concentrated in a rotary evaporator and the concentrates were tested at 1000, 2000 and 3000 μg $mL^{-1}$ for their antifungal efficacy *in vitro* and at 3000 μg $mL^{-1}$ against silkworm pathogen *Nomuraea rileyi in vivo*. The data obtained were recorded for each trails in a Randomized Complete Block Design (RCBD) and was subjected with the *Indostat* software analysis for its significance. **Results:** Among the tested extracts maximum zone of inhibition of 16 mm was observed in *Gracilaria edulis* and *Ulva fasciata* treated batches at 3 mg $mL^{-1}$ against *N. rileyi*. *In vivo* results revealed maximum effective rearing rate of 79% in batches treated with *Gracilaria edulis* against *Nomuraea rileyi* followed by *Ulva fasciata* of 75% effective rearing rate and found to be significant at $p<0.01$. **Conclusion:** Both *Gracilaria edulis* and *Ulva fasciata* crude methanol extracts can be used as antifungal agents in preparing eco-friendly disinfectants. These disinfectants can be used to treat silkworms infected by *Nomuraea rileyi* in silkworm rearing for the better crop yield without any limitations in sericulture industry.

Key words: Antifungal activity, *Nomuraea rileyi*, silkworm, sea weed, disinfectant, crude extract

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

## INTRODUCTION

Silkworm is susceptible to a number of diseases caused by different infectious agents such as fungal, bacterial, viral and protozoan diseases. In addition, *Bombyx mori* is the model organism for *Lepidoptera*, the order with second most numerous species in insects, including many species important for agriculture and forestry[1]. In India, sericulture is an important part of agriculture and has developed a complete system of silk industry. There are different approaches for the management of silkworm diseases during rearing. Disease prevention, disease suppression and development of disease resistant/tolerant breeds are such approaches presently used for the control of diseases in silkworm. Most of the technologies recommended earlier were based on preventive approaches, which include use of disinfectants for eliminating pathogens from the rearing environment and use of bed disinfectants to prevent the spread of diseases during rearing.

Silkworms are susceptible to a number of diseases caused by different infectious agents and mixed infections[2]. The cocoon loss due to diseases in India was estimated to be about 15-20 kg per 100 Disease Free Layings (DFLs) which account of about 30% of the total loss. However, fungal diseases often lead to great loss in silkworm industry. High rate of multiplication and spread are the main characteristic of the fungal diseases in silkworm and muscardine develops into an epizootic within a short period, if the conditions are congenial. Surveyed and recorded silkworm diseases such as Grasserie, Flacherie and Muscardine. Among fungal diseases, white muscardine, green muscardine, yellow muscardine and aspergillosis are caused by *Beauveria bassiana*, *Nomuraea rileyi* (*Spicaria prasina*) *Metarhizium anisopliae*, *Paecilomuces farinosus*, *Aspergillus flavus*, *Aspergillus oryza* and *Aspergillus tamari* etc[3].

The green muscardine incidence was sporadic in India without causing any damage to the sericultural crops. However, no systematic study was available on this disease. Recently conducted 1 year survey covering all the three seasons in five sericultural areas of Karnataka, India and reported the prevalence of green muscardine disease throughout the year along with white muscardine[4]. The prevalence of green muscardine was more during rainy season followed by winter and summer. This disease is caused by *Nomuraea rileyi*, an entomopathogenic fungus which invades mainly through the integument and infects the hemolymph and digestive tract of silkworm[5]. The disinfectants which are in regular use in sericulture industry against the fungal cultures are Sanitech, Asthra, bleaching solution, 2% formalin and 70% alcohol[6,7].

Marine halophytes are the specialised group of plants adapted for high saline conditions which included mangroves, seaweeds, sea grass and blue green algae. Marine resources are an unmatched reservoir of biologically active natural products, many of which exhibit structural features that has not been found in terrestrial organism[8]. There are numerous reports on compounds derived from macro algae with a broad range of biological activities such as the antimicrobial, antiviral, anti-tumour and anti-inflammatory as well as neurotoxins and its uses from seaweeds[9-11]. They are also proven to have rich source of structurally diverse bioactive compounds with valuable pharmaceutical potential[12-13].

The marine algae has rich source of structurally diverse bioactive compounds with valuable pharmaceutical potential. In this context, the study aims to find effective and eco-friendly disinfectant from marine algae in prevention and control of silkworm disease by antifungal activity on crude extracts of seaweeds *Caulerpa serrulata*, *Gracilaria edulis*, *Ulva fasciata* against fungal silkworm pathogen, *Nomuraea rileyi* which in turn helps in exploring marine algae to control silkworm diseases and other beneficial insects.

## MATERIALS AND METHODS

This whole study was carried out at Indian Institute of Chemical Technology (IICT), Hyderabad, India during the period of October, 2015 to March, 2016, seasons prevailing for muscardine diseases in India.

**Chemicals:** All chemicals viz., acetone, methanol, potato dextrose agar, dimethyl sulphoxide used for this study were of analytical grade and procured from the Himedia Laboratories, Mumbai, India.

**Fungal culture collection:** *Nomuraea rileyi* (MTCC No.4171) was obtained from the Institute of Microbial Technology, Chandigarh, India. Culture test of *Nomuraea rileyi* was maintained on Potato Dextrose Agar (PDA) slants and were sub-cultured in petri dishes prior to testing for *in vitro* and *in vivo.*

**Algal Sample collection:** *Caulerpa serrulata* (*Caulerpaceae*), *Gracilaria edulis* (*Gracilariaceae*) and *Ulva fasciata* (*Ulvaceae*) were collected from Mandapam coast, on the South east coast of India at a latitude 9°45'N and longitude 79°45'E during winter season. These samples were identified at the Marine Algal Research Station, Central Salt and Marine Chemicals Research Institute, Mandapam, Tamilnadu, India.

**Preparation of extracts from algal seaweeds:** The collected seaweeds were washed thoroughly with seawater and allowed to dry in the shade for 3-4 days. The dried samples were brought to laboratory and again washed thoroughly with distilled water for 2-3 times for removal of excess salts and debris. Each sample of 200 g of was chopped into fine pieces and packed in Soxhlet Extractor (Model No. 3840029, Borosil Glass Works Ltd., India) and extracted with methanol for 36-48 h at the temperature of 50-55°C. The extracts were concentrated and dried under reduced pressure in rotary evaporator (Model: RE 2001, Series No. 2012034, *Aditya Scientifics*, India).

***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 petri dishes 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 *Nomuraea rileyi*. The medium was poured into sterile petri dishes under aseptic conditions in laminar flow chamber. When the medium was poured in the plate solidified, $1\times10^8$ spores $mL^{-1}$ of *Nomuraea rileyi* was inoculated and uniformly spread over the agar surface with a sterile L-shaped rod. Solutions were prepared by dissolving compounds in Dimethyl sulphoxide (DMSO) and three concentrations were made. After incubation, cups were scooped out with 6mm sterile cork borer and the lids of the dishes were replaced. Three different seaweed extracts were added separately in each cup of 1000, 2000 and 3000 μg $mL^{-1}$, respectively. All the samples were kept at room temperature. After 24 h inhibition zones were observed, measured and diameter was calculated in mm as described in the Microbiological Methods and Bacteriological Analytical Manual with slight modification[14].

**Silkworm rearing:** Ten Disease Free Layings (DFLs) of (PM×CSR2), a popular polyvoltine x bivoltine hybrid was used for the study. These layings were incubated at 25±1°C temperature and 70-80% Relative Humidity (RH) after surface treatment with 2% formalin solution. The silkworm rearing was conducted under standard rearing conditions[15]. 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 process 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 seaweed extracts:** The most effective concentration of the three extracts was evaluated for bioassay studies on silkworm. Acetone was used for dissolving all the three extracts. Extracts were prepared with distilled water of 3000 μg $mL^{-1}$ concentration and were treated to silkworms. At first day of 5th instar, nine batches with 100 silkworms in two replications were kept separately. One batch as control, one batch with only *Nomuraea rileyi* spores[16] ($1\times10^8$ spores $mL^{-1}$), one batch with only acetone (2%), three batches inoculated with three extracts and other three batches with *Nomuraea rileyi* infected silkworms exposed to three different seaweed extracts. The silkworms were first inoculated with the fungal suspension and after 2 h different algal extracts were swapped over the silkworm. Data on mortality of silkworm larvae and cocoon yield due to fungal pathogen with different treatments of algal extracts were recorded everyday and statistically analyzed. The survived silkworm larvae were mounted on plastic collapsible mountage after attain ripening stage and allowed for spinning. On 5th day the silkworm cocoons were harvested and cocoon assessment was carried out[17].

**Statistical analysis:** The data obtained on *in vitro/in vivo* study and silkworm economical traits over control were recorded for each trails in a Randomized Complete Block Design (RCBD) and subjected for bio-statistical analysis with assistance of the computer software developed by Indostat Service Pvt. Ltd., India.

## RESULTS

***In vitro* assay:** Antifungal activity of all the three crude extracts against *Nomuraea rileyi* (1000, 2000 and 3000 μg $mL^{-1}$) were assessed on the agar plates (Fig. 1).

***In vivo* bioassay on silkworm larvae:** Three different seaweed extracts *Gracilaria edulis*, *Ulva fasciata* and *Caulerpa serrulata* at 3000 μg $mL^{-1}$ were assessed for its activity on silkworm larvae *in vivo* against *Nomuraea rileyi* with control batches. The average cocoon and shell weight did not show much difference among all the algal treatments alone and also with the *Nomuraea rileyi* treated groups. There was a slight difference in the shell ratio (%) among all the treatments (Table 1).

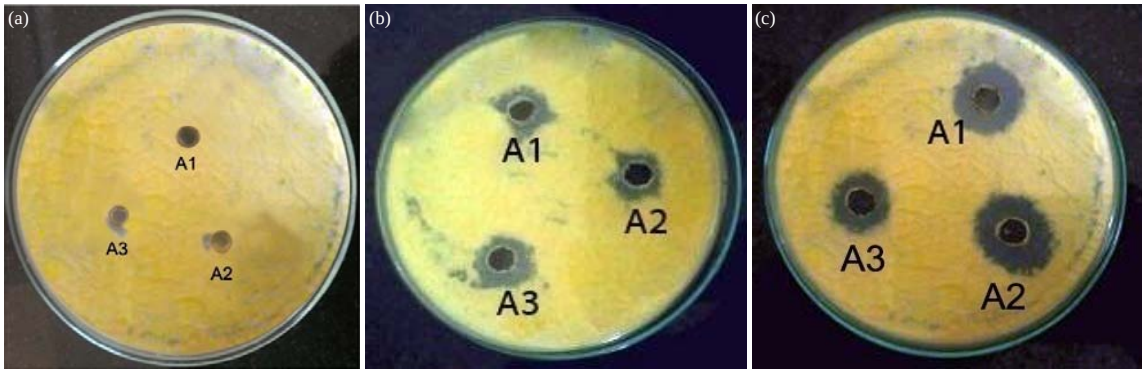


Fig. 1: Effect of seaweed extracts on *Nomuraea rileyi in vitro* by agar well diffusion method

Table 1: Effect of seaweeds extracts on the rearing parameters of *Nomuraea rileyi* infected silkworm larvae

| Treatments | Effective rearing rate (%) | Good cocoon (%) | Cocoon weight (g) | Shell weight (g) | Shell ratio (%) |
|---|---|---|---|---|---|
| 2% Acetone | 92.00±1.00 | 86.66±1.50 | 1.736±0.05 | 0.296±0.03 | 17.08±1.79 |
| Treated Control (*Nomuraea rileyi*) | 20.66±1.50 | 1.40±1.00 | 1.576±0.04 | 0.243±0.03 | 15.43±1.98 |
| Untreated Control | 90.00±2.00 | 88.66±2.08 | 1.720±0.01 | 0.286±0.01 | 16.66±0.75 |
| *Gracilaria edulis* | 91.00±1.00 | 87.00±1.15 | 1.726±0.04 | 0.290±0.02 | 16.81±1.74 |
| *Ulva fasciata* | 92.33±1.50 | 86.33±0.57 | 1.733±0.02 | 0.306±0.03 | 17.70±1.93 |
| *Caulerpa serrulata* | 90.33±1.50 | 85.00±1.00 | 1.706±0.02 | 0.293±0.02 | 17.20±1.73 |
| *Nr+Gracllaria edulis* | 79.00±1.70 | 72.33±1.50 | 1.720±0.04 | 0.290±0.03 | 16.88±1.92 |
| *Nr+Ulva fasciata* | 75.00±1.00 | 70.33±1.50 | 1.703±0.04 | 0.293±0.02 | 17.24±1.70 |
| *Nr+Caulerpa serrulata* | 69.00±2.00 | 61.00±2.00 | 1.686±0.07 | 0.286±0.03 | 16.96±1.65 |
| SE± | 0.1342 | 0.7026 | 0.0808 | 0.0911 | 0.4007 |
| CD at 5% | 0.3864 | 0.2332 | 0.2606 | 1.0233 | 0.0851 |

Each data collected in three replications: ±SE, Nr: *Nomuraea rileyi*

Table 2: Zone of inhibitions on seaweed extracts against *Nomuraea rileyi*

| | Zone of inhibition | | |
|---|---|---|---|
| Seaweed extracts | 1000 (µg mL$^{-1}$) | 2000 (µg mL$^{-1}$) | 3000 (µg mL$^{-1}$) |
| *Gracilaria edulis* | Nil | 14.0±1.00 mm | 16.0±1.10 mm |
| *Ulva fasciata* | Partial | 13.3±0.57 mm | 16.0±1.00 mm |
| *Caulerpa serrulata* | Partial | 12.0±1.00 mm | 13.6±0.57 mm |
| ±SE | - | 0.0162 | 0.0141 |
| CD at 5% | - | 0.0454 | 0.0401 |

Each data collected in three replications: ±SE

Bio-statistical analysis on anti fungal activity of seaweed extracts against the silkworm pathogens of both *in vitro* and *in vivo* established to be significant at $p<0.05$ (Table 2). Further, the mean squares values obtained on biostatical analysis against the economical traits of the silkworm rearing performances over the control was also found to be significant at $p< 0.01$ (Table 1).

## DISCUSSION

In the current study, green muscardine is one of the fungal diseases in silkworm which cause severe economic loss to the industry. Three seaweeds were tested for their antifungal efficacy against silkworm fungal pathogen *Nomuraea rileyi*. Among the tested extracts maximum zone of inhibition of 16 mm was observed in *Gracilaria edulis* and *Ulva fasciata* treated batches at 3 mg mL$^{-1}$ against *Nomuraea rileyi*. *In vivo* results revealed maximum effective rearing rate of 79% in batches treated with *Gracilaria edulis* against *Nomuraea rileyi* followed by *Ulva fasciata* of 75% effective rearing rate.

Even though, prevalence of muscardine is confined to rainy and winter seasons, it accounted for 43% of the total disease occurrence in a year[18]. Several chemical fungicides like bavistin, mancozeb, zineb, dithane M-45, captan etc., were evaluated against muscardine by various researchers and the most effective among them are recommended for the prevention of the diseases[19]. An eco-friendly and plant based

bed disinfectant formulation named 'Ankush was developed at CSRTI, Mysore for preventing the spread of all silkworm diseases including white muscardine[20]. Biological control of silkworm disease pathogens by algal derived compounds has not much 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 bio control agents and improved formulations for use under the Integrated Pest Management (IPM) throughout the world[20]. Marine macro algae are considered as an excellent source of bioactive compounds which has a broad range of biological activities including antibacterial[21], antifungal[22-24], antiviral[25-27], antioxidant[28-30] and anti-inflammatories[31-33]. The potential contribution of marine organisms to the discovery of new bioactive molecules is remarkably increasing[34].

Apart from the disinfectants which are in regular use in sericulture industry against the fungal disease have been carried out on the effect of crude extracts of marine macro algae against green muscardine *Nomuraea rileyi in vitro* and *in vivo*[3]. In the present study, methanolic extracts of *Gracilaria edulis* (Brown algae), *Caulerpa serrulata* and *Ulva fasciata* (Green algae) were tested for their antifungal activity against green muscardine *Nomuraea rileyi*. A little or no information is available on the antifungal effect of seaweed extracts against *Nomuraea rileyi*. These present study revealed observable antifungal activity of all the three seaweed extracts against *Nomuraea rileyi* at 2 and 3 mg $mL^{-1}$. Further these extracts were tested on *Nomuraea rileyi* infected silkworm larvae to find out the maximum control of pathogen on silkworm without effecting both qualitative and quantitative traits of silkworm. It was observed that with the effect of different seaweed extracts the mortality of silkworm was in the range of 8-31% where as in *Nomuraea rileyi* infected silkworm 79.34% of mortality was observed. Maximum effective rearing rate of 92.33% was observed in *Ulva fasciata* treated batch followed by 92.00% in 2% acetone treated batch and untreated control of 90%. Among the batches treated with algal extracts against *Nomuraea rileyi* maximum effective rearing rate of 79% observed in G*racilaria eludis* treated batch. While observing the qualitative characters like cocoon weight and shell ratio % there was no much difference between the control and treated batches. However maximum cocoon weight was observed in batch treated with 2% acetone (1.736 g) followed by control (1.720 g) and *Ulva fasciata* treated batch (1.733 g). It also revealed that, these extracts have no effect on the quantitative and qualitative characters of silkworm and could be used safely for the control of muscardine in commercial silkworm rearing. The antifungal property of the algal extracts evaluated against silkworm pathogen may be due to presence of phytochemicals like tannins and phenolic compounds. Phenolic compounds may affect growth and metabolism of fungus. Tannins were used therapeutically as antiviral, antibacterial, antiulcer and antioxidant agents in pest and pathogen interaction. During the interaction of pathogens and bio control agents hyphal coiling, granulation, distortion, vacuolation and bulging may be promoted[3]. Further detailed studies are required for isolation of particular active molecule from these algal samples, which is acting as an antifungal agent.

However, the present study stated to discover the antifungal activity of certain seaweed extracts against silkworm pathogen *Nomuraea rileyi* that can be beneficial to formulate disinfectant against fungal pathogen.

Hence, the result significantly showed the antifungal activity of seaweed extracts against fungal pathogen *Nomureae rileyi*. Further study is needed to identify the bioactive molecules of seaweed extracts and to characterize their acting mechanism on other fungal and bacterial pathogens of silkworm. In the present study, crude extracts of seaweeds were tested against silkworm pathogen *in vivo* and could able to control the disease with significant rearing rate ($p<0.01$). Effective crude extracts can be used as disinfectants in silkworm rearing for better crop yield without any limitations in sericulture industry.

## CONCLUSION

*In vitro* and *in vivo* studies on the utilization of three seaweed extracts proved that the methanolic extracts of *Gracilaria edulis*, *Ulva fasciata* and *Caulerpa serrulata* at 3000 μg $mL^{-1}$, showed varied degree of antifungal activity against *Nomuraea rileyi in vitro* and inhibited the growth of fungus *in vivo* with effective rearing rate of 79, 75 and 69%, respectively. Based on the study it can be recommended that these three seaweed extracts can be used as antifungal agents in preparing of eco-friendly disinfectants/pharmacological agent to treat silkworms infected by *Nomuraea rileyi*.

## SIGNIFICANCE STATEMENTS

This study discovers the antifungal activity of seaweed extracts that can be beneficial for controlling silkworm fungal pathogens in the field level. This study will also help the researcher to uncover the critical area of utilizing seaweed extracts in controlling silkworm diseases that many researchers were not able to explore. A new theory on making eco-friendly and effective disinfectant formulations with seaweed extracts in controlling silkworm diseases was arrived without any limitations in the sericulture industry.

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