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Evaluation of different solvent extracts of *Sargassum wightii* (brown algae) for its anti-fungal efficacy against silkworm pathogens

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Abstract
The present study was carried out in Indian Institute of Chemical Technology, Hyderabad during November, 2016-March, 2017 to investigate the antifungal activity of the three solvent extracts of seaweed *Sargassum wightii* against silkworm fungal pathogens. The crude seaweed compounds extracted with ethyl acetate, dichloromethane and diethyl ether solvents were tested at 1000, 2000 and 3000 µg mL^{-1} for their antifungal efficacy *in vitro* and at 3000 µg mL^{-1} against silkworm pathogens *Aspergillus flavus, Nomuraea rileyi, Aspergillus oryzae* and *Beauveria bassiana in vivo*. The data obtained was recorded for each trail in a one way ANOVA and was subjected with the *Graph pad prism* software analysis for its significance. Among the tested extracts maximum zone of inhibition of 20mm was observed in diethyl ether treated batches at 3mg/mL^{-1} against *Aspergillus flavus* and 19.66 mm against *Beauveria bassiana. In vivo* results revealed maximum effective rearing rate of 84% in batch treated with diethyl ether against *Aspergillus flavus* followed by *Nomuraea rileyi* of 82 % and found to be significant at $p<0.01$. *Sargassum wightii* proved that the diethyl ether extract and ethyl acetate extract at 3000 µg mL^{-1}, showed varied degree of antifungal activity against silkworm fungal pathogens. Based on the results both diethyl ether and ethyl acetate extract of *Sargassum wightii* can be used as antifungal agents in preparing eco-friendly disinfectant in sericulture industry.

Keywords: *Sargassum wightii*, antifungal efficacy, pathogen, crude extract, *in vitro, in vivo*

1. Introduction
Centuries of domestication of mulberry silkworm, *Bombyx mori* has resulted in loss of certain wild characters which made it susceptible to several diseases caused by different infectious agents such as bacterial, fungal, protozoan and viral diseases [1]. 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 [2, 3]. 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 [4]. 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 [5].
Silkworms are susceptible to a number of diseases caused by different infectious agents and mixed infections [5]. The cocoon loss due to diseases in India was estimated to be about 15-20kg 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 characteristics of the fungal diseases in silkworm and muscardine develops into an epizootic within a short period, if the conditions are congenial [6]. Among fungal diseases white muscardine, green muscardine, yellow muscardine and aspergillosis are caused by *Beauveria bassiana*, *Nomuraea rileyi*, *Spicaria prasina*, *Metarhizium anisopliae*, *Paecilomyces farinosus*, *Aspergillus flavus*, *Aspergillus oryzae* and *Aspergillus tamari* etc. [7, 8]. They invade mainly through the integument and infects the hemolymph and digestive tract of silkworm [9]. The disinfectants which are in regular use in sericulture industry against the fungal infections are Sanitech, Asthra, bleaching solution, 2% formalin and 70% alcohol [10-12].

Marine halophytes are the specialized 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 [13]. There are numerous reports on compounds derived from macro algae with a broad range of biological activities such as the antimicrobial, antiviral, anti-tumor and anti-inflammatory as well as neurotoxins and its uses from seaweeds [14-17]. They are also proven to have rich source of structurally diverse bioactive compounds with valuable pharmaceutical potential [18, 19].

In this context, the present study aims to find effective and eco-friendly disinfectant from marine algae in prevention and control of silkworm fungal diseases by antifungal activity of crude extract of *Sargassum wightii* against silkworm fungal pathogens which include helps in exploring marine algae to control silkworm diseases and other beneficial insects.

2. Materials and Methods

This whole study was carried out at Indian Institute of Chemical Technology (IICT), Hyderabad, India during the period of November, 2016 to March, 2017 seasons prevailing for fungal diseases in India.

2.1 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.

2.2 Fungal culture collection

Beauveria bassiana (MTCC NO 984), *Nomuraea rileyi* (MTCC NO 4171), *Aspergillus flavus* (MTCC NO.8654) and *Aspergillus oryzae* (MTCC NO1846) were obtained from the Institute of Microbial Technology, Chandigarh, India. These Culture test were maintained on Potato Dextrose Agar (PDA) slants and were sub-cultured in petri dishes prior to testing for *in vitro* and *in vivo*.

2.3 Algal Sample collection

Sargassum wightii was collected from Mandapam coast, on the South-east coast of India at a latitude $9^0$45'N and longitude $79^0$45'E during winter season. These samples were identified at the Marine Algal Research Station, Central Salt and Marine Chemicals Research Institute, Mandapam, Tamil Nadu, India.

2.4 Preparation of extracts from *Sargassum wightii*

The collected seaweed was washed thoroughly with seawater and allowed to dry in the shade for 3-4 days. The dried sample was brought to laboratory and again washed thoroughly with distilled water for 2-3 times for removal of excess salts and debris. Dried seaweed of 200 g was chopped into fine pieces and packed in Soxhlet Extractor (Model No. 3840029, Borosil Glass Works Ltd., India) and extracted with Ethyl acetate temperature 76-78^{0}C, Dichloromethane temperature 35-40^{0}C and Diethyl ether temperature 35-40^{0}C, for 36-48 hours. The extracts were concentrated and dried under reduced pressure in rotary evaporator (Model: RE 2001, Series No. 2012034, *Aditya Scientifics*, India).

2.5 *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 15lb inc^{-2} for 20 min. Further, the agar cups bioassay was employed for testing antifungal activity of the extracts on *Beauveria bassiana*, *Nomuraea rileyi*, *Aspergillus flavus* and *Aspergillus oryzae*. The medium was poured into sterile Petri dishes under aseptic conditions in laminar flow chamber. When the medium was poured in the plate solidified, 1×10^8 spores mL^{-1} of these fungal spores were 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 6 mm sterile cork borer and the lids of the dishes were replaced. Three different solvent 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 [20].

2.6 Silkworm rearing

Ten Disease Free Layings (DFLs) of ($PM\times CSR_2$), a popular polyvoltine x bivoltine hybrid was used for the study. These layings were incubated at 25±1^{0}C temperature and 70-80% Relative Humidity (RH) after surface treatment with 2% formalin solution. The silkworm rearing was conducted under standard rearing conditions [21]. The young larvae (1st-3rd instars) reared at 26-28^{0}C with 80-90% RH and late age larvae (4th and 5th instars) maintained at 24-26^{0}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.

2.7 *In vivo* bioassay of seaweed extracts

The most effective concentration of the three solvent 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, 17 batches with 100 silkworms in three replications were kept separately. Four batches with *Beauveria bassiana*, *Nomuraea rileyi*, *Aspergillus flavus* and *Aspergillus oryzae* spores [22] (1×10^8 spores mL^{-1}) separately, one batch with only acetone (2%), 12 batches with four different fungal (*Aspergillus flavus*, *Nomuraea rileyi*, *Aspergillus oryzae* and *Beauveria bassiana*) pathogens infected silkworms exposed with Ethyl acetate (EA), Dichloromethane(DCM) and Diethyl ether(DEE) solvent extracts of *Sargassum wightii*. The silkworms were first inoculated with the fungal suspension and after 2 hrs different solvent 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 every day and statistically analyzed. The survived silkworm larvae were mounted on plastic collapsible mount age after attain ripening stage and allowed for spinning. On 5th day the silkworm cocoons were harvested and cocoon assessment was carried out [23].

2.8 Statistical analysis

The data obtained on *in vitro/in vivo* study and silkworm economical traits over control were recorded for each trail analysis of variance (ANOVA) followed by Dunnett's multiple comparison test (*Graph Pad Prism* 7.01, USA). The

experiments for which, the 'P' value is < 0.01 in comparison to the control were considered as statistically significant.

3. Results and Discussion

3.1 *In vitro* assay: Antifungal activity of all the three crude extracts against *Aspergillus flavus, Nomuraea rileyi, Aspergillus oryzae* and *Beauveria bassiana* spores (1000, 2000 and 3000 µg mL^{-1}) were assessed on the agar plates (Table-1).

Table 1: Zone of Inhibition of different solvent extracts against fungal pathogens.

Seaweed extracts	*Aspergillus flavus*			*Nomuraea rileyi*			*Aspergillus Oryzae*			*Beauveria bassiana*		
	1mg/ml	2mg/ml	3mg/ml	1mg/ml	2mg/ml	3mg/ml	1mg/ml	2mg/ml	3mg/ml	1mg/ml	2mg/ml	3mg/ml
EA	-	11.66±0.6	18±1	-	13±1	14.33±0.6	-	12±1	13.66±0.5	-	14.66±0.5	19±1
DCM	-	12.66±0.5	14.66±0.5	-	10.66±0.5	12.66±0.5	-	10.33±1.5	12.33±0.6	-	13.33±1.1	16.66±0.5
DEE	9.66±0.5	16±1	20.33±0.6	7.66±0.5	12.66±1.1	15.33±0.5	-	11.66±0.5	14±1	11.33±1	17±1	19.66±0.5

Note: Each data collected in three replications: ±SD, EA: *Sargassum wightii* extracted with Ethyl acetate, DCM: extracted with Dichloromethane and DEE: extracted with Diethyl ether.

3.2 *In vivo* bioassay on silkworm larvae

Three different solvent extracts EA, DCM, DEE *of S.wightii* at 3000 µg mLG^{1} were assessed for its activity on silkworm larvae *in vivo* against *Beauveria bassiana, Nomuraea rileyi*, *Aspergillus flavus* and *Aspergillus oryzae* with control batches based on the *in vitro* results (Fig. 1). The average cocoon and shell weight did not show much difference among all the algal treatments alone and with the fungal pathogens treated groups. There was a slight difference in the shell ratio (%) among all the treatments (Fig. 2).

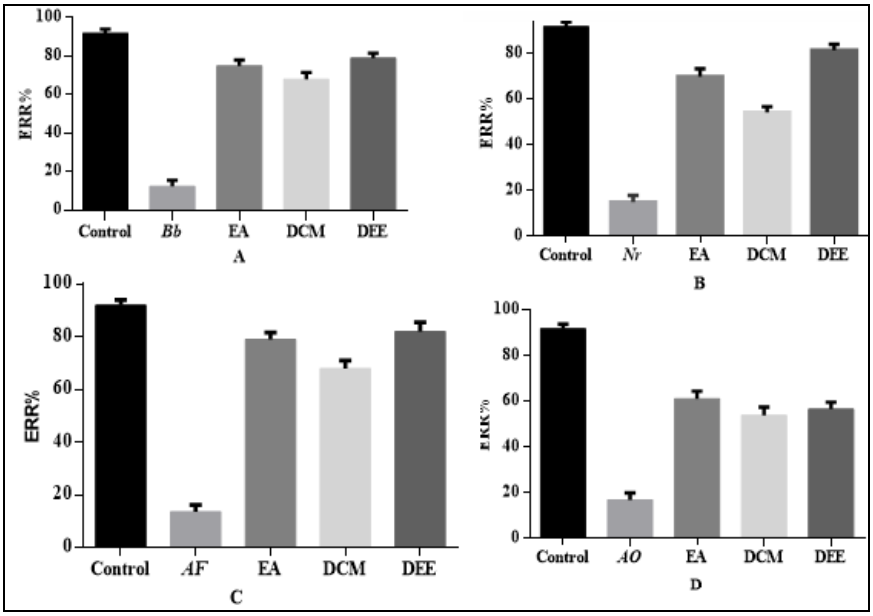

Fig 1: Effect of different solvent extracts of *Sargassum wightii* on ERR of treated Silkworms.

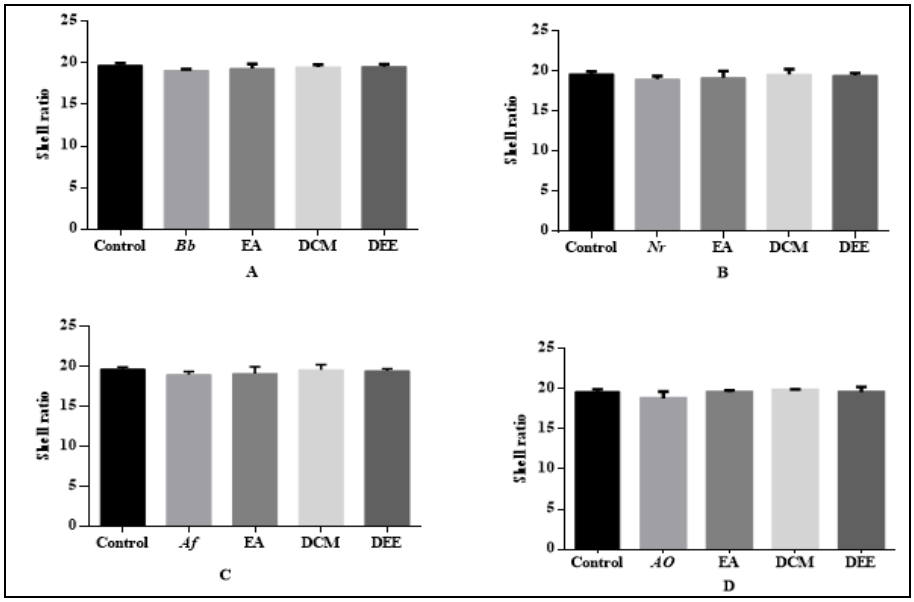

Fig 2: Effect of different solvent extracts of *Sargassum wightii* on shell ratio of treated silkworm.

Note: [*Bb: Beauveria bassiana, Nr: Nomuraea rileyi, Af: Aspergillus flavus, Ao: Aspergillus oryzae,* EA: Ethyl acetate, DCM: Dichloromethane, DEE: Diethyl ether extract, *S. wightii*: *Sargassum wightii*, ERR: Effective Rearing Rate]

In this study, three solvent extracts of *Sargassum wightii* were tested for their antifungal efficacy against silkworm fungal pathogens. A little or no information is available on the antifungal activity of different solvent extracts of *Sargassum wightii* against silkworm fungal pathogens. *In vitro* studies revealed maximum zone of inhibition of 20.33 mm and 19.66 mm in diethyl ether treated extract against *Aspergillus flavus* and *Beauveria bassiana* at 3 mg/ml respectively. Further, these extracts were tested on the fungal pathogen infected silkworms to find out the maximum control of the pathogen on silkworm without effecting both qualitative and quantitative characters. *In vivo* results revealed maximum effective rearing rate of 84% and 82% in batches treated with diethyl ether extract against *Aspergillus flavus* and *Nomuraea rileyi* respectively at 3mg/ml. It was observed that, with the effect of diethyl ether extract and ethyl acetate extract of *Sargassum wightii* against fungal infected silkworms the mortality was around 15-30%, where as in the negative control of fungal infected silkworms the mortality was about 85-90%.

Even though, prevalence of muscardine is confined to rainy and winter seasons, it accounted for 43% of the total disease occurrence in a year [24]. 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 [25]. 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 [26].

While pursuing research on disease 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 [26]. Marine macro algae are considered as an excellent source of bioactive compounds which has a broad range of biological activities including antibacterial [27], antifungal [28-30], antiviral [31-33], antioxidant [34-36] and anti-inflammatories [37-39]. The potential contribution of marine organisms to the discovery of new bioactive molecules is remarkably increasing [40].

Four seaweeds *Sargassum wightii, Stocheospermum marginatum, Gracilaria foliifera* and *Padina boergesenii* extracted in four solvents acetone, methanol, chloroform and diethyl ether were tested for their antimicrobial activity against 12 bacterial pathogens and also against five fungal pathogens. All the extracts, in particular brown seaweeds extracted in acetone exhibited the significant antimicrobial activity. This study established acetone extracts of brown seaweed were highly effective, against bacteria and fungi [41]. Organic solvent extracts of *Sargassum wightii* and *Kappaphycus alwarezii* were tested for their antimicrobial activity successfully showed the maximum zone of inhibition against most of the tested microorganisms. All the four solvent extracts were found to be effective against the pathogens. The maximum zone of inhibition was shown by the acetone extract of *Kappaphycus alwarezii* against *Pseudomonas aeruginosa* and diethyl ether extract of *Sargassum wightii* against *E.coli* [42]. Antifungal activity of *Tubenaria conoides* against *Beauveria bassiana* was studied and maximum control of *B. bassiana* on silkworm was observed at concentration of 2000µg mL^{-1} *in vivo* [23]. Crude methanol and water extracts of 19 marine algal species from Chlorophyta, Phaeophyta and Rhodophyta collected from the western coast of Libya were evaluated for antibacterial activity. Methanol extracts showed higher antibacterial activity than aqueous extracts [43].

Effect of antifungal activity of seaweed extracts against soil borne pathogens in pulses revealed that methanol extract of *Sargassum myricocystum* at 30 per cent concentration showed significant antifungal activity against soil borne pathogens followed by *Caulerpa racemosa* and *Gracilaria edulis* [44]. *Caulerpa serrulata, Gracilaria edulis and Ulva fasciata* were tested for their antifungal activity against silkworm fungal pathogen *Nomuraea rileyi in vitro* and *in vivo*. Among the tested extracts maximum zone of inhibition of 16 mm was observed in *Gracilaria edulis* and *Ulva fasciata* treated batches at 3 mg/mLG^{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 [17].

The present study revealed observable antifungal activity of different solvent extracts of *Sargassum wightii* against silkworm fungal pathogens at 3 mg/ml. However, there was no significant difference observed in shell ratio among all the treated batches and control batches. Maximum shell ratio of 19.8% was observed in batch treated with dichloromethane extract against *Aspergillus oryzae*. It also reveals that, *Sargassum wightii* solvent extracts has no effect on the quantitative and qualitative characters on silkworm and could be utilized safely for the control of fungal diseases in commercial silkworm rearing. The brown algae have naturally high secondary metabolites compared to red and green. Extracts of marine brown algae have been reported to exhibit antibacterial activity and antimicrobial activity [45]. The result obtained from the study was corroborated with earlier reports emphasized on antifungal activity of marine brown algae [23, 41, 44]. Further detailed studies are required for isolation of the active molecules from this algal sample which is active on the pathogen and need to elucidate the structure and molecular mechanism of action of this seaweed extract by molecular analysis.

4. Conclusion

In vitro and *in vivo* studies on the utilization of three solvent extracts of *Sargassum wightii* proved that the diethyl ether extract and ethyl acetate extract at 3000µg mL^{-1}, showed varied degree of anti-fungal activity against silkworm fungal pathogens *in vitro* and inhibited the growth of fungus *in vivo* with effective rearing rate ranging from 70-84% against *Aspergillus flavus*, *Nomuraea rileyi* and *Beauveria bassiana*. Based on the study it can be recommended that these two solvent extracts can be used as anti-fungal agents in preparing eco-friendly disinfectants to treat silkworms infected by fungal pathogens. This study discovers the antifungal activity of seaweed extract that can be beneficial for controlling silkworm fungal pathogens in the field level.

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