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Effect of marine extracts on the microbial pathogens causing flacherie in the mulberry silkworm, *Bombyx mori* L.

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ABSTRACT

Objective: Silkworms are invertebrate animals that are killed by bacteria pathogenic against humans, such as *Staphylococcus aureus*, *Streptococcus* pyogenes, *Pseudomonas aeruginosa* and *Vibrio cholera*. Biochemical characterization of the microbes in the haemolymph of diseased silkworm collected during the survey indicated the presence of *Bacillus* sp., *Streptococcus* sp., *Staphylococcus* sp. and *Pseudomonas* sp. in the culture. **Methods:** Studies were carried out in vitro to assess the efficacy of some marine extracts for the containment of these microbes through turbidimetry analysis and zone of inhibition test. **Results:** The observations made during this study revealed that the ethyl acetate crude extracts of two marine samples are *Aurora globostellata* and *Spirostella inconstans* var. moendrina Dendy effective against these microbes causing flacherie diseases in silkworm. The comparison of their effects indicated that ethyl acetate extracts were generally more effective Extensive studies using these extracts on the growth and cocoon production of the mulberry silkworm, *Bombyx mori* L. are likely to throw much light on the possibility of using such extracts as a prophylactic measure during silkworm rearing to improve silk production. **Conclusions:** Also, the results indicate that maybe plays a possible role in the contamination of humans and animals, in particular silkworms, while marine extracts showed a potential to control the contamination caused by bacterial diseases.

1. Introduction

Silkworm diseases caused by Protozoa, bacteria, fungi and viruses are among the major constraints in silk production. The infectious diseases of silkworm cause considerable loss in cocoon production. Sericulturists are striving constantly to minimize the incidence of diseases and maximize cocoon production [5]. Among these diseases bacteria plays the prime role in causing diseases and affect the rearing of silkworm. The main diseases attacking the silkworms are flacherie, grasserie, muscardine and pebrine[3]. Flacherie constitutes 70% of loss due to diseases in the study area. However, the role of bacterial pathogens in silkworm flacherie are documented from early times, generally observed that the amount of research information generated on these appears to be relatively less except *Bacillus thuringiensis* when compared to viral, fungal and protozoan pathogens of silkworm. Bacteria have active participation in silkworm flacherie as individual pathogen or in combination with viruses, thereby probably adding in causing disease epizootics.

Silkworm *Bombyx mori* is domesticated for silk production and are reared for income generation. One of the major constraints in silk production is the diseases in the silkworm rearing. Flacherie is an important disease-affecting silkworm. Flacherie may be caused by microbial and microbial agents. Microbial flacherie is caused by both bacteria and viruses[5,17]. First reported bacteria as etiological agents of silkworm flacherie. The bacterial diseases are called as flacherie diseases because the corpuses of silkworms that die of them are soft and rot. During the initial stages of infection, the larva became lethargic and stops feeding. There are many reports on bacterial flacherie[7, 19, 15, 12, 2].

[21] Reported that crop loss due to flacherie might be about 70 percent of the total crop loss due to diseases. [2] Estimated annual crop loss of 20–40 percent from flacherie. [2] broadly

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distinguished bacterial diseases into two classes (i) those are restricted to the intestine and (ii) those that enter the haemolymph.

Although much information is available on the etiological agents of bacterial flacherie, no attempt has been made in the study area to isolate and identify, the flacherie causing bacteria, to develop suitable antibiotic remedy to curtail their pathogenicity and to assess the damage that these pathogen impose on sericulture crop. In the socio-economic survey, the farmers had reported flacherie as a serious threat to their crops and many farmers are resistant to involve themselves in sericulture. Hence, a study was planned to identify the different bacterial pathogens in flacherie-infected worms collected from fields. In the previously reported bacterial sensitivity assays, many of the bacterial species identified in the present study are missing. Further, the drug resistant for bacterial isolates changes often, hence it is essential to identity a broad-spectrum antibiotic that can cure or prevent the infection. In the present investigation, about 50 bacterial species had been isolated from flacherie-infected worms. Hence, it is imperative to identify a suitable antibiotic that can protect the infected larvae. With these concerns, antibiotic sensitivity assay had been developed for 6 species of most abundant pathogen in the gut.

2. Material and methods

2.1. Sample collection

The bacterial flacherie infected larvae were collected from sericulture farms in Tenkasi of Tirunelveli district in the month of October-2011 (LXCSR2 Variety). From the information of farmers about disease in their farms, the farms were visited and 200 disease developed larvae in the age group III -V instars were collected in aseptic containers and transported to the laboratory. The morphological characteristics and activities of the larvae were noted. About 50 larvae in IV instar stage, which had failed to develop further and stopped feeding, were chosen for bacteriological study.

2.2. Haemolymph Collection

Using peptone water the worms were dissected out in aseptic room and the gut, silk gland and haemolymph were collected carefully in sterile eppendrof tubes and labelled. The foregut, midgut and hindgut were isolated separately. From the gut and the silk gland, about one gram tissue was taken out separately and homogenized separately using known volume of sterile 1% peptone water. Then the homogenates were made to 100ml using 1% sterile peptone water. Further serial dilutions were made using 9ml of the same dilution. 1ml of aliquots of approximate dilution was pipetted out into sterile petriplates. About 15-20 ml of sterile nutrient molten agar was poured aseptically into the petriplates; the plates were rotated in clock wise and antilock wise directions, and the nutrient agar medium was (Hi-Media) allowed to solidify. For each dilution, duplicate plates were maintained and the inoculated plates were incubated at 37℃ for 72hours. For enumerating the bacterial load in the haemolymph, the collected haemolymph was serially diluted with 1% sterile peptone water, and the culture was carried out as mentioned above. After 72 hours, the numbers of bacterial colonies formed were counted by using bacteriological colony counter. The plates, which contain 30-300 colonies, were selected for enumeration of total plate count. The bacterial populations were expressed as number of Colony Forming Units (CFU) per gram of sample analyzed.

2.3. Microbial Analysis

Microbial analysis of the haemolymph was carried out and this included the culture of the microbes in Nutrient agar for bacteria by streak plate method followed by their morphological and biochemical characterization by various standard methods. Gram stain method, Hanging drop method and Colony morphology observation were followed for the morphological characterization. Biochemical characterization of the isolated microbial pathogen was done by Indole production test, Methyl red test, Voges proskauer test, Citrate utilization test, Nitrate reduction test and Casein hydrolysis test. [6]

2.4. Antibacterial assay

The effectiveness of these extract on the microbial pathogens was then assayed by Zone of inhibition test and Turbidimetry analysis. Zone of inhibition test was carried out in petriplates on Muller-Hinton agar medium. For this, the culture was poured into the petriplates, incubated and suitable wells were created. The marine sponge extracts were then poured in to these wells and the zone of inhibition formed around the well was measured.

2.5. Turbidimetry assay

Turbidimetry analysis was carried out by following the time course growth to determine the inhibition of the growth of microbial pathogens by the plant extracts [16]. For this, the standard nutrient broth was prepared and its optical density was read in the spectrophotometer at 550nm as the initial and 10 ml of the broth was taken in a clean test tube, inoculated with the microbial culture prepared from the haemolymph of the diseased silkworm using inoculation loop and maintained as control. Similarly, nutrient broth was taken in different test tubes and was inoculated with the microbial culture. All these test tubes were incubated at

37^0c overnight. They were taken out for the experiment and different volumes of the marine sponge extract was added into the cultures followed by the addition of distilled water to maintain the volume equal in all test tubes. The tubes were kept in a shaker for a while and the optical density of the cultures was measured in the spectrophotometer at 550 nm. They were then kept incubated at 37^0C and the optical density was measured again at different time intervals. The decrease in the optical density of the culture was taken as an indication of the effectiveness of the herbal extract against the growth of the microbial pathogen. [6].

4. Results

In the present investigation, the pathogenicity study of 5 bacterial species viz., *Serratia marcescens*, *E.coli*, *Pseudomonas aeroginosa*, *Staphylococcus aureus* and *Bacillus thuringiensis* were determined. LC_{50} doses of the five bacterial isolates to IV Instar larvae of *B.mori* were calculated 24 h after bacterial administration. LC_{50} was determined separately for all five bacterial isolates. The mortality rate was high in *Pseudomonas aeroginosa* followed by *E.coli*, *Serratia marcescens*, *Staphylococcus aureus*, and *Bacillus thuringiensis*. According to [4] the septic bacteria of *Serratia marcescens* is the most dangerous pathogen to the silkworm than the other bacterial isolates *Proteus* sp., *Aeromonas aeroginosa* sp., *Streptococcus faecalis* and *Bacillus* sp. Various bacteria such as *Bacillus thuringiensis*, *Streptococcus faecalis*, *Staphylococccus* and *Serratia marcescens* have already been reported to be associated with silkworm diseases either singly or in combination [20].

The circumference and area of the zone of inhibition formed in the bacterial culture plate (Table 1) were taken as an index for the ability of the sponge extracts to deal with the respective microbes causing diseases in the silkworm. It could be observed that ethyl acetate extracts are comparatively more effective. The sponge extracts are comparatively much effective against the bacterial pathogens causing diseases in the silkworm *B. mori*. The changes in the optical density of the nutrient broth inoculated with the bacterial culture prepared from the haemolymph of the diseased silkworm with the addition of sponge extracts were noted to find out the susceptibility of the bacterial culture to the effect of the sponge extracts both with the increase in concentration and time (Table 2).

Discussion

Silkworm is died after injection of pathogenic bacteria such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Vibrio cholera* and others but it is survived by injection of antibiotics including chloramphenicol [9]. Unlike bacteria and viruses that need to be ingested or infected through

Table 1

Marine sponge extracts of sensitivity in microbes isolated from flacherie infected silkworm samples

S.No	Microorganism	Zone of inhibition (mm)					
		Aurora globostellata			*Spirostella inconstans* var. moendrina Dendy		
		20 μl	50 μl	100 μl	20 μl	50 μl	100 μl
1	*Serratia marcescens*	0.4	1.6	3.2	1.2	2.4	4.1
2	*Staphylococcus aureus*	1.5	1.4	5.4	2.2	2.8	3.5
3	*Bacillus thuringiensis*	1.9	2.8	3	2.1	2.4	2.9
4	*Bacillus magatorium*	R	1.1	3.4	1.3	3.1	3.4
5	*Enterobacter cloacae*	1.3	3.1	1.6	R	3.2	3.2
6	*Pseudomonas* aerogenosa	0.9	1.5	2.1	2	2.4	3.5

Table 2

The change in the optical density of the nutrient broth inoculated with the bacterial culture prepared from the haemolymph of diseased silkworm with the addition of increasing quantities of the sponge extracts

Marine sponge extract	Optical density measured at 550nm						
	Time	Control	1 ml	2 ml	3ml	4ml	5ml
Aurora globostellata sponge extracts	0hour						
	1hour	0.05	0.08	0.08	0.11	0.07	0.08
	2hour	0.10	0.10	0.11	0.14	0.10	0.09
	3hour	0.12	0.12	0.15	0.05	0.12	0.08
	4hour	0.15	0.14	0.11	0.13	0.15	0.05
	5hour	0.18	0.05	0.05	0.06	0.18	0.09
Spirostella inconstans var. moendrina. *Dendy marine* sponge extracts	0hour	0.05	0.12	0.09	0.11	0.08	0.07
	1hour	0.10	0.10	0.10	0.12	0.10	0.09
	2hour	0.12	0.12	0.13	0.09	0.08	0.08
	3hour	0.15	0.14	0.12	0.13	0.14	0.05
	4hour	0.18	0.15	0.14	0.11	0.13	0.09
	5hour	0.17	0.19	018	0.16	0.09	0.07

wounds to cause a disease, entomopathogenic fungi can kill insects by direct penetration of the cuticle, followed by multiplication in the hemocoel [1,10]. Chitosan solution at different concentrations showed effective antibacterial activity against *S. marcescens* strains isolated from both the cactus plant and silkworms. In addition, chitosan solutions up to 0.10 mg/ml showed stronger antibacterial activity against *S. marcescens* compared with the remainder treatment, which is consistent with the result of [11].

[18] Reported that multiplication of flacherie infected bacterium leads to poor intake of food that are occupied in the larval gut lumen. The poor food intake leads to biochemical changes, bacterial toxins, and histopathological changes in gut epithelium had affected the energy budget in the silkworms. The larval weight, pupal weight, shell weight, cocoon weight and filament length are affected due to changes in energy budget. The poor supply of energy damage the silk protein in the silk gland of silkworm. The sericin and fibroin content also slightly reduced.

Conclusion

The pathogenic microbes had caused a breakdown in protein synthetic machinery in the silk gland. Hence, it is imperative to develop management strategies to prevent bacterial flacherie. It was planned to study about the enhancement of immunity by administrating suitable immune modulating agents thereby safeguarding silkworms from the attack of disease causing microorganisms.

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