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Effect of herbal extracts on the microbial pathogens causing flacherie and muscardine diseases in the mulberry silkworm, *Bombyx mori* L.

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ABSTRACT

A preliminary survey on the incidence of silkworm diseases in ten selected sericulture farms near Srivilliputhur, a hub of sericulture activity in the Virudhunagar district of Tamilnadu, India showed that the incidence of the bacterial diseases, flacherie is quiet common in this region followed by the fungal diseases, muscardine and the viral diseases, Grassarie. 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. The nature of fungal species however, could not be ascertained due to several handicaps including the difficulties encountered during their culture. Studies were carried out *in vitro* to assess the efficacy of some herbal extracts for the containment of these microbes through turbidimetry analysis and zone of inhibition test. The observations made during this study revealed that the aqueous and alcoholic crude extracts of three herbs such as, *Acalypha indica*, *Ocimum sanctum* and *Tridax procumbens* are effective against these microbes causing flacherie and muscardine diseases in silkworm. The comparison of their effects indicated that alcoholic extracts were generally more effective than aqueous extracts and that the extracts of *Tridax procumbens* are very effective against these microbial pathogens followed by the extracts from *Ocimum sanctum* and *Acalypha indica*. 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.

Key words: Sericulture, herbal extracts, flacherie, muscardine, *Bombyx mori*

INTRODUCTION

The mulberry silkworm, *Bombyx mori* is of great economic importance as a foreign exchange earner for many silk producing countries of the world (Krishnaswami *et al*., 1992). In tropical countries, mulberry silkworm is continuously reared and this makes it highly susceptible to pathogens and hence occurrence of diseases is a major constraint (Samson *et al*., 1998). Four silkworm diseases namely Grasserie (viral), Flacherie (bacterial), Muscardine (fungal) and Pebrine (protozoan) are common in China and India. These diseases which used to cause heavy loss to silkworm crops in the past are now under control in China through proper forecasting and integrated management, but in India, more than 40 per cent of crop losses still occur due to these diseases (Veeranna, 1999). Medicinal and aromatic plants constitute a major source of natural organic compounds widely used in human health care. These plants produce many compounds as secondary metabolites that have no apparent metabolic, physiologic and structural role in the producer, but often have effects on other organisms. In many cases they are believed to function as biochemical defense (Jain *et al*., 2004). The present study was undertaken to find out the possibility of using the extracts of three medicinal plants such as *Acalypha indica*, *Ocimum sanctum* and *Tridax procumbens* for controlling the bacterial and fungal pathogens causing flacherie and muscardine diseases in the mulberry silkworm, *Bombyx mori*.

MATERIALS AND METHODS

Haemolymph Collection

The information on the incidence of silkworm diseases was obtained from ten leading farmers practising sericulture in this region. The healthy and diseased silkworms were sampled from their farms and brought to the laboratory under cool condition. The haemolymph from the normal and diseased silkworms were then collected in pre- chilled test tubes. They were then centrifuged at 1500 rpm for 10 minutes and the supernatant were transferred to the storage tubes and were kept stored at -20°C.

Herbal Extract Preparation

The leaves of the three herbal plants such as*Acalypha indica*, *Ocimum sanctum* and *Tridax procumbens* were collected; shade dried and pounded separately using mortar and pestle to get a fine powder. Ten grams each of these powders were packed separately in a burette column keeping glass wool at the bottom and filter paper rings on top. Aqueous and alcoholic extracts were collected from these leaf powders by pouring double distilled water and ethyl alcohol on top of the burette column. The collected extracts were then concentrated in hot water bath and made up to 10 ml with distilled water and kept stored in the deep freezer. Required quantities of these extracts were taken and diluted with distilled water to get the required concentrations.

Microbial Analysis

Microbial analysis of the haemolymph was carried out and this included the culture of the microbes in Nutrient agar for bactetia 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. Fungal cultures were prepared for analysis in Potato Dextrose Agar (PDA) medium.

Efficacy 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 plant extracts were then poured in to these wells and the zone of inhibition formed around the well was measured. 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 (Palombo and Semple, 2001). 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 ^{o}C overnight. They were taken out for the experiment and different volumes of the herbal 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 ^{o}C 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.

Table 1. Observation of the incidence of diseases in ten selected sericulture farms near Srivilliputhur in the virudhunagar district of Tamilnadu state.

Name of the farmer/farm	Location	Predominant disease noticed
Chinnathambi	Ramalingapuram	Flacherie
Gursamy	Ramalingapuram	Flacherie
Nallasivam	Ramalingapuram	Flacheire
Perumal	Ramalingpuram	Flacherie
Gowndar Thoppu	Poovani	Flacherie
Sudha Nursery	Krishnankoil	Flacherie
Rengasamy	Krishnankoil	Flacherie
Martin	Nachiarpatti	Muscrdine
Govindammal	Ramalingapuram	Muscardine
Nagaraj	Nachiarpatti	Grasserie

Table 2. The circumference and area of the zone of inhibition formed in the test for the bacterial culture prepared from the hamolymph of the diseased silkworm with the extracts of *Acalypha indica*, *Ocimum sanctum* and *Tridax procumbens.*

Name of the plant extract	50µl		100µl		150µl	
	Circum (mm)	Area (mm^2)	Circum (mm)	Area (mm^2)	Circum (mm)	Area (mm^2)
Acalypha indica-Aqueous	18.8± 4.1	28.3±5.8	20.1±1.7	36.3±6.8	26.1 ± 1.7	46.3 ± 6.8
Acalypha indica-Ethanolic	21.9±3.1	29.9±9.7.	22.1±3.4	39.7±8.9	22.8 ± 3.9	43.4±1.8
Ocimum sanctum-Aqueous	21.9±4.2	38.8±8.2	24.1±4.8	47.4±7.8	27.2 ± 6.5	61.6±9.8
Ocimum sanctum-Ethanolic	23.6±1.8	42.4±6.8	30.4±1.8	73.5±8.6	30.4 ± 1.8	73.5±8.6
Tridax procumbens-Aqueous	25.1±3.1	50.8±5.7	27.2±1.8	59.1±7.7	28.3 ± 3.1	64.1±9.4
Tridax procumbens-Ethanolic	30.4±4.8	74.6±6.6	37.7±3.1	113.6±5.9	40.8 ± 3.1	133.2±7.4

Each value is the average of three to five observations ± Standard deviation.

RESULTS AND DISCUSSION

The information collected from the ten leading sericulture farmers in this region (Table 1) revealed that flacherie, muscardine and grasserie are the silkworm diseases that are prevalent in this region. Of these, flacherie, caused by bacterial infection is predominant, followed by muscardine (fungal) and grasserie (viral) diseases. Data made available from various sources on the incidence of silkworm diseases in tropical regions put the average annual loss to be around 30 per cent. If the diseases are controlled below the economic threshold level then there will be an increase of 25 per cent silk production without any increase in the area under mulberry sericulture (Nagarajan and Radha, 1990).

Microbial analysis of the subcultures developed from the haemolymph of the silkworm that showed symptoms of flacherie and grasserie revealed that the microbes were gram positive, rod shaped bacteria (*Bacillus* sp.), cocci shaped (*Streptococcus* sp, *Staphylococcus* sp) and gram negative, rod shaped (*Pseudomonas* sp.). Biochemical characterization tests of the cultures showed positive results confirming the nature of bacteria as mentioned above. 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 (Sengupta *et al*., 1990). *Bacillus thrungiensis* is a widely distributed facultative entomogenous bacterium with as much as 34 varieties and are well known as bio-pesticide and employed extensively against insect pests in agriculture. Unfortunately they are also pathogenic to the silkworm (Selvakumar *et al*., 1999). The nature of fungus could not be ascertained due to practical difficulties. However, since the silkworm showed symptoms of white muscardine, it was inferred that the fungus could be *Beauveria bassiana*, the most common one causing white muscardine in India (Chandrasekaran and Nataraju, 2008).

The circumference and area of the zone of inhibition formed in the bacterial culture plate (Table 2) and fungus culture plate (Table 3) were taken as an index for the ability of the herbal extracts to deal with the respective microbes causing diseases in the silkworm. It could be observed that alcoholic extracts are comparatively more effective than aqueous extracts and that this difference is only marginal in the case of the extracts from *Ocimum sanctum*, slightly intense for *Aclypha indica* and very intense for *Tridax procumbens.* Further, it could be noticed that both the aqueous and alcoholic extracts of *Tridax procumbens* are comparatively much effective against the bacterial and fungal 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 herbal extracts were noted to find out the susceptibility of the bacterial culture to the effect of the herbal extracts both with the increase in concentration and time (Table 4).

Plants act as the richest source of organic chemicals and phytochemicals influence the life and behaviour of several insects (Eid *et al.*, 1989). Plant extracts are also considered as cheap and abundant source to increase the silk yield.

Table 3. The circumference and area of the zone of inhibition formed in the test for the fungal culture prepared from the hamolymph of the silkworm with muscardine disease treated with varying quantities of the extracts of *Acalypha indica*, *Ocimum sanctum* and *Tridax procumbens*

Name of the plant extract	50µl		100µl		150µl	
	Circum (mm)	Area (mm^2)	Circum (mm)	Area (mm^2)	Circum (mm)	Area (mm^2)
Acalypha indica-Aqueous	12.7±3.1	13.1±6.3	13.7±1.8	22.5± 4.9	17.8±1.8	25.4±4.9
Acalypha indica-Ethanolic	12.7±3.1	13.1±6.3	17.9±4.6	26.4± 1.3	18.8± 3.1	28.8± 9.4
Ocimum sanctum-Aqueous	14.8±1.6	14.9±4.1	16.9±3.1	23.1±9.1	17.9±4.6	26.4±3.5
Ocimum sanctum-Ethanolic	15.8± 2.9	23.6±3.4	17.8±1.8	25.4±4.9	19.9±1.8	31.7±5.9
Tridax procumbens-Aqueous	15.7±5.4	21.2±7.3	19.9±1.8	31.7±5.8	21.5±3.1	42.9±7.6
Tridax procumbens-Ethanolic	16.9±3.4	23.1±9.1	22.1±3.1	38.9±9.7	26.2±1.8	54.7±7.7

Each value is the average of three to five observations ± Standard deviation.

Table 4. 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 extracts of *Tridax procumbens.*

Name of the plant extract	Optical density measured at 550nm					
	Time	Control	1 ml	2ml	3ml	4ml
Acalypha indica-Aqueous	0 hour	0.05	0.05	0.05	0.05	0.05
	1 hour	0.10	0.08	0.06	0.05	0.04
	2 hours	0.12	0.10	0.08	0.06	0.06
	3 hours	0.15	0.12	0.11	0.10	0.10
	4 hours	0.18	0.16	0.15	0.14	0.15
Acalypha indica-Ethanolic	0 hour	0.05	0.05	0.05	0.05	0.05
	1 hour	0.10	0.06	0.04	0.03	0.02
	2 hours	0.12	0.08	0.08	0.08	0.06
	3 hours	0.15	0.11	0.10	0.08	0.06
	4 hours	0.18	0.15	0.12	0.10	0.08
Ocimum sanctum-Aqueous	0 hour	0.05	0.05	0.05	0.05	0.05
	1 hour	0.10	0.08	0.06	0.05	0.04
	2 hours	0.12	0.10	0.08	0.06	0.06
	3 hours	0.15	0.12	0.11	0.10	0.08
	4 hours	0.18	0.16	0.13	0.11	0.10
Ocimum sanctum-Ethanolic	0 hour	0.05	0.05	0.05	0.05	0.05
	1 hour	0.10	0.08	0.06	0.05	0.04
	2 hours	0.12	0.10	0.08	0.06	0.06
	3 hours	0.15	0.12	0.11	0.10	0.08
	4 hours	0.18	0.15	0.12	0.11	0.10
Tridax procumbens-Aqueous	0 hour	0.05	0.05	0.05	0.05	0.05
	1 hour	0.10	0.06	0.04	0.03	0.02
	2 hours	0.12	0.08	0.06	0.05	0.06
	3 hours	0.15	0.12	0.11	0.10	0.10
	4 hours	0.18	0.14	0.13	0.10	0.10
Tridax procumbens-Ethanolic	0 hour	0.05	0.05	0.05	0.05	0.05
	1 hour	0.10	0.06	0.04	0.03	0.02
	2 hours	0.12	0.08	0.08	0.08	0.06
	3 hours	0.15	0.10	0.08	0.08	0.05
	4 hours	0.18	0.10	0.12	0.10	0.08

Rajasekaragouda *et al* (1997) noticed the growth promoting effect of the water and ether extracts of the plants such as *Tribulus terrestis* and *Psoralea corylifolia*. Extracts of some commonly occuring weed plants such as *Tribulus terrestries, Phylanthus niruri, Boerhavia diffusa, Psoralea corylifolia,, Caesalpinia coriaria* and *Parthenium hystrophorus* were reported to improve the silk and egg production in the silkworm, *Bombyx mori* (Murugan *et al.*, 1998). These silkworm larvae fed on mulberry leaves treated with *Coffea arabica* leaf extracts recorded significantly higher shell weight (0.296g) than control (Jeyapaul *et al*., 2003). Dietary supplementation of the leaf, flower and pod extracts of *Moringa oleifera* elicited varied responses in the final instar larvae of *B. mori* (Rajeswari and Isaiarasu, 2004).

Plant derived medicines have been part of our traditional health care in most parts of the world and there has now been an increasing interest in using plants as the sources of agents to fight microbial diseases (Sandhya *et al*., 2006). Over the last 20 years, a large number of plant species have been evaluated for their antimicrobial activity and many natural compounds derived from plant and their crude extracts have been proved to be protective against the toxicity of many chemicals (Shyamprasad *et al*., 2002). In this context all the three plants used for the preparation of the extracts in the present study have proven antimicrobial activity (Joshi, 2000). The extracts of *Acalypha indica* showed antifertility effect in albino rats with the petroleum ether and ethanol extracts being most effective (Hiremath *et al*., 1999). They also showed antimicrobial activity against four bacterial and fungal strains that is comparable to the effect of standard antibiotics antifungal drugs (Somchit *et al*., 2010). The leaf extracts of *Ocimum sanctum* have been shown to contain constituent such as eugenol that has antimicrobial and antimycotic effects (Kishore *et al*., 1982). The extracts of *Tridax procumbens* possess antiseptic, insecticidal and parasiticidal properties (Saxena and Sosanna, 2005) Perhaps a good understanding of the disease, its mode of transmission and control of the microbial pathogens causing silkworm diseases using plant extracts could be another possibility of improving silk productivity by reducing the incidence of silkworm diseases. 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.

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Bombyx mori, the domestic silk moth, is an insect from the moth family Bombycidae. It is the closest relative of Bombyx mandarina, the wild silk moth. The silkworm is the larva or caterpillar of a silk moth. It is an economically important insect, being a primary producer of silk. A silkworm's preferred food are white mulberry leaves, though they may eat other mulberry species and even the osage orange. Domestic silk moths are entirely dependent on humans for reproduction, as a result of millennia of The In-vitro evaluation acetone extract of medicinal plants showed significant effect on inhibition zone. Among the nine medicinal plants extracts, Ocimum tenuiflorum recorded maximum zone of inhibition against Bacillus sp., Asparagus officinalis against Staphylococcus sp. and Phyllanthus emblica against Streptococcus sp., respectively in the concentrations 2, 4 and 6 per cent on 24 and 48 hours of observation for the 10-4 and 10-6 dilutions compared to control. Introduction.Â The important diseases affecting silkworm are flacherie, grasserie, muscardine and pebrine. Among these four diseases, the flacherie is most devastating disease of silkworm accounting for the cocoon crop loss to the tune of 33.88 per cent (Tayal and Chauhan, 2017). Various pathogens like fungi, bacteria,viruses and nematode cause diseases in mulberry. Among the pests few sap suckers and defoliators are considered to be major as they cause extensive damage to the mulberry. These diseases and pests cause around 12-25% leaf yield loss either by depletion in nutritive value or defoliation.