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Antibacterial Property of Chloroform Extracts of Plants Against Pathogens of Mulberry Silkworm, *Bombyx mori L.*

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Mulberry silkworm, *Bombyx mori* L. domesticated for more than 5000 years is highly susceptible to diseases and adverse environmental conditions. Hence, it is appropriate to use plant products, which have been well proved for their antimicrobial properties. Among the various diseases affecting *B. mori,* bacterial pathogens alone inflict loss to the tune of 70 per cent. Hence, *in vitro* and *in vivo* studies were conducted for the management of bacterial pathogens with chloroform extracts of plants, *viz., Thuja orientalis* (L)*, Aegle marmelos* and *Asparagus gonoclados* (Baker) along with standard antibiotics. The plant extracts were tested against pathogenic forms of bacteria, *Staphylococcus aureus* and *Bacillus thuringiensis* infecting the commercially exploited silkworm breeds, PM x CSR2 and CSR2 x CSR4. The plant products not only reduced the rate of disease incidence but also enhanced the economic parameters, *viz.,* larval weight, cocoon weight, shell weight and shell ratio.

Key words: *Bombyx mori*, Bacterial pathogens, Plant extracts, Antibacterial property, Enhanced economic parameters.

Tropical sericulture encounters lower cocoon productivity due to continued rearing, improper disinfection and occurrence of diseases. Among these factors that affect the success of cocoon production in mulberry silkworm, *Bombyx mori* L., the menance of diseases caused by viral, bacterial and fungal pathogens gain prime importance. One of the major diseases affecting *B. mori* is bacterial flacherie. Varying degrees of crop losses due to bacterial pathogens are reported from time to time by several authors. Samson *et al.,* (1990) and Savanurmath *et al.,* (1992) reported that incidence of bacterial flacherie was the highest (57.2%, 47.9 %) among the diseases.

Plants serve as good sources of useful compounds for the development of new chemotherapeutic agents and their potential is yet to be explored. Many plants have been used because of their antimicrobial traits, which are due to compounds synthesized by the process of secondary metabolism (Jansen *et al.,* 1987). Among the estimated 5, 00, 000 plant species, only a small percentage has been investigated phytochemically and the fraction submitted for biological screening is even smaller (Kroschwitz *et al.,* 1992). Considering the vast potential of plants as sources for antimicrobial principles, especially against bacteria, a systematic study was undertaken to screen the easily available local flora against the pathogenic strains of bacteria affecting *B. mori,* an economically important insect, most valued for lustrous and fabulous fibre, the silk.

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Materials and Methods

Collection of plant material

Fresh disease free plant material from four plants *Acorus calamus* (rhizome), *Aegle marmelos* (leaf). *Asparagus gonoclados* (leaf) and *Thuja orientalis* (bark) were collected from Coimbatore region of Tamil Nadu, India. The materials were thoroughly washed two to three times with running tap water and once with sterile distilled water and air dried on sterile blotter paper under shade.

Solvent extraction

The air dried plant samples were powdered with a blender and 25 gm of shade dried powder was filled in thimble soaked overnight in chloroform and then extracted with chloroform in soxhlet extractor for six hours (Khatune, 2000). The filtrate was collected in a rained bottom flask which was transferred to a rotary flash vaccum evaporator for evaporation of the solvent. The residue was dissolved in acetone and preserved at 5°C in airtight bottles until further use.

Isolation and maintenance of bacterial pathogens for antimicrobial studies

Flacherised cadavers were collected from various places from Tamil Nadu. Pure colonies of different bacterial species isolated from diseased cadavers other than *Bacillus thuringiensis* were maintained on Nutrient Agar medium and *Bacillus thuringiensis* on T3 medium. The bacterial species were characterized based on morphological studies, biochemical tests and observing the cells, spores

and crystals under phase contrast microscope. Species specific tests were carried out for correct identity of *Staphylococcus aureus*. Pathogenicity studies were conducted against two silkworm breeds, PM x CSR2 (Cross breed) and CSR2 x CSR4 (Bivoltine hybrid) to select the virulent pathogens for further works.

Most virulent strain of *S. aureus,* OM Sta 2 causing a mortality of 52. 79 per cent and 68.58 per cent during two different seasons against the cross breed, PM x CSR2 and 72.00 and 77.20 per cent against bivoltine hybrid, CSR2 x CSR4 was taken for the study Similarly, *B. thuringiensis* strain, SLM Bt 2 causing 80.84 per cent, 75.14 per cent mortality of PM x CSR2 and 94.69 per cent, 100 per cent mortality against CSR2 x CSR4 was taken for further studies (Table 1).

Efficacy of botanicals against flacherie under in vitro condition

Disc diffusion method

Plant extracts @ 500 ppm concentration were prepared in acetone through serial dilution. The bacterial pathogens were seeded into respective medium @ 10^7 cells/ml for *S. aureus* and 10^6 spores/ml for *B. thuringiensis* with 24h old cultures. After solidification, 5 mm diameter sterile filter paper discs impregnated with the plant extracts were placed on the test organisms seeded plates. Streptomycin sulphate @ 100 ppm for *S. aureus* and Erythromycin @ 100 ppm for *B. thuringiensis* served as positive control and acetone served as negative control. The antimicrobial assay plates were incubated at 37°C for 24h. The diameters of inhibition zone (DIZ) were measured in mm.

Table 1. Pathogenicity of bacterial pathogens against silkworm cross breed, PM x CSR2 and bivoltine hybrid, CSR2 x CSR4

Bacterial pathogens	Season wise larval mortality (%)			
	PM x CSR2		CSR2 x CSR4	
	Season I	Season II	Season I	Season II
KI Bc 1 (*Bacillus cereus*)	71.56	65.60	78.00	100.00
BM Bc 1 (*B.cereus*)	75.40	60.92	84.00	100.00
AM Bc 1 (*B. cereus*)	67.20	66.62	72.00	81.51
SLM Bt 1 (*Bacillus thuringiensis*)	75.30	72.00	86.00	92.79
AR Sta 1 (*Staphylococcus aureus*)	49.20	38.17	71.20	64.29
DM Sta 1 (*S. aureus*)	47.00	60.07	72.00	74.29
PI Sta 1 (*S. aureus*)	47.90	46.59	62.00	64.27
OM Sta 1 (*S. aureus*)	46.20	65.69	58.00	71.50
AM Sta 1 (*S. aureus*)	49.20	59.20	61.00	64.29
OM Sta 1 (*S. aureus*)	52.79	68.58	72.00	77.20
BM Sta 1 (*S. aureus*)	47.40	54.55	51.00	51.65
DM Sta 1 (*S. aureus*)	32.76	37.23	47.00	58.25
PI Sta 1 (*S. aureus*)	54.55	50.37	67.00	64.20
SLM Bt 2 (*B. thuringiensis*)	80.84	75.14	94.69	100.00
AR Sta 2 (*S. aureus*)	45.95	35.94	58.67	64.29
Untreated control	0.00	0.00	0.00	0.00
SEd	2.98	2.49	2.07	1.34
CD (0.05)	6.11	5.09	4.23	2.73

Efficacy of botanicals against bacterial strains under in vivo condition

Fresh mulberry leaves dipped in bacterial suspension of 10^7cells/ml for *S. aureus* and 10^6 spores/ml for *B. thuringiensis* were used for bioassay studies. The worms after second moult were fed with mulberry leaves treated with bacterial suspension. The treated leaves were provided as first feed on first day and thereafter the larvae were fed with normal leaves. On the next day, the leaves treated with 500 ppm extracts of *A. marmelos*, *A. gonoclados* and *T. orientalis* and 100 ppm of antibiotics were fed to the worms. Fresh leaves were dipped in required concentration of extracts and shade dried before feeding silkworms. Botanical treatments were administered twice, once on the second day of third instar and the other on the first day of the fourth instar. The worms fed with bacterial suspension alone served as treated control. Untreated control was also maintained.

Statistical analysis

Analysis of variance (ANOVA) for different observations made on the treatments were performed and made were compared using least significant difference (LSD) (Panse and Sukhatme, 1957).

Results and Discussion

The results on the pathogenicity of bacterial pathogens against two commercially exploited

silkworm breeds, PM x CSR2 and CSR2 x CSR4 during two different seasons are presented in Table 1.

During both seasons the highest mortalities were caused by the bacterial strains, SLM Bt 1 (75.30 %, 72.0 %) and SLM Bt 2 (80.84%, 75.14%) against PM x CSR2 and CSR2 x CSR4 (86.00%, 92.79%, 94.69%, 100%), followed by different strains of *B. cereus,* which produced mortality ranging from 60.92 to 100 per cent.

Though many pathogenic strains were available in *S. aureus*, the highest mortality (52.79%, 68.58%) was produced during two different seasons by the strain, OM Sta 2 against PM x CSR2 and CSR2 x CSR4 (72.00%, 77.20%), followed by the strain, Dm Sta 1 against PM x CSR2 (47.00%, 60.07%) and CSR2 x CSR4 (72.00%, 74.29%), respectively.

The mortality caused by other strains varied from 32.76 to 71.50 per cent. Irrespective of the seasons and the silkworm breeds, the spore and crystal producing bacterium, *Bacillus thuringiensis* was found to be highly pathogenic followed by the spore producing *B. cereus*. Certain strains of *S. aureus* were also pathogenic, but comparatively lesser than *B. thuringiensis* and *B. cereus.*

Efficacy of botanicals against pathogenic bacteria

In vitro *efficacy of chloroform extracts of botanicals against* S. aureus *and* B. thuringiensis

All the botanicals showed antibacterial properties against *S. aureus* producing an inhibition zone varying from 16.0 to 17.5 mm diameter against 17.0 and 15.0 mm in control (Table 2).

Table 2. Growth inhibitory effect of botanicals against *Staphylococcus aureus* and *Bacillus thuringiensis*

Botanicals *	*Inhibition zone (mm)*			
	Bac erial pathogens			
	S. aureus (OM Sta 2)	*S. aureus* (DM Sta 1)	*B. thuringiensis* (SLM Bt 1)	*B. thuringiensis* (SLM Bt 2)
Thuja orientalis	16.0± 0.08	16.0± 0.08	22.5	22.5
Acorus calamus	17.0	16.0± 0.08	15.5	17.5
Asparagus gonoclados	17.5	18.0	18.0	18.0
Aegle marmelos	17.0	16.0± 0.08	19.0	18.5
Streptomycin sulphate (Standard)	17.0	15.0	NA	NA
Erythromycin (Standard)	NA	NA	24.0	23.0
Chloroform	0.0	0.0	0.0	0.0

*500 ppm of Chloroform extract ; NA – Not applicable ; Figures in parantheses indicate the strain of pathogen

Similarly, antibactericidal effect was exhibited against *B. thuringiensis* with zone of inhibition varying from 15.5 mm to 22. 50 mm against 24.0 and 23.0 mm in control (Table 2).

In vivo antibacterial activity of plant extracts against silkworm cross breed

Studies on antibacterial effect of chloroform extracts of *Aegle marmelos* and *Asparagus gonoclados* against *S. aureus* revealed that both were effective producing 25.00 per cent mortality and 61.80 per cent reduction in *A. marmelos* and 28.50 , 56.50 per cent respectively in *A. gonoclados* against 65.50 per cent mortality in untreated control. The botanicals were also not detrimental to silkworm, which was shown by enhanced economic parameters (Table 3). *A. marmelos* showed equal effectiveness to the standard, Streptomycin sulphate (3.05g, 1.70g, 0.28g and 16.6 %) with respect to larval weight (3.30 g), cocoon weight (1.72 g), shell weight (0.28g) and shell ratio (16.2%). Similarly in *A. gonoclados,* the economic parameters recorded were 2.95g, 1.65g, 0.28g and 16.90 per cent, respectively.

In vivo *antibacterial activity of plant extracts against silkworm cross breed*

The silkworm larval mortality due to be *B. thuringiensis* got reduced due to treatment with *T. orientalis* and *A. gonoclados* (34.0%, 35.0%) against erythromycin (36.00 %) and 96.00 per cent in treated control.

The enhancement in economic parameters were also observed due to botanical treatment. The following measures of larval weight, cocoon weight, shell weight and shell ratio, respectively were recorded in various treatments *viz., T. orientalis* bark (3.53g, 1.76g, 0.35g and 19.89%), *A. gonoclados* (3.50g, 1.70g, 0.35g and 20.59%), Erythromycin (3.52g, 1.72g, 0.36g and 20.93%), treated control (2.49g, 1.45g, 0.26g and 17.93%) and untreated control (3.00g, 1.64g, 0.30g and 18.29%).

Pathogenicity studies indicated that certain strains of *Staphylococcus aureus,* all the strains of *Bacillus cereus* and *B. thuringiensis* were found to be pathogenic to commercially exploited silkworm breeds, PM x CSR2 and CSR2 x CSR4. According to Steinhaus(1949), certain strains of *B. cereus* are among the virulent bacterial pathogens of *Bombyx mori*. The spore forming bacteria were found to be highly toxic to *B. mori* (Angus, 1956; Aizawa *et al.,* 1961; Anitha *et al.,* 1994; Selvakumar *et al.,* 1999; Pramanik and Somchoudhury, 2001; Manimegalai, 2003). The occurrence of *Staphylococcus* in

Table 3. *In vivo* efficacy of botanicals against *Staphylococccus aureus* (DM Sta 1) infecting silkworm cross breed, PM x CSR2

Treatments	Per cent mortality	Per cent reduction from control	Larval weight (g)	Cocoon weight (g)	Shell weight (g)	Shell Ratio (%)
Aegle marmelos + *S. aureus*	25.00[b]	61.80[a]	3.30[a]	1.72[a]	0.28[a]	16.20[a]
Asparagus gonoclados + *S. aureus*	28.50[b]	56.50[a]	2.95[b]	1.65[a]	0.28[a]	16.90[a]
Streptomycin sulphate + *S. aureus*	27.00[b]	58.70[a]	3.05[b]	1.70[a]	0.28[a]	16.60[a]
Treated control *S. aureus* (1x 10^6 spores/ml)	65.50[c]	0.00[c]	2.25[c]	1.19[b]	0.18[b]	12.90[b]
Untreated control (Sterile water)	0.00 [a]	0.00[a]	3.10[a]	1.59[a]	0.27[a]	16.90[a]

In a column, means followed by similar letters are not statistically different (P = 0.05) by DMRT

silkworm has been reported by Chitra *et al.,* (1973) and Patil (1994). The rate of mortality varied in silkworm infected by different bacterial species. Variations in pathogenicity between breeds against *B. thuringiensis* were reported by Manimegalai and Chandramohan (2005a). In the present investigation, chloroform extracts of plants, *Thuja orientalis*, *Asparagus gonoclados* and *Aegle marmelos* were found effective against *S. aureus* and *B. thuringiensis* to be in addition to enhancing the economic parameters.

Though the occurrence of substances in plants as protectors against diseases was reported by Gaumann, (1950) and Walker and Stahmann, (1956), the role of such plant products in insect resistance to diseases was reported by Kushner and Harvey (1962). The efficacy of *T. orientalis* and *Piceae mariana* (Smirnoff and Hutchinson, 1965), aqueous extract *T. orientalis* (Manimegalai and Chandramohan, 2005b) against *B. thuringiensis* has also been reported.

The efficacy of *Goiaba* and *Coccus aureus* against *S. aureus* (Gnan and Demellos, 1999), curcumin against *Staphylococcus* sp, *Streptococcus* sp (Ishita chattopadhyay *et al.,* 2004) and garlic preparations against *Escherichia, Staphylococcus,*

Table 4. *In vivo* efficacy of botanicals against *Bacillus thuringiensis* (SLM Bt 1) infecting silkworm cross breed, PM x CSR2

Treatments	Per cent larval mortality	Per cent reduction from control	Larval weight (g)	Cocoon weight (g)	Shell weight (g)	Shell Ratio (%)
Thuja orientalis + *B. thuringiensis*	34.00[b]	64.60[a]	3.53[a]	1.76[a]	0.35[a]	19.89[a]
Asparagus gonoclados + *B. thuringiensis*	35.00[b]	63.50[a]	3.50[b]	1.70[a]	0.35[a]	20.59[a]
Erythromycin + *B. thuringiensis*	36.00[b]	62.50[a]	3.52[b]	1.72[a]	0.36[a]	20.93[a]
Treated control (*B. thuringiensis* (1x 10^6Spores/ml))	96.00[c]	-	2.49[c]	1.45[b]	0.26[b]	17.93[b]
Untreated control (Sterile water)	0.00 [a]	-	3.00[a]	1.64[a]	0.30[a]	18.29[a]

In a column, means followed by similar letters are not statistically different (P = 0.05) by DMRT

Streptococcus, Proteus and *Bacillus* (Ellen Tattleman, 2005) were reported earlier.

The antibacterial activity in plants are due to the inhibition of crystal formation in *B. thuringiensis* (Kushner and Harvey, 1962). Chadha (1976) attributed the antibacterial activity is mainly due to the presence of tannin and flavonoids, which caused aggregation of bacterial cells (Pell *et al.,* 2000) by acting either on the cell wall or cell membrane or on the extra cellular elements such as cell secreted enzymes. According to Kumeswara Rao (2000), the activity is due to agglutination of pathogenic bacteria. The increase in larval weight and cocoon parameters in *B. mori* due to administration of plant products were reported by several authors (Rajashekhar Gouda, 1991; Mamadapur, 1994; Mahesha *et al.,* 1999 and Manimegalai and Chandramohan, 2005b).

The present study demonstrated that the plant products are equal in their effectiveness as that of antibiotics, besides many positive effects such as safety, no resistance development, easy availability and cost effectiveness. Inspite of the greater potential of plants for therapeutic treatments and their added benefits, the potentiality has been explored to a limited extent only.

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