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Bioefficacy of some plant extracts against pathogenic bacteria isolated from diseased silkworm larvae

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Abstract: During the time of rearing, the larvae of the silkworm, *Bombyx mori* come in contact with pathogenic bacteria which accounts for considerable loss to cocoon production. In this connection, the bacterial pathogens were isolated from diseased silkworm larvae by growing them in a nutrient agar medium, and bio-efficacy of plant extracts against pathogenic bacteria was the main focus of the study. Morpho-physiological characteristics, biochemical properties and DNA sequencing of the 16S rRNA gene indicated that the pathogenic bacterium was *Klebsiella pneumoniae.* Growth of bacterium was observed in the presence of the extracts of *Cinnamomum zeylenicum*, *Azadirachta indica*, *Curcuma longa* and *Zingiber officinale* using the disc diffusion method. It was found that bioefficacy of the ethanol extracts of *C. zeylenicum* (22mm zone diameter of inhibition), *C. longa* (21mm zone diameter of inhibition), *Z. officinale* (20mm zone diameter of inhibition) and *A. indica* (10mm zone diameter of inhibition) showed against *K. pneumoniae* respectively. The test plant extracts can be used as the source of antibiotic substances for possible controlling of *K. pneumoniae* causing flacherie disease of silkworm larvae.

Key words: Silkworm, *Klebsiella pneumoniae,* Plant extracts

Introduction

Sericulture is an agro-based industry practiced in the greater Rajshahi, Chapai Nawabganj, Natore, Bogra and Naogaon and certain nontraditional areas in Bangladesh. It is grouped under village and small enterprises sector that plays major role for the creation of sustainable employment and income. The poor people of the society, the landless, and the poor woman in particular, can be involved in sericulture activities. According to an estimate, livelihood of about 0.1 million people in Rajshahi region are directly or indirectly involved with sericulture industry.

The mulberry silkworm, *Bombyx mori* has been domesticated for silk production for more than 5,000 years and provides the major source of income for 30 million families globally. Geographically, Asia is the main producer of mulberry silk in the world and produces over 98% of the total global output (Rahman, 2011). During the period of rearing, the silkworm comes in contact with pathogenic agents (*viz.* Protozoa-microsporidians, Virus, Fungi and Bacteria). Rearing silkworm free from diseases is a major constraint to silkworm rearers. About 34-40% the total crop in a year has been reported to be lost due to diseases (Vaidya, 1960).

Bacterial diseases were of common occurrence in Bangladesh. This is a disease of high temperature (above 30°C) and low relative humidity below 80%. It is reported to be responsible for 20-40% of total cocoon loss in Karnataka (Chitra *et al.,* 1975) every year.

Bacterial flacherie is a common disease of mulberry silkworm. The aetiology of bacterial diseases is not fully understood because of the multiplicity of bacterial types involved in bacterial infections (Choudhury *et al*., 2002). Generalized symptoms of insects associated with bacterial infections are loss of appetite, diarrhoea (discharge of watery feces), and vomiting, then the larvae softening and die emitting a foul odor (Tanada & Kaya, 1993).

In Jammu and Kashmir State, Flacherie occurred 3-8% if the worms were reared even hygienically, where as, under infective stage the rate a mounts to 12-35%. In other sericultural areas of India also this disease is equally prevalent. The disease flacherie was found more prevalent among multivoltines compared to that of bivoltine (Samson, 1995). Barman (1989) recorded about 11.5% mortality due to bacterial reasons during May-June month out of 30.51% total loss category 5-15% loss due to septicemia. This is a serious disease in Bangladesh from economic point of view, since it is prevalent mostly in the ripen

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mounted worms and causing death within 24 hours. Since there are no specific preventive measures for the occurrence and spread of disease other than sanitized rearing methods, the only commercial practice today is to discard large stocks of worms in case of infection to avoid the spread of disease (Acharya *et al.,* 2002). Antibiotics are widely used in sericulture industry as a component of bed disinfectants and as therapeutic applications against bacterial diseases (Subramanian *et al*., 2009). Broad spectrum antibiotics *viz*. penicillin, streptomycin, tetracycline and chloramphenicol were already tried on silkworm and found successful (Venkatesh & Srivastava, 2010). Antibiotics in silkworm are approved for four different purposes: disease treatment, disease prevention, disease control and for health maintenance or growth promotion (Phillips *et al*., 2004).

The aim of this study was to isolate the pathogenic bacteria from diseased silkworm and to evaluate the antibacterial activity of plant extracts on the pathogen.

Materials and Methods

Collection of silkworms: Diseased silkworm larvae were collected from Bangladesh Sericulture Research and Training Institute (BSRTI), Rajshahi. There were then used as a source of inocula for the isolation of the pathogenic microorganisms.

Isolation, purification and characterization of the microbes

The bacteria were isolated from silkworm haemolymph. One loopful of haemolymph was directly inoculated into nutrient broth, which was incubated at 37°C for 2 days with shaking at 120 rpm. Control flask without inoculates was incubated at 37°C with shaking. After 2 days the cultures were found turbid and then it was used as inocula for further experiments.

Microscopic examination and identification of bacterial cells: For the identification of the pathogenic bacteria, morphological characterizations, microscopic observations, growth characteristics, biochemical and antibiotic sensitivity tests were performed. The microorganisms were identified according to *Bergey's Manual of Systematic Bacteriology*.

Identification of the pathogen by 16S rRNA gene sequence: Genomic DNAs of the bacterial isolates were extracted according to Sambrook *et al*. (1989). Gene fragments specific for the highly variable region of 16S rRNA gene was amplified by PCR. PCR was performed using a set of primer of 16SF (5'-GAGTTTGATCCTGGCTCAG-3') and 16SR (5'-GAAAGGAGGTGATCCAGCC-3') according to Loffler *et al*. (2000). The PCR products were subjected to 1% agarose gel electrophoresis, stained with ethidium bromide and visualized on a UV transilluminator for the presence of about 1500 bp PCR products. PCR products were purified using StrataPrep PCR purification kit (Stratagene, USA) according to the manufacturer's protocol. Sequencing reactions were carried out using ABI-Prism Big dye terminator cycle sequencing ready reaction kit and the PCR products were purified by a standard protocol. The purified cycle sequenced products were analyzed with an ABI Prism 310 genetic analyzer. The chromatogram sequencing files were edited using Chromas 2.32. The homology of the 16S rRNA gene sequences was checked with the 16S rRNA gene sequences of other organisms that had already been submitted to GenBank database using the BLASTN (http://www.ncbi.nih.gov/BLAST/) algorithm.

Preparation of plant extracts and antibacterial activity test

Four medicinal plants were selected for the present investigation *viz*. *C. zeylenicum* (Cinnamon), *A. indica* (Neem), *C. longa* (Turmeric), and *Z. officinale* (Ginger). Ten gram of air dried powder was placed in 100ml of methanol in a conical flask and kept in rotary shaker at 120 rpm for 24h. After 24h it was filtered off through whatman filter paper number-1 and the solvent was evaporated to make the final volume one-forth of the original volume.

Disc diffusion method was used for the antibacterial activity test of the extracts against *K. pneumoniae*. Dried and sterilized filter paper discs (6mm diam.) were then impregnated with methanol extracts in different concentrations (200mg/10µl, 100mg/10µl, 50mg/10µl, 25mg/10µl) using micropipette and residual solvents were completely evaporated and then the paper discs of selected plant extracts were set on the nutrient agar medium uniformly sprayed with the said bacteria. Standard disc of Azithromycine (15µg/disc) and blank discs (impregnated with solvents followed by evaporated) were used as positive or negative control, respectively. The plates were then incubated at 37ºC for 24 hours to

allow maximum growth of the bacterium. Observations were noted of bactericidal effectiveness on the basis of inhibitory zones.

Results and Discussion

Isolation of bacteria: Bacteria were isolated by plating onto an agar solidified nutrient medium. The plates were incubated at 37°C for 24 hours and bacterial colonies were found to grow on the medium. Results of microscopic analysis of bacterial cells and their growth characteristics are presented in Table 1(a) while the biochemical and antibiotic sensitivity tests of the bacteria are presented in Table 1(b) and 1(c), respectively. Isolated bacterial strain was identified by both morphological and biochemical tests and this was further confirmed by 16S rRNA gene sequence analysis. The strain showed 99% homology with *K. pneumoniae* 5.3A (gene bank accession on JN 644581.1).

Table 1(a). Culture media dependent characteristics and microscopic observations of the isolated bacterial strain (*K. pneumoniae*)

Agar plates	Characters	Results
Nutrient agar slant	Abundance of growth	Moderate
	Colour	Creamy White
Nutrient broth culture		Uniform with fine turbidity
Microscopic observations	Gram staining	Gram-negative
	Motility	Motile

Table 1(b). Biochemical test results for the isolated bacterial strain (*K. pneumoniae*)

Sugar utilization	
Carbon sources	**Bacteria**
Glucose	+
Arabinose	-
Lactose	+
Xylose	+
Malonate	+
Rhamnose	+
Raffinose	-
Catalase	+
Oxidase	+
Nitrate reduction	-
Indole	-
Methyl Red	-
VP	+
Dulcitol	-
Lysine decarboxylase	+
Citrate	+
Urease	+
H_2S production	-
MacConkey	Pink
XLD	Yellow

+ sign indicate the growth of microorganisms while; - sign indicate no growth.

Table 1(c). Antibiotic sensitivity tests

Antibiotics	Disc distance (mm)	R	S and I
Vancomycin	5	R	-
Pefloxacin	12	-	I
Cefuroxime sodium	5	R	-
Penicillin	5	R	-
Cephardine	5	R	-
Mecillinam	5	R	-
Nitro furantoin	5	R	-
Vancomycin	5	R	-
Azithromycine	18	-	S
Gentamycin	16	-	S
Ciprofloxacin	16	-	S

(5-10mm) = Resistant to antibiotics (R); (15-20mm) = Sensitive to antibiotic (S), (10-15mm) = intermediate resistance (I).

Antibacterial activities of plants extract against *K. pneumonia*

Extracts in organic solvents (ethanol) of important medicinal plants such as *C. zeylenicum, A. indica, C. longa,* and *Z. officinale* were evaluated for their antibacterial activities against *Klebsiella pneumoniae* by estimating zones of inhibition as produced by disc-diffusion method on nutrient agar medium. The bacterial strain of *K. pneumonia* was found to be sensitive and was inhibited by the ethanol extracts of the mentioned plants at high concentration only (20mg/10µl). It was found that antibacterial activity of the ethanol extracts of *C. zeylenicum* (22mm), *C. longa* (21mm), *Z. officinale* (20mm) and *A. indica* (10mm) showed significant effects against *K. pneumoniae* respectively (Fig. 1 and Table 2).

Table 2. Measurement of the inhibition zones of plant extracts against *K. pneumoniae*

Sl. No.	Name of the medicinal plants	Concentration of the plant extracts	Zone of inhibition (mm in diameter)
1.	*C. zeylenicum*	200mg/10µl 100mg/10µl 50mg/10µl 25mg/10µl	22mm 17mm 13mm 5mm
2.	*A. indica*	200mg/10µl 100mg/10µl 50mg/10µl 25mg/10µl	10 mm 5 mm 5 mm 5 mm
3.	*C. longa*	200 mg/10µl 100 mg/10µl 50 mg/10µl 25 mg/10µl	21 mm 16 mm 10 mm 7 mm
4.	*Z. officinale*	200 mg/10µl 100 mg/10µl 50 mg/10µl 25 mg/10µl	20 mm 12 mm 17 mm 5 mm

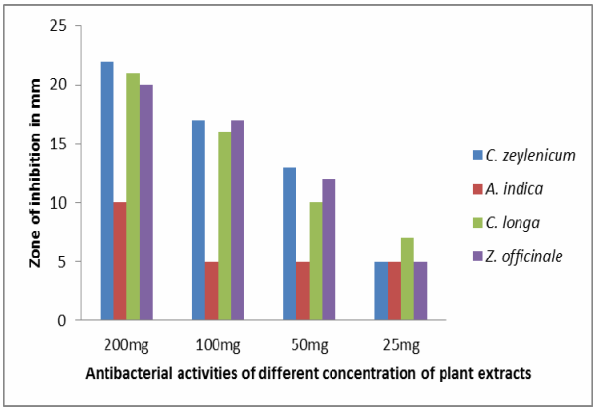

Fig. 1. A comparative study of antibacterial activities of different doses of four plant extracts.

The economic status of Bangladesh mainly depends on agriculture. Silk is the tradition of Rajshahi, Bangladesh. Many people earn their livelyhood by silk worm rearing. During the silk worm rearing, the silk worm comes in contact with various pathogenic bacteria. About 34 to 40 percent of total crop in a year has been reported to be loss due to diseases like flacherie. Flacherie is known to be caused the assembly of various pathogenic bacteria in silkworms and are commonly known as bacterial flacherie.

In this study the microorganism was identified as a number of the genera *K. pneumoniae* bacterium. Physiological and biochemical tests revealed that the microorganisms were Gram-negative, rod-shaped and motile bacteria. After a 16S rRNA gene sequencing and BLAST search, 99% similarity was observed with *K. pneumoniae*. Similar methods were used by Anitha *et al*. (1994) and Nangia *et al*. (1999) who identifying *Bacillus* and *Staphylococcus* species respectively from the diseased worms.

Chitra *et al*. (1973) reported seven pathogenic bacteria from various tissues of the diseased silkworms were isolated. The isolates were *Acrobacter cloacae*, *Achromobacter superficialis*, *Achromobacter delmarvae*, *Pseudomonas boreopolis, P. ovalis*, *Escherichia freundii* and *Staphylococcus albus*. A number of pathogenic bacteria such as *Bacillus subtilis*, *B. cereus, Staphylococcus albus, S. aureus* and *Klebsiella*

cloacae were isolated from diseased silkworm (Priyadharshini *et al*., 2008). Earlier on *Streptococcus faecalis*, *Bacillus thuringienisis* and *B. bombysepticus* were reported by Patil (1994); Nataraju *et al*. (1991); Hartman (1931). Various species of *B. thuringiensis*, *B. megaterium*, *B. ellenbachi*, *B. bombysepticus*, *B. bombycoides*, *B. mycoides* and *B. laterosporus* as the etiological agent of flacherie causing diseases in silkworm (Ishiwata, 1902; Hartman, 1931). *Bacillus* has been identified as one of the most important pathogenic organism involve in flacherie (Paillot, 1942; Steinhuaus, 1949).

The prevalence of *Bacillus* as major disease causing organisms is further strengthened by the findings of Nataraju *et al.* (1991) who have isolated six spores forming *Bacillus* from silkworm litter samples and found two of them to be pathogenic. Chishti *et al.* (1991) have isolated Bacilli group of bacteria which were gram positive, and spore forming rods. Manimegalai & Chandramohan (2006) have reported that among the bacterial isolates identified, 4.65 percent were found to be *Bacillus thuringiensis.*

The occurrence of *Staphylococcus* in silkworm has been reported by Chitra *et al.* (1973) and Patil (1994). Anitha *et al.* (1994) have isolated *Streptococcus* and *Bacillus* species. The occurrence of *K. pneumoniae*, gram negative bacteria in the silkworm haemolymph is being reported for the first time through this study. However there are few reports on the occurrence of gram negative bacteria in silkworm such as *Serratia* sp. (Anitha *et al.*, 1994); *Pseudomonas* (Bucher, 1963) and *Aerobacter cloacae* (Chitra *et al.,* 1973).

Antibiotics are used in order to find out their effectiveness against pathogenic bacteria (Mahmoud *et al*., 2012). A part of the present work showed that *in vitro* control of the bacterium like *K. pneumoniae*, eleven types of antibiotic discs were used in order to find out their effectiveness against the bacterium. Three antibiotic discs *viz*. azithromycine, gentamycin and ciprofloxacin were showed strongly effective against the said bacteria.

Samson (1987) suggested Streptomycin, Tetracycline are very effective against *K. pneumoniae*. Nahar (1995) suggested that the Ampicillin, Ciprofloxacine and Gentamicin were effective against *K. pneumoniae*.

Antibiotics showed promising results for controlling of silkworm diseases. The effects of antibiotics upon the infection with bacterial disease were studied. Ciprofloxacin, Azithromycin and Gentamycin as a cheap antibiotic in Bangladesh can be used easily by farmers to control of silkworm diseases.

Extracts of *C. zeylenicum*, *C. longa*, *Z. officinale* demonstrated a strong activity against gram negative bacteria. This investigation can be used in the folk of medicine and source of antibiotic substances for possible treatment of flacherie diseases. If the mentioned plant extracts are appropriately applied against pathogen in the silk industry, the silkworm rearing will be a thriving agriculture to farmers as well as Bangladesh. China, Japan reaches at the top of the list among the silk exporting countries. It is our believe that, indigenous medicinal plant extracts are successfully applied through isolation of bacteria from diseased silk worm against pathogens; Bangladesh will achieve the equal status of these countries.

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