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In vivo antibacterial effect of chitosan against *Staphylococcus aureus* and *Bacillus thuringiensis* and its impact on economic parameters of silkworm, *Bombyx mori.* L.

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Abstract
Background: Silkworm pupae is one of the major by-products of silk industry and considered as waste in silk reeling. Reelers after reeling out silk are generally used to throw the dead pupae at the outskirts of the city, creating nuisance and health hazards. Hence, disposal of silkworm pupae is a very big challenge. But this pupae contains numerous biological constituents which could be utilized in many industries including pharmaceuticals.
Aim of the study: Silkworm pupae are used to extract chitosan which is having many biological properties. The present study aimed to evaluate *in vivo* antibacterial effect of chitosan against bacterial pathogens, *Staphylococcus aureus* and *Bacillus thuringiensis* in silkworm.
Materials and Methods: Chitosan was extracted by deproteinization, demineralization and deacetylation. The different concentrations ranged from 0.5 to 4.5 percent were used for this study. Antibacterial bioassay was conducted and observations on larval mortality, larval weight, cocoon weight and shell weight were calculated.
Results: The antibacterial activity of chitosan extracted from silkworm pupae were maximum with 2.5 per cent against *Staphylococcus aureus* and *Bacillus thuringiensis* and economic parameters were also found to increased when compared to negative control.
Conclusion: The present study concluded that chitosan extracted from silkworm pupae showed antibacterial activity, its application to silkworm reduced larval mortality which indicated disease resistance and increased economic parameters. The unused pupae was effectively reutilized and converted into useful bioproduct.

Keywords: silkworm pupae, chitosan, antibacterial activity, *Staphylococcus aureus* and *Bacillus thuringiensis*

Introduction
Silkworm pupae is one of the major by-products of silk industry which contains 55.60 per cent of total protein and 32.2 per cent lipid content by dry weight. This protein is boosted with high level of essential amino acids, namely methionine, valine, and phenylalanine. 100 g of silkworm pupae contains various bio chemicals such as protein (55 g), fat (8.5 g), fiber (6.0 g), carbohydrates (25.43 g) and energy contents (389.60 Kcal/100 g). Mineral compositions (mg/100 g) such as calcium (102.31 mg), potassium (1826.59 mg), magnesium (287.96 mg), phosphorus (1369.94 mg), sodium (274.57 mg), iron (9.54 mg), zinc (17.75 mg), manganese (2.08 mg) and selenium (0.08 mg) respectively. Additionally, it also comprises a number of vitamins such as Vitamin A (273.99 µg), Vitamin E (51.45 IU/kg), Vitamin C (<5.78 mg), Vitamin B1 (1.91 mg), Vitamin B2 (5.43 mg), Vitamin B3 (15.20 mg), Vitamin B5 (12.49 mg), Vitamin B7 (144.51 µg), Vitamin B9 (0.41 mg), and Vitamin B12 (0.5 mg/100 g) (Rao, 1994; Zho and Han, 2006; Longvah *et al.*, 2011) [1, 2, 3]
As exoskeleton and internal organs such as spiracle and tracheae of silkworm pupae are lined by chitin, the chrysalides are used as an alternative source of chitin and consequently chitosan. β-1, 4-N-acetyl-D-glucosamine (chitosan) is a derivative of chitin after deacetylation. Silkworm pupae chrysalides having anticancerous property (Kaizer *et al.*, 1989; Hursting *et al.*, 1990; Caygill *et al.*, 1996 and Sasaki *et al.*, 1993) [4-7]. The fatty acids present in the silkworm chrysalis oil also has high antitumor activity. Hirano (2001) [8] processed chitin in the form of films and fibers from silkworm pupae skin. However, the major development of chitin film and fiber is in pharmaceutical and medical applications as wound dressing material (Yusof *et al.*, 2003) [9] and controlled drug release (Kato *et al.*, 2003) [10]. In addition, an interesting application of chitosan is composite bone filling material, which forms a

self-hardening paste for tissue regeneration in treatment of periodontal bony defects [Ito *et al.,* 1998) [11] and its oligomers have been claimed as anticancer drugs. Wattanathron *et al.* (2012) [12] identified that silk- worm pupae protect against Alzheimer's disease. Biological properties of chitosan are biocompatibility, biodegradability, hemostatic, fungistatic, spermicidal, anticholestermis, wound healing ability, reducing scars, retention of fibroblast growth factors, release of glucosamine, N-acetyl glucosamine monomers, oligomers, and stimulation of human dermal fibroblast cellular activities as well as inhibition of a wide variety of bacteria. It stimulates cell adhesion and proliferation and helps in the organization of the extracellular matrix. Pharmacological studies showed that silkworm pupae are alimental for increasing immunity, protecting the liver and preventing cancer. Consumption of silkworm pupae could supplement Vitamin B2 intake, which prevents the serious effects of Vitamin B2 deficiency (Kwon *et al.,* 2012) [13].

Silkworm pupae is considered as waste in silk reeling unit and disposal of silkworm pupae was a very big challenge for them. Reelers after reeling out silk are generally used to throw the dead pupae at the outskirts of the city, creating nuisance and health hazards. In China, human consumption of silkworm pupae has been practiced since the very earliest times and has been approved as a new source by the Ministry of Health of the Republic of China (Zhou and Han, 2006) [2]. The indigenous population in northeast India uses a variety of insects as food, one of which is the pupae of mulberry (*Bombyx mori*) eri silkworm (*Samia ricini*) and muga silkworm (*Antheraea assamensis*). Though the silkworm pupae consist of numerous biological constituents which are of great value in many industries including pharmaceuticals, it is not properly utilized. Hence, the present study aimed to make an attempt to explore the utilization of silkworm pupae which are discarded as waste for the extraction of chitosan. Also, the antimicrobial property of extracted chitosan was validated *in vivo* against bacterial pathogens of silkworm, *Staphylococcus aureus* and *Bacillus thuringiensis* in silkworm.

Materials and Methods

Collection and cleaning of silkworm pupae

Silkworm pupal wastes were collected from reeling units of Coimbatore district. The silkworm pupae were cleaned by removing the pelade layer of the silkworm cocoon. The silkworm pupae were dried in Hot air Oven at 85° C for 4 hours to remove 80 per cent of moisture for extraction of chitosan.

Extraction of chitosan

The chitin and chitosan extraction involved mainly three steps *viz.,* Deproteinization, Demineralization and Deacetylation. Deproteinization of silkworm pupae were carried out by using 4 per cent dilute sodium hydroxide at 70° C for 4 hours. Silkworm pupae to NaOH ratio of 1:10 (w/v) were maintained. After the treatment, the materials were washed with running tap water for 4-5 times to remove excess alkali and subsequently rinsed in deionized water. Demineralization of silkworm pupae were carried out by treating 3 per cent Hydrochloric acid at ambient temperature for two hours with deproteinized pupae to liquid ratio of 1:10 (w/ v). The material was washed with running water and rinsed in deionized water. The product obtained was chitin. Deacetylation was carried out by treating chitin with 45 per cent concentration of sodium hydroxide at 95° C for 4 hours and the solid to liquid ratio was maintained at 1:12 (w/v). After the treatment, the material was washed with water and rinsed in deionized water. The final product obtained was chitosan. The chitosan was dried in hot air oven for 10 h at 50° C for further use (Suresh *et al.,* 2012) [14].

Antibacterial bioassay

Bacterial suspension @ 10^7 cells / ml of *Staphylococcus aureus* and *Bacillus thuringiensis* were measured in Neubauer haemocytometer and used for bioassay studies. Mulberry leaves were freshly collected, dipped in bacterial suspension of 10^7 cells / ml and the leaves were allowed to shade dry for some time. Silkworms were reared under standard recommended condition at 26±2ºC temperature, 75% relative humidity (Krishnaswami, 1973) [15]. Thirty third instar silkworm larvae (Double hybrid) after second moult were fed with bacterial pathogen treated leaves. The treatments were replicated thrice. The bacterial pathogen treated leaves were provided during the first feed on first day and thereafter the larvae were provided with normal leaves. On next day, the leaves were treated with the different concentrations of chitosan ranging from 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4. 0 to 4.5 per cent and allowed to dry for some time before feeding to silkworms. Observations on larval mortality, larval weight, cocoon weight and shell weight were calculated. The control batches were also maintained for comparison. Statistical analysis was done by using the standard procedures of Gomez and Gomez (1984) [16].

Results and Discussion

Among the different concentrations, chitosan of 2.5 per cent recorded significantly lower mortality of 10.50 per cent on larvae inoculated with *Staphylococcus aureus.* The mortality of 73.00 per cent was noticed in negative treated control was significantly high. Chitosan of 2.5 per cent showed highest larval weight (22.99 g), cocoon weight (1.62 g), pupal weight (1.34 g) and shell weight (0.27 g). This was followed by 2 per cent chitosan recorded larval weight (21.02 g), cocoon weight (1.52 g), pupal weight (1.25 g) and shell weight (0.26 g). The negative control showed highest mortality (73.00%), lowest larval weight (17.12g), cocoon weight (0.94g), pupal weight (0.75g) and shell weight (0.19g). The reduced mortality of silkworm larvae in treatments was due to the antibacterial activity of chitosan solution fed to silkworm through leaves which indicated disease resistance against pathogens. The economic parameters were also increased due to chitosan application when compared to control (Table 1).

Table 1: *In vivo* effect of antibacterial activity of chitosan against *Staphylococcus aureus* in silkworm

S. No.	Treatments	Economic Parameters of silkworm				
		Larval mortality	Larval weight (g)	Cocoon weight (g)	Pupal weight (g)	Shell weight (g)
1.	0.5	32.00^{e}	18.22^{def}	1.11^{ef}	0.89^{ef}	0.22^{cde}
2.	1	28.00^{d}	18.84^{cde}	1.22^{de}	0.98^{de}	0.23^{abc}
3.	1.5	24.50^{c}	19.42^{cd}	1.31^{cd}	1.05^{cd}	0.25^{ab}
4.	2	22.00^{b}	21.02^{b}	1.52^{ab}	1.25^{ab}	0.26^{a}
5.	2.5	10.50^{a}	22.99^{a}	1.62^{a}	1.34^{a}	0.27^{a}

6.	3	25.00^{c}	20.21^{bc}	1.22^{de}	0.97^{de}	0.24^{ab}
7.	3.5	28.00^{d}	19.06^{cde}	1.13^{ef}	0.91^{ef}	0.22^{bcd}
8.	4	38.00^{f}	18.78^{cde}	1.00^{fg}	0.79^{fg}	0.21^{cd}
9.	4.5	48.00^{h}	17.49^{ef}	0.92^{g}	0.72^{g}	0.19^{d}
10.	Positive control	41.00^{g}	21.00^{b}	1.41^{bc}	1.14^{bc}	0.26^{a}
11.	Negative control	73.00^{i}	17.12^{f}	0.94^{g}	0.75^{g}	0.19^{d}
SEd		1.1333	0.7638	0.0733	0.0661	0.0201
CD		2.3391	1.5759	0.1513	0.1365	0.0415

Similarly antibacterial activity of chitosan against *Bacillus thuringiensis* on silkworm was also studied (Table 2). The results revealed that chitosan of 2.5 per cent recorded significantly lower mortality (17.00 %), highest larval weight (23.49 g), cocoon weight (1.82 g), pupal weight (1.50 g) and shell weight (0.32 g) respectively. This was followed by 2 per cent chitosan which showed larval mortality (18.00%), larval weight (21.53 g), cocoon weight (1.62 g), pupal weight (1.31g) and shell weight (0.31g). The highest mortality (92.00 %) and lowest larval weight (17.63 g), cocoon weight (1.15g), pupal weight (0.90 g) and shell weight (0.25 g) was observed in negative treated control. These results revealed that chitosan showed disease resistance and increased economic parameters of silkworm when compared to control.

Table 2. *In vivo* effect of antibacterial activity of chitosan against *Bacillus thuringiensis* in silkworm

Sl. No.	Treatments	Economic Parameters of silkworm				
		Larval mortality	Larval weight	Cocoon weight	Pupal weight	Shell weight
1.	0.5	30.50^{f}	18.73^{def}	1.32^{de}	1.05^{efg}	0.27^{bcde}
2.	1	28.00^{e}	19.35^{cde}	1.42^{cd}	1.13^{cde}	0.29^{abcd}
3.	1.5	22.00^{c}	19.92^{cd}	1.51^{bc}	1.21^{bcde}	0.30^{ab}
4.	2	18.00^{b}	21.53^{b}	1.62^{b}	1.31^{b}	0.31^{a}
5.	2.5	17.00^{a}	23.49^{a}	1.82^{a}	1.50^{a}	0.32^{a}
6.	3	19.00^{b}	20.71^{bc}	1.42cd	1.13^{cde}	0.29^{abc}
7.	3.5	25.00^{d}	19.50^{cde}	1.33^{d}	1.07^{def}	0.27^{bcde}
8.	4	36.00^{g}	19.28^{cde}	1.14^{ef}	0.88^{g}	0.26^{cde}
9.	4.5	52.00^{h}	18.00^{e}	1.13^{f}	0.89^{g}	0.24^{e}
10.	Positive control	30.00^{ef}	21.50^{b}	1.60^{b}	1.28^{bc}	0.32^{a}
11.	Negative control	92.00^{i}	17.63^{f}	1.15^{ef}	0.90^{g}	0.25^{de}
SEd		1.1906	0.7610	0.0845	0.0811	0.0199
CD		2.4572	1.5707	0.1744	0.1673	0.0411

The antibacterial activity of chitosan was studied and reported by several workers. The antibacterial activity of chitosan might be the interaction between positively charged chitosan and negatively charged microbial membranes, which prevents the transport of essential solutes into the cell and results in leakage of proteinaceous and intracellular components thereby killing the bacterial cell (Chung *et al.,* 2011) [17].

The chitosan concentrations *viz.,* 3.0, 3.5, 4.0 and 4.5 showed poor performance on mortality and economic parameters of silkworm when compared to 2.0 and 2.5 per cent. Higher concentrations of chitosan caused increased viscosity and decreased solubility of chitosan (Liu *et al.,* 2006) [18]. No *et al.* (2002) [19] reported that the antibacterial activity of chitosan is effective in inhibiting growth of bacteria. The antimicrobial properties of chitosan depend on its molecular weight and the type of bacterium. Chitosan generally showed stronger bactericidal effects for positive bacteria than negative bacteria. The antimicrobial activity is associated with molecular weight, degree of acetylation, concentration of chitosan and load of pathogen (Fernandes *et al.,* 2008) [20].

Bacterial pathogens, *Staphylococcus aureus* and *Bacillus thuringiensis* used in this study are gram positive bacteria, The cell membrane of gram positive bacteria is covered by a cell wall consisting of layers of peptidoglycans which contains acetylmuramic acid as well as D- and L-amino acids and techoic acid (Tortora, 2010) [21]. to which the positively charged amino groups of chitosan binds, result in cell wall distortion – disruption and expose cell membrane to osmotic shock and exudation of cytoplasmic contents (Vishu kumar *et al.,* 2005) [22]. The binding of chitosan to techoic acids coupled with a potential extraction of membrane lipids results in bacterial death.

Conclusion

The present study concluded that chitosan extracted from silkworm pupae found to possess not only antibacterial activity against bacterial pathogens in silkworm but also increased economic parameters. Hence, effective reutilization of unused pupae could be used as a high potential raw material in various biomedical industries and protects the environment by converting it into useful bio product.

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