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In Vivo Antibacterial Effect of Thymoquinone-Encapsulated Chitosan Nanoparticles in *Bombyx mori* L. Infected by Pathogenic Bacteria

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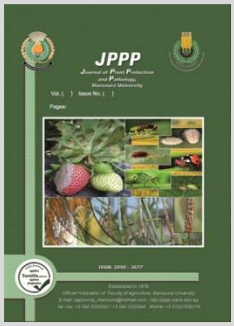

ABSTRACT

The present work studied the antibacterial effect of different formulations of Chitosan (Chs); free Thymoquinone (Tq), and Thymoquinone-loaded chitosan nanoparticles (Tq-Chs NPs) on *Bombyx mori* L. infected by pathogenic bacteria, by evaluating some biochemical aspects of larval haemolymph. *Bombyx mori* larvae classified into three groups; each group was fed on mulberry leaves treated with one of the formulations (free Tq, free Chs and Tq-encapsulated Chs) with drug concentrations (0.05% and 0.1%). Before being used, the prepared formulations were characterized by size, morphology, encapsulation efficiency; in addition, in vitro drug release experiment was performed. The biochemical results revealed an improvement in the antibacterial and antioxidant activity of the studied formulations, especially with increasing the concentrations compared to control; (Tq-encapsulated Chs) followed by free Chs and free Tq revealed highly significant decreases in mixed function oxidase (MFO) activity, especially larvae treated with the high concentration (0.1%). The activity of acid phosphatase, total lipid content and carbohydrates pointed to a highly significant increase. Similarly, the qualitative analysis of silkworm proteins showed an obvious variation in the number and position of the detected protein bands from one treatment to another. It can be concluded that Tq and Chs showed an inhibitory impact against pathogenic bacteria infecting *Bombyx mori* larvae, proved their effects as antioxidant and anti-inflammatory agents which could be improved by loading Tq on Chs nanoparticles (Tq-Chs NPs).

Keywords: Thymoquinone; Chitosan nanoparticles; *Bombyx mori*; pathogenic bacteria.

INTRODUCTION

Silkworm belongs to Lepidoptera, Family: Bombycidae, it is a complete metamorphic insect having four stages: egg, larva, pupa and adult. Larvae being monophagous insects, they utelize absorbed nutrients to provide energy for growth. *Bombyx mori* larvae synthesize and secrete a large amount of silk proteins during metamorphosis (Truman and Riddiford, 1999; Tanaka *et al*., 2009).

Keeping silkworm larvae free from diseases all over the rearing period is a major target to silkworm rearers, because annually, 34-40% of the total crop of silk production is lost due to silkworm diseases. Among these diseases; Bacterial flacherie which is a common disease of mulberry silkworm. The etiology of this disease is not clear owing to the diversity of bacterial types involved in (Choudhury *et al*., 2002).

The bacteria which represent the etiological agent of flacherie in silkworms can be *Bacillus subtilis, Bacillus cereus, Streptococcus pneumoniae, Staphylococcus aureus, E. coli, Pseudomonas fluorescence, and Klebsiella cloacae* as reported by Chitra *et al.* (1973); Anitha *et al.* (1994) and Sakthivel *et al.* (2012).

Antibiotics are commonly used in sericulture industry as bed disinfectants and therapeutic agents against bacterial diseases, but, the usage of antibiotics and antibacterial chemotherapeutics is becoming more restricted, because of: (i) Resistance of bacteria to antibiotics and. (ii) The side effects exhibited by most antibiotics. Therefore, newer drugs must be discovered with lesser rate of resistance development and lesser toxicity (Neu, 1992; Okeke *et al*., 2005; Subramanian *et al*., 2009).

Researchers in sericulture are recently using nanoparticles to upgrade silk production; these particles may be utilized during insufficient leaf production (Pandiarajan *et al*., 2016). The scientific community was willing to investigate the physiological and biochemical influence of nanoparticles on silkworm *B. mori* (Patil *et al*., 2016).

Thymoquinone (Tq) is the major constituent of *Nigella sativa* volatile oil; it has evidently proved by its medical activities such as hepatoprotective, anti-inflammatory, antioxidant, antibacterial and anti-cancer agent. These valuable effects of (Tq) support the use of this natural compound as a drug in medical purposes (Kokoska *et al*., 2008; Khader and Eckl, 2014).

Chitosan (Chs), is a derivative of chitin, which is obtained from shrimp shells, crabs, lobsters, and other insects as silkworms (Paulino *et al*., 2006 and Zhou *et al*., 2009). It is a natural polymer used as a carrier for drugs or cross-linked by various reagents and has excellent properties such as hydrophilicity, biocompatibility, biodegradability and antibacterial activity, so it is used for various applications (Lei *et al*., 2009). Chs also has antimicrobial, antioxidant properties besides its potency to control drug release (Yang and Shao, 2000; Kumar *et al*., 2004; Prashanth & Tharanathan, 2007; Sonia & Sharma, 2011).

The present study has therefore been carried out to evaluate the antibacterial potency of the thymoquinone-loaded Chitosan (Tq-Chs), free thymoquinone (Tq) and free Chitosan (Chs) nanoparticles with two concentrations on some biochemical aspects of *Bombyx mori* L. Infected by bacterial pathogenic.

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MATERIALS AND METHODS

1. Silkworm *Bombyx mori* L.

Eggs of mulberry silkworm *Bombyx mori* L. hybrid ($G_2 \times V_2 \times Kk \times M_2$) were procured from the Sericulture Research Department, Plant Protection Research Institute (PPRI), Agricultural Research Center, Giza, Egypt.

2. Chemicals

Chitosan (Chs) (medium molecular weight, degree of deacetylation 96%) was bought from Tecno-Pharmachem. Glacial acetic acid (99.7%) was acquired from Biochemfor laboratory chemicals. Thymoquinone (Tq), trypolyphosphate (TPP) were purchased from Sigma Aldrich.

3. Preparation of Sodium tripolyphosphate Chitosan nanoparticles (TPP- Chs Nps) and Thymoquinone-loaded chitosan nanoparticles (Tq-Chs NPs)

Chitosan nanoparticles (Chs) have been synthesized using the Patil and Surana method (2017) previously described with some alterations. For the preparation of blank TPP-Chitosan nanoparticles, 0.1 g of chitosan was added to 50 ml of 3% acetic acid solution and stirred overnight at room temperature on a MS300HS magnetic stirrer (Misung Scientific Co., Ltd, Korea). Then, 0.06 g of TPP was dissolved in 4 ml deionized water and added to the chitosan solution dropwise. For chitosan nanoparticles loaded with thymoquinone (Tq-Chs) preparation, the same steps were followed except that before the formation of Chs, 0.1 g of Tq was added (dissolved in 2.5 ml of ethanol and 2.5 ml of deionized water). The dispersion of all the prepared formulations was then centrifuged to collect nanoparticles as pellets at 8500 rpm (VS-18000 M, Korea, energy 220 V/50 HZ) for 105 min at 10ºC. The collected pellets were washed and re-dispersed in 20 ml of deionized water and sonicated for 2 min with a water bath sonicator (CD-4820, CODYSON, China).

4. Physical and chemical aspects of the prepared formulations

Transmission electron microscopy (TEM)

TEM (JEM 1230 electron microscope Jeol, Tokyo, Japan) was used to determine the morphology and size of the preparations.

Particle size and zeta potential

The particle size and zeta potential of the prepared formulations (Chs and Tq-Chs) were measured at 25 ° C through dynamic light scattering (DLS) analysis (Zetasizer Nano ZS90, Malvern Instruments, UK).

Encapsulation efficiency (EE %), loading capacity (LC) and process yield (Y)

The encapsulation efficiency (EE %) of Tq was calculated for (Tq-Chs). The sample was centrifuged for the separation of the free drug (supernatant) from the encapsulated one (pellet) at 12,000 rpm (VS-18000 M, Korea, power 220V/50 HZ) for 30 minutes. The clear supernatant (KMC-1300V, BIONEX, Korea) was then collected and vortexed to get a homogeneous mixture, after centrifugation the gained pellets were diluted with deionized water and sonicated for 10 minutes for another use. These procedures were repeated 3 times for the same sample. The amount of free Tq in the supernatant was dissolved in ethanol and sonicated for 10 minutes, then the absorbance was measured at 330 nm using a visible UV spectrophotometer (Bhattacharya et al., 2015) (Jenway 6405, U.K.). From the Tq calibration curve, the concentration of the free drug was estimated in the supernatant. The encapsulation efficiency and the loading capacity were calculated for (Tq-Chs) from the following equations:

$$\textbf{Encapsulation Efficiency } \% = \frac{\textbf{Initial concentration} - \textbf{Final concentration}}{\textbf{Initial concentration}} \times 100\ \%$$

$$\textbf{Loading capacity } \% = \frac{\textbf{Initial concentration} - \textbf{Final concentration}}{\textbf{Nanoparticles weight}} \times 100\ \%$$

The process yield of the prepared nanoparticles was calculated from the next equation:

$$Process\ yield(Y) = \frac{W1}{W2} x\ 100$$

Where: W1 is the weight of dried nanocapsules recovered, W2: is the sum of the initial dry weights of the starting materials. The pellet was dried and weighed after centrifugation at 10,000 rpm, 4 ºC for 15 min. The analysis of all samples in triplicates was done to avoid mistakes in the calculation of the EE%, LC and Y.

In vitro drug release

A dialysis bag (MWCO 12.000 g / mole; Sigma-Aldrich) was employed for in-vitro release profiling of (Tq-Chs). The in-vitro release experiment was carried out using a flask containing 80 ml of PBS (pH 6.5), at 37 ° C ± 0.5 ° C. The equivalent amounts of formulations loaded with drugs (5 ml) were packed and immersed in a dialysis bag. At pre-established time intervals (0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 7, 8, 9, 10, 11, 12, 18, 24, 30, 36, 42, 48, 54, 60, 66, 72 hrs), a 3 ml sample was removed and substituted by fresh PBS and then, the absorbance was measure at 330 nm using a UV-Vis spectrophotometer.

5. Infection of silkworm larvae with bacteria

According to Aneja (2003), pathogenic bacteria were collected from diseased black thorax of Septicemia infected larvae and cultured overnight in nutrient agar medium at 37°C. Then, some colonies were selected and transferred into sterile 10 ml (0.85%) NaCl (stock solution). Serial dilutions with different concentrations of bacterial spore suspension (100%, 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20% and 10%) was made according to Cockerill et al., (2012). Silkworm B. mori larvae were artificially infected by spraying the concentration (50%) of bacterial spore suspension on mulberry leaves which were then fed to larvae once on the 2nd day of the 5th instar larvae.

6. In vivo experiments

Silkworm rearing technique

After egg hatching, larvae were reared in the laboratory at 25 ± 2º C and 75 ± 5% relative humidity according to Krishnaswami (1978). The newly hatched larvae were fed on fresh clean mulberry leaves until the 5th instar, which is the stage that was used in our bioassays.

Schedule of application

Larvae were divided into three groups fed on mulberry leaves treated with (free Tq, free Chs and Tq-encapsulated Chs). Each group was divided into two subgroups representing drug concentrations (0.05% and 0.1%) with 3 replicates (50 larvae for each). Mulberry leaves were washed and let dry. Then, leaves were immersed in each concentration for 5 minutes and left to dry, then, offered to larvae (1 diet/day) twice; at the beginning of the 5th larval instar and at the middle of the same instar. Two control groups (positive control with infection and without treatment and negative group without infection or treatment) were fed on leaves and were only treated with distilled water.

7. Biochemical aspects

Samples of haemolymph were collected at the 7th day of the 5th larval instar by incising one of the thoracic legs of larvae and bending the body to expose the sternum at the position of the incised leg. This ensured proper drainage of the haemolymph avoided any risk of internal organs injury. The haemolymph of each treatment was collected in 1.5 ml Eppendorf tubes with small crystal of phenyl thiourea (PTU) to prevent melanization of sample and to be prepared for biochemical assays according to Mahmoud (1988) protocol.

Oxidase activity: Mixed function oxidases (MFO)

p-nitroanisole o-demthylation was assayed to evaluate the mixed function oxidase activity according to the method of Hansen and Hodgson (1971).

Determination of phosphatases activity: Acid phosphatase activity

Acid phosphatase activity was measured according to the method of Laufer and Schin (1971).

Determination of total lipid content

The total lipid content of the haemolymph was determined by the phosphovanillin method of Baronos and Blackstock (1973).

Determination of total carbohydrate content

The total carbohydrate content of the haemolymph was determined according to Singh and Sinha (1977).

8. Identification and determination of proteins by sodium dodecyl sulfate polyacrylamide gel-electrophoresis (SDS-PAGE)

Polyacrylamide gel electrophoresis (PAGE) was used for the separation of protein subunits and the determination of protein molecular weights in fresh samples of haemolymph of mulberry silkworm *Bombyx mori* (5th larval instar) which electrophoresed at the same time in the same gel as described by Laemmli (1970).

9. Statistical Analysis

Obtained data was subjected to analysis of variance (ANOVA) as one way to find the differences among different treatments. Statistical analysis was conducted using Proc ANOVA in SAS (SAS Institute, 1988). Means separation was conducted using Duncan Multiple Range Test using the same program.

RESULTS AND DISCUSSION

Results

1. Physical and chemical aspects of the prepared formulations

Transmission electron microscopy (TEM)

Transmission electron micrographs for Chs NPs and Tq-Chs NPs are illustrated in Figure 1 (a and b, respectively). The TEM images showed the presence of regular and relatively homogeneous particles. All formulations have morphology almost spherical in shape with a smooth surface and few aggregations.

Particle size and zeta potential

The calculated hydrodynamic particle sizes of the different formulations: Chs Nps and Tq-Chs NPs were 200±25, 390±70, respectively. The polydispersity index distribution (PDI) was 0.36 and 0.51 for Chs and Tq-Chs, respectively. The mean zeta potentials for Chs and Tq-Chs were found to be 16±6 mV, 32±3 mV, respectively.

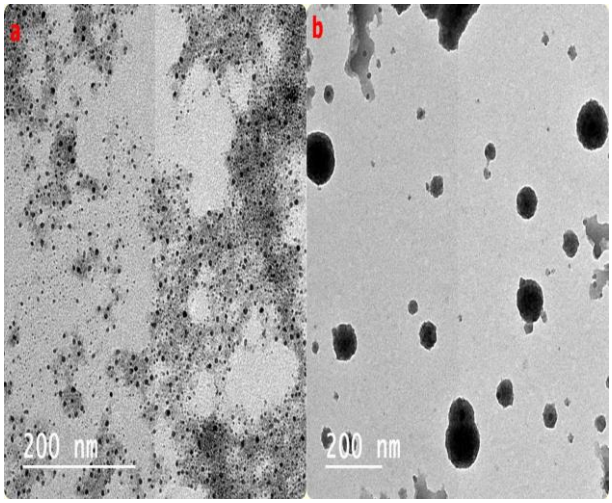

Figure 1. Transmission electron microscopy (TEM) micrographs of a: Chitosan nanoparticles (ChsNps) and b: Thymoquinone-loaded Chitosan nanoparticles (Tq-Chs NPs).

Encapsulation efficiency measurements (EE %), loading capacity (LC %) and process yield

The Tq encapsulation efficiency for Tq-Chs was 76%±16. The loading capacity for Tq-Chs was 15%±3. The process yield for Tq-Chs was 7% ± 0.4.

In vitro drug release

The in-vitro release of Tq-Chs has been assessed to provide some indication of drug delivery fulfillment. The release profile of Tq from Tq-Chs revealed that approximately 31.5 % of Tq were released at a relatively rapid rate through the first 54hrs; the formulation maintained then persistent release up to 72 hours (Figure 2).

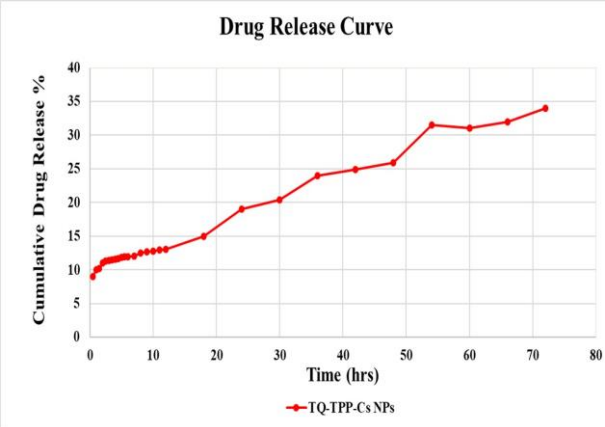

Figure 2. Drug release profile of Thymoquinone (Tq) from Thymoquinone-loaded Chitosan nanoparticles (Tq-Chs NPs).

2. In vivo results

Oxidase activity: Mixed function oxidases (MFO)

As seen in Figure 3, a significant decrease was noticed among means of MFO activity ($p<0.0001$, LSD0.05= 3.182) in case of larvae treated with the 2nd concentration (0.1%) of both (Tq-Chs) and Chs (20.67±0.87 & 21.63±0.87 mmole sub. oxidized/min/ml) respectively, followed by the 2nd concentration (0.1%) of Tq (24.82±0.87 mmole sub. oxidized/min/ml). Meanwhile, means of the 1st concentration (0.05%) of (Tq-Chs), Tq, Chs and positive control recorded (25.92±1.44, 26.52±1.27, 26.4±1.10& 28.11±0.98 mmole sub. oxidized/min/ml), respectively.

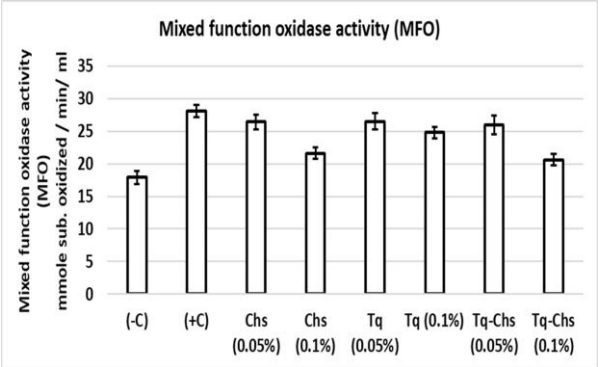

Figure 3. Effect of thymoquinone-loaded Chitosan (Tq-Chs), free thymoquinone (Tq) and free Chitosan (Chs) nanoparticles with different concentrations on mixed function oxidase activity (MFO) of the infected fifth larval instar of *Bombyx mori* L.

Acid phosphatase activity

As illustrated in Figure (4), the results of acid phosphatase activity pointed to highly significant increases (p<0.0001, $LSD_{0.05}$= 1.224) in enzyme activity means with different concentrations of treatments compared with the positive control (3.49±0.31µg phosphate/min/ml); the 2nd concentration (0.1%) of (Tq-Chs) followed by Chs (0.1%) recorded high acid phosphatase activity (12.94± 0.18 & 11.53±0.25 µg phosphate/min/ml), respectively. By the same way, the 2nd concentration (0.1%) of Tq showed a higher level of the enzyme activity (9.92±0.21 µg phosphate/min/ml) than the 1stconcentration (0.05%) of (Tq-Chs), Chs then Tq (9.48±0.15, 9.09±0.18 then 8.31±0.18 µg phosphate/min/ml), respectively.

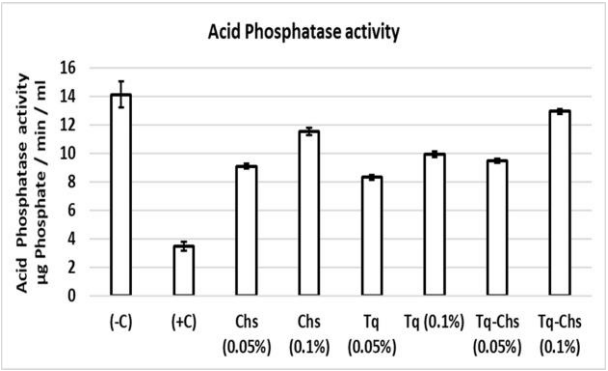

Figure 4. Effect of thymoquinone-loaded Chitosan (Tq-Chs), free thymoquinone (Tq) and free Chitosan (Chs) nanoparticles with different concentrations on acid phosphatase activity of the infected fifth larval instar of *Bombyx mori* L.

Total lipid content

The obtained results in Figure (5) indicate that, a highly significant increase (p<0.0001, $LSD_{0.05}$= 2.859) was noticed in total lipid content as a result of treating infected larvae with different concentrations of treatments under investigation as compared with control group (9.48±0.31 mg/ml); the 2nd concentration (0.1%) of both (Tq-Chs) and Chs showed high significant lipid content than other treatments (18.30±1.21 & 16.27±0.22 mg/ml), respectively. Also, Tq (0.1%) and the 1st concentration (0.05%) of (Tq-Chs), Chs and Tq showed high lipid content (12.68±0.80, 12.55±0.89, 12.28±0.52 & 12.20±0.60 mg/ml) compared with the positive control.

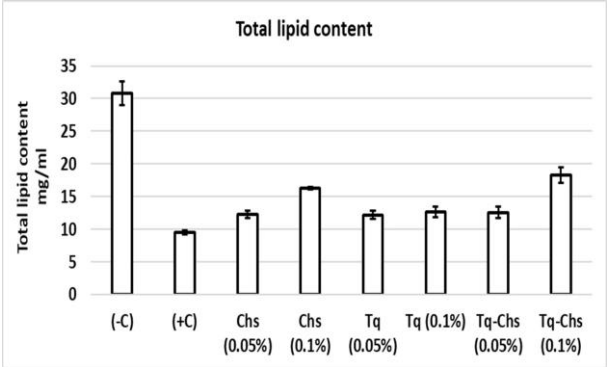

Figure 5. Effect of thymoquinone-loaded Chitosan (Tq-Chs), free thymoquinone (Tq) and free Chitosan (Chs) nanoparticles with different concentrations on total lipid content of the infected fifth larval instar of *Bombyx mori* L.

Total carbohydrate content

All treatments with different concentrations exhibited high significant increases in total carbohydrate content (p<0.0001, $LSD_{0.05}$= 2.744) compared with the control group (Figure 6). Treated larvae with the 2nd concentration (0.1%) of (Tq-Chs), Chs and Tq resulted in high significant increases in carbohydrate content in haemolymph (61.55±1.21, 61.35±0.91& 60.39±1.37 mg/ml), respectively. Similarly, the 1st concentration (0.05%) of the same treatments showed high significantcontents of carbohydrates (54.4±1.18, 53.82±0.54 & 42.97±0.02 mg/ml), respectively, while the positive control group recorded (26.36±0.83 mg/ml).

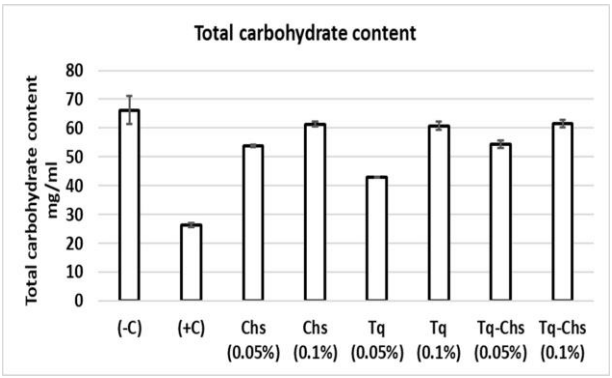

Figure 6. Effect of thymoquinone-loaded Chitosan (Tq-Chs), free thymoquinone (Tq) and free Chitosan (Chs) nanoparticles with different concentrations on total carbohydrate content of the infected fifth larval instar of *Bombyx mori* L.

Qualitative analysis of silkworm proteins

The proteins of *Bombyx mori* larvae treated with Thymoquinone encapsulated Chitosan (Tq-Chs), Thymoquinone (Tq) and Chitosan (Chs) with different concentrations were analyzed by SDS-PAGE. The electrophoretic pattern is shown in Figure (7) and Table (1); forty-six polymorphic bands were detected with molecular weight ranging from 108 to 8 KDa. The treatments under investigation share the band with molecular weight 8 KDa.

Both concentrations of (Tq-Chs) 0.05 and 0.1% share the band with molecular weight 65 KDa. The concentration 0.1% of (Tq-Chs) was distinguished by the bands has molecular weights 23 & 20 KDa. While the 1st concentration of the same treatment (0.05%) was marked by the bands have molecular weights 72, 59 & 43 KDa.

Both concentrations of Tq share the bands with molecular weights 66 & 29 KDa while being absent in the control groups. The concentration 0.1% of Tq was characterized by the bands with molecular weights 57, 46 & 24 KDa. The 1st concentration of the same treatment (0.05%) was characterized by the band with molecular weight 48 KDa.

The two concentrations of Chs share the band with molecular weight 60 KDa, the concentration of Chs (0.05%) demonstrated some characteristic bands with molecular weights 84, 74, 68 & 39 KDa.

The protein band with molecular weight 75 KDa was present in control groups, while absent in other treatments under investigation.

Table 1. Effect of Thymoquinone-loaded Chitosan (Tq-Chs), free Thymoquinone (Tq) and free Chitosan (Chs) nanoparticles with different concentrations on the protein-banding patterns of the infected fifth larval instar of *Bombyx mori* L.

T.	Tq-Chs		Tq		Chs				T.	Tq-Chs		Tq		Chs			
Mwt	0.05%	0.1%	0.05%	0.1%	0.05%	0.1%	-C	+C	Mwt	0.05%	0.1%	0.05%	0.1%	0.05%	0.1%	-C	+C
108	0	0	0	0	0	0	0	+	51	0	0	0	0	0	0	+	0
101	0	0	0	0	0	0	+	0	49	+	+	0	0	+	+	0	0
100	+	0	0	0	0	+	0	0	48	0	0	+	0	0	0	0	0
99	0	+	0	+	0	0	0	0	46	0	0	0	+	0	0	0	0
98	0	0	+	0	+	0	0	0	43	+	0	0	0	0	0	0	0
88	0	0	0	0	0	0	+	0	42	0	0	0	0	0	0	0	+
84	0	0	0	0	+	0	0	0	41	0	0	0	0	0	+	+	0
75	0	0	0	0	0	0	+	+	39	0	0	0	0	+	0	0	0
74	0	0	0	0	+	0	0	0	38	0	+	+	0	0	+	0	0
72	+	0	0	0	0	0	0	0	36	+	0	0	+	0	0	0	0
71	0	0	0	+	0	+	0	+	32	0	0	0	0	0	0	0	+
70	0	0	0	0	0	0	0	0	30	+	+	0	0	+	0	+	0
69	0	0	0	0	0	0	+	0	29	0	0	+	+	0	+	0	0
68	0	0	0	0	+	0	0	0	28	0	0	0	0	0	0	0	0
66	0	0	+	+	0	+	0	0	24	0	0	0	+	0	0	0	0
65	+	+	0	0	0	0	0	0	23	0	+	0	0	0	0	0	0
63	0	0	0	0	0	0	0	+	20	0	+	0	0	0	0	0	0
61	0	0	0	0	0	0	+	0	13	0	0	0	0	+	0	+	+
60	0	0	0	0	+	+	0	0	12	+	0	0	+	0	+	0	0
59	+	0	0	0	0	0	0	0	11	0	+	+	0	0	0	0	0
58	0	+	+	0	0	0	0	0	10	0	+	0	0	+	0	+	+
57	0	0	0	+	0	0	0	0	9	+	0	0	+	0	+	+	+
53	0	0	0	0	0	0	0	+	8	+	+	+	+	+	+	0	0

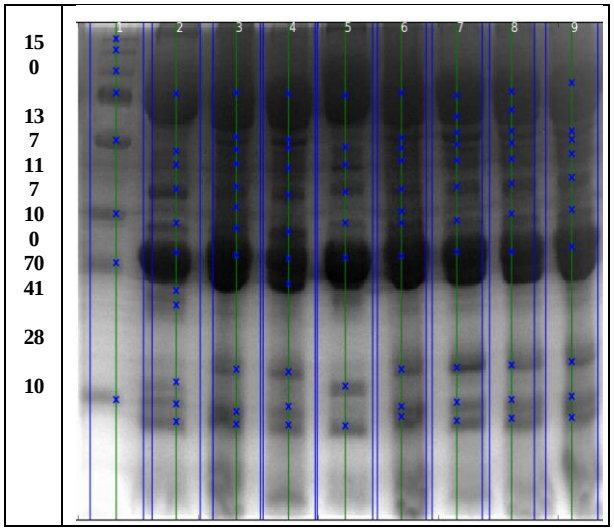

Figure 7. Protein banding patterns of the infected fifth larval instar larvae of *Bombyx mori* treated with thymoquinone-loaded Chitosan (Tq-Chs), free thymoquinone (Tq) and free Chitosan (Chs) nanoparticles with different concentrations.

Lane 1: Marker with M.wt. 150, 137, 117, 100, 70, 41, 28 & 10 KDa.
Lane 2: Protein bands of larvae treated with Tq-Chs (0.05%).
Lane 3: Protein bands of larvae treated with Tq-Chs (0.1%).
Lane 4: Protein bands of larvae treated with Tq (0.05%).
Lane 5: Protein bands of larvae treated with Tq (0.1%).
Lane 6: Protein bands of larvae treated with Chs (0.05%).
Lane 7: Protein bands of larvae treated with Chs (0.1%).
Lane 8: (-) Control.
Lane 9: (+) Control.

Discussion

Nigella sativa (black cumin) contains an active component called thymoquinone (Tq) to which the therapeutic effects of *N. sativa* was imputed. Regrettably, one major dysfunction of the Tq is its low biological availability which, due to its low water solubility, can reduce its therapeutic effectiveness. Therefore, the present research aims to increase Tq bioavailability by using chitosan (Chs) nanoparticles as a nanocarrier.

Chs is the fundamental construction unit on which we rely in the present study. Chs is considered as a medicinal material extracted from a substance that develops harsh external shells in crustaceans and has remarkable advantages just as: being indigestible to keep the long-term effect of the drug, biodegradable because it is extracted from natural products, vitally compatible and has no harmful impacts on living tissue and as well non-toxic impacts. Polyanion sodium triphosphate (TPP) is added to the manufacturing of chitosan nanoparticles through the development of ion interconnections through attractions of the TPP with the positive-chitosan amine group between the negative-charged phosphate groups and these physical connections have reversible characteristics and improve the characteristics of nanoparticles (Yang *et al*., 2009).

The increased size of (Tq-Chs) NPs can be owing to an increase in the amount of negatively charged Tq in the solution in comparison to chitosan polymer, leading to a decrease in the positively charged Chs and negatively charged

TPP interactions by vying with TPP, leading to an increase in NP size from 200 nm to more than 390 nm (Calvo *et al*., 1997).

The current data revealed that the rate of release of Tq from (Tq-Chs) NPs was rapid throughout the first hours, then followed by a prolonged release that last for 72 hours. The initial quick release from Tq might be attributable to its release from nanoparticles' surface and to its deferred release due to the release of Chs from the core of nanoparticles (Alam *et al*., 2012).

Nanoparticles have tendency to repel each other and provide diffusion stability with elevated positive or negative zeta-potentials > 30 mV. If the zeta potential values are low (less than 5 mV), no force is present to prevent the particles from agglomerating resulting in diffusion instability (Paolino *et al*., 2006; Gumustas *et al*., 2017). The mean zeta potentials for Chs Nps and (Tq-Chs) NPs were found to be 16±6 mV, 32±3 mV, respectively.

The biochemical results clarified that, there was an obvious improvement in the tested biochemical parameters, when treating infected larvae with the different formulations under investigation, compared with the positive control group. Treating infected larvae with Tq-Chs especially the concentration (0.1%) exhibited a highly significant decrease in oxidase activity, while activity of acid phosphatase was significantly increased, followed by free Chs and free Tq with the same concentration and then, the concentration 0.05%. This enhancement may be attributed to the antioxidant and antibacterial properties of Tq-Chs, which rely on the inhibitory activity against bacteria due to the methanolic part of Tq which inhibitsthe synthesis of the cell wall by inducing changes in the structure of the membranes through inhibiting bacterial protein synthesis (Khsai, 2002; Randhawa and Al-Ghamdi, 2002). As well, the antibacterial activity of Chs depends on the interaction between Chs and bacterial cell membranes, which inhibit the transfer of essential solutes into the cell and leads to leakage of proteinaceous and intracellular components leading to kill the cell of bacteria, and the minimum inhibitory concentration (MIC) of Chs ranged from 0.005 to 0.1% building on the species of bacteria and the molecular weight of Chs as documented by No *et al.* (2002) and Chung *et al.* (2011).

The study revealed also significant elevations in the lipid and carbohydrates contents as a result of treating larvae with Tq-Chs followed by free Chs and free Tq with the concentration 0.1% then the concentration 0.05%, compared with control group. The improvement of these biochemical aspects may be owing to disease resistance against pathogen as a result of the significant antimicrobial activity of Tq extract of *N. sativa* on Gram positive and negative bacteria. Also, Tq is a major active lipophilic component of *N. Sativa* and have many pharmacological characteristics just as its immu-nomodulation, anti-inflammatory and antioxidant effects (Mansour *et al*., 2002; Aljabre *et al*. 2005; Al-Majed *et al*., 2006). Furthermore, Chs showed a strong bactericidal effect for positive bacteria than negative bacteria. It is known that the cell membrane of gram positive bacteria is covered by a cell wall composed of layers of peptidoglycans containing acetylmuramic acid also D- and L-amino acids and teichoic acid whereby the positively charged amino groups of Chs binds, leading to cell wall distortion or disruption and resulting in expose the cell membrane to osmotic shock and exudation of cytoplasmic constituents and potential extraction of membrane lipids resulting in bacterial death (Vishukumar *et al.,* 2005; Tortora, 2010).

For the generation of molecular markers based on protein polymorphisms, the most common technique is the electrophoretic separation of proteins, then specific staining of a unique protein subclass. Changes in haemolymph proteins of the 5th instar infected larvae were mentioned qualitatively due to the therapeutic technique based on treating infected *B. mori* larvae with treatments; (Tq-Chs) followed by free Chs and free Tq using two concentrations 0.1% then 0.05% of each of them. The band with molecular weight 65 KDa was evident in (Tq-Chs) treatment. Chs is characterized also by the presence of the band with molecular weight 60 KDa, meanwhile the bands with molecular weights 66 & 29 KDa were detected with Tq. The appearance of these specific new bands which are characteristic for each treatment while absent in the control groups, may be attributed to changing into one or more of the other protein components of the blood, or to the increase of the rate of digestive (amylase) and oxidizing (succinate dehydrogenase) enzymes which help in the utilization of exogenous food materials, leading finally to more production. While, absence of protein bands may point to either failure of production, utilization or breakdown of blood proteins to preserve amino acid concentration in the blood which might be consumed to form silk proteins, as documented by LakshmiKumari *et al.* (1997) and Mahesha *et al.* (2000). These changes in haemolymph proteins may be also owing to levels of bombyxin hormone (an insulin-like protein) in blood which is modulated by the brain as a result to variation in nutrients, and consists a part of the mechanism harmonizing the growth of internal organs with overall somatic growth; it also has a role in carbohydrate metabolism as documented by Nijhout and Grunert (2002). Therefore, the results of the protein analysis are positively correlated with the biochemical qualities of larvae.

CONCLUSION

The promotion of silk protein synthesis and enhanced silk production of silkworm larvae depend on the healthy growth of larval instar and with being pathologically free. From the results obtained in the present study, it can be concluded that Thymoquinone (Tq) and Chitosan (Chs) were found to have an inhibitory impact against pathogenic bacteria attacking *Bombyx mori* larvae, and proved their activity as antioxidant and anti-inflammatory, especially with higher concentration (0.1%), with various mechanisms. Moreover, the antibacterial effect of Tq against pathogenic bacteria might be increased by loading it into Chs nanoparticles.

Disclosure of potential conflict of interest

The authors declare no conflict of interest.

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التأثير المضاد للبكتيريا لمادة الثيموكينون المغلفة داخل جسيمات الشيتوسانانانونية على دودة الحرير "بومبيكسموراى" المصابة ببكتيريا ممرضة: من داخل الجسم الحى

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تهدف الدراسة الحالية إلى تقييم التأثير المضاد للبكتيريا لتركيبات مختلفة من:الشيتوسان (Chs)؛ الثيموكينون الحر (Tq) بالإضافة إلى الثيموكينون المغلف داخل جسيمات الشيتوسانانانونية (Tq-Chs NPs) فديدان الحرير "بومبيكسموراى" المصابة بالبكتيريا المسببة للأمراض, من خلال دراسة بعض الصفات البيوكيميائية لدم يرقات الحرير المعالجة كدراسة مستوى العوامل المؤكسدة (mixed function oxidase), المحتوى الكلى للدهون والكربوهيدرات وأيضا التحليل الكهربى للبروتين. وقد تم تقسيم اليرقات عشوائيا إلى ثلاث مجموعات؛ تم تغذية كل مجموعة على أوراق التوت المعاملة بالتحضيرات المختلفة السابق ذكرها بتركيزين مختلفين (0.05%, 0.1%). وقد تم توصيف التحضيرات المختلفة المذكورة قبل استخدامها عن طريق تعيين حجمها وكفاءة التغليف وبالإضافة إلى ذلك تم تنفيذ تجربة إطلاق الأدوية فى المختبر. وقد أشارت النتائج إلى تحسن الصفات البيوكيميائية للحشرة حيث لوحظ زيادة كفاءة النشاط المضاد للبكتيريا والنشاط المضاد للأكسدة للمعاملات المستخدمة خاصة مع زيادة التركيز مقارنة بالكنترول,وخاصة Tq-Chs NPs)الذى أظهر نقص معنوىعالى لمستوى أنزيم (MFO) فى دم اليرقات التى تم معالجتها بتركيز 0.1% ثم اليرقات التى تم معاملتها بالشيتوسان (Chs) والثيموكينون الحر(Tq) حيث انخفض مستوى الأنزيم أيضا إنخفاضا معنويا. كذلك أظهرت النتائج زيادة معنوية ملحوظة فى نشاط أنزيم acid phosphatase والمحتوى الكلى لكل من الدهون (lipid) والكربوهيدرات.وذلك مع إستخدام التركيز الأعلى 0.1%فى جميع المعاملات قيد الدراسة مقارنة بالكنترول. كما أظهر التحليل الكهربى للبروتين فى دم اليرقات المعالجة تباينا واضحا فى عدد ومواضع شرائح البروتين بين المعاملات المختلفة قيد الدراسة مع ظهور شرائح بروتينية مميزة لكل معاملة غير متواجدة فىالكنترول مما يوضح وجود تحسن فى تكوين البروتين وكذلك الحالة الصحية لليرقات مما يؤدى إلى زيادة كفاءة وإنتاج الحرير التى تعتبر الهدف الأساسى لهذه الدراسة. مما سبق نستخلص فاعلية الثيموكينون (Tq) والشيتوزان (Chs) كمثبطات للبكتيريا الممرضة التى تصيب يرقات الحرير وتؤثر بالسلب فى جودة وإنتاج الحرير ويمكن زيادة فاعلية وتأثير هذه التركيبات بتحميل الثيموكينون على جزيئات الشيتوسان النانونية (Tq-Chs).