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In vitro antimicrobial activity of some medicinal plant and propolis extracts against mulberry silkworm, *Bombyx mori* L. pathogens

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Abstract

This study has been conducted to determine the antimicrobial activity of some medical plant extracts against mulberry silkworm, *Bombyx mori* L. pathogens. A total of 22 bacterial and 9 fungal isolates were successfully isolated from the external and internal silkworm larvae. Four bacterial species isolated from the infected larvae in this study were identified as follows: *Staphylococcus aureus* M., *Escherichia coli* M., *Bacillus licheniformis* C. and *Bacillus thuringiensis* B., while 6 fungal species were isolated as follows: *Aspergillus niger* V., *Aspergillus flavus* L., *Aspergillus terreus* T., *Aspergillus fumigatus* F., *Pencillium citrinum* F. and *Fusarium oxysporum* F. The highest activity among medicinal plant extracts against all pathogenic bacterial and fungal isolates was *Cinnamomum zeylanicum* J. while, *Curcuma longa* L. and *Foeniculum vulgare* M., showed no activity against tested pathogenic microorganisms. It is our hope that the data generated will be an addition to the existing pool of biocontrol silkworm pathogens in Egypt.

Keywords: silkworm, bacteria, fungi, antimicrobial agent, plant extract, propolis.

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Introduction

Silk is one of the nature's gifts to mankind produced by silkworm. Among silkworms the most commercially exploited one are mulberry silkworm, *Bombyx mori* L. (Thirumalaisamy et al., 2009). Silkworm, *B. mori* is a typical insect belonging to Family Bombycidae, Order Lepidoptera, Class Insecta of Phylum Arthropoda and is one of the genetically well-characterized insects next only to the fruit fly Drosophila. It has emerged as Lepidopteran molecular model system (Goldsmith, 1995). The mulberry silkworm is a great economic importance as a foreign exchange earner for many silk producing countries of the world (Krishnaswami et al., 1992). Four silkworm diseases namely Grasserie (viral), Flacherie (bacterial), Muscardine (fungal) and Pebrine (protozoan) are common in China and India. These diseases caused 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 percent of crop losses still occur due to these diseases (Veeranna, 1999). Bacterial infection is more prevalent in the silkworm, *B. mori* among the protozoan, viral and fungal pathogens and constitutes about 60-70% of total silk crop loss in Japan (Aruga & Tanada, 1971) and India (Chitra et al., 1975). The beta endotoxin of *Staphylococcus aureus* causes toxidermia, a septicemia and death in the silkworm larvae (Muktadir et al., 2006), *Bacillus thuringiensis* B. is linked to produce endotoxin causing mortality due to damage of gut lining and paralysis in response to starvation reported during several investigations (Selavakumar et al., 1999; Aizawa, 1971a,b; Aizawa & Fujiyoshi, 1968). Aspergillosis or *Aspergillus* disease is a mycosis or a fungal disease caused by *Aspergillus* fungi and it is one of the important diseases of silkworm, *B. mori* (Yu et al., 2002). Among the diseases, root rot caused by soil borne fungi like *Fusarium oxysporum* F. due to the ability to thrive well in soil and fast spread of (Dhahira Beevi & Qadri, 2010**)**. White muscardine in tasar silkworm is caused by the infection of *Penicillium citrinum* T. distributed all over the world infecting significant crop loss in all tasar culture countries (Kiran Kumar et al., 2011**)**. Medicinal 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). There are many edible and medicinal plants with high antimicrobial effects, such as thyme garlic (*Allium sativum* L.), turmeric (*Curcuma longa* L.), propolis, *Zingiber officinalis* R. and cinnamon (species belonging to *Cinnamomun* genus) (Nabavi et al., 2015; Arash et al., 2015; Mahmoud, 2012; Koc et al., 2011; Simoes et al., 2009). The present study was undertaken to find out the possibility of using the extracts of *Cinnomomum zeylanicum* B. and other medicinal plants for controlling the bacterial and fungal pathogens causing flacherie and muscardine diseases in the mulberry silkworm, *B. mori* . Only in vitro methods were conducted in assessing the antibacterial and antifungal potential of the crude extracts of cinnamon and other medicinal plants.

Materials and methods

Collection of larvae: Diseased larvae of silkworms, *B. mori* were collected from Sericulture Laboratory of the Plant Protection Department, Faculty of Agriculture, Assiut University, then preserved in aseptic plastic containers and transported to botany and microbiology department, Faculty of Science, Al-Azhar University, Assiut branch, Egypt to complete the isolation and identification of associated bacteria and fungi with silkworm larvae.

Selection of medicinal plants: Five different medicinal plants, *C. zeylanicum, Z. officinalis, C. longa, F. vulgare,* and *Allium sativum* L. and bee propolis for screened potential antibacterial and antifungal activity. The plant materials were obtained from local market at Assiut governorate, Egypt.

Preparation of plant extracts: The collected plant material was washed with distilled water and shade dried at room temperature, and then was grinded to fine powder with grinder. The powdered materials were used for preparation of methanolic extracts by using 100g powder in 500ml methyl alcohol 99% for 48hrs. The mixtures was stirred every 24 hrs using a shaker apparatus. At the end of extraction, each extract was concentrated in rotary evaporator at 60 ^{o}C and stored at 4 ^{o}C until further uses.

Isolation of fungal and bacterial pathogens from silkworm: Mulberry silkworm, *B. mori* showing microbial infection was surface sterilized with 0.1%mercuric chloride and then washed with distilled water. The bacteria and fungi that were isolated from the Mulberry silkworm were streaked nutrient and potato dextrose agar media respectively. Using streak plate technique, the bacterial and fungal colonies were further purified, after attaining good growth; slants were stored in refrigerator at 4^{o}C for further studies and used as stock cultures. The bacterial pathogens of silkworm were identified based on biochemical and morphological characteristics such as colony morphology and staining techniques, while fungal pathogens of silkworm were identified based on morphological characteristics such as colony and microscopic morphology.

Anti-microbial assay: Screening of five medicinal plant extracts and bee propolis for their antimicrobial activity was done by well diffusion method based on diameter inhibition zone of the microorganisms.

Well diffusion analysis: Screening of antimicrobial activity of the medicinal plant extracts and propolis were performed by well diffusion technique. For this, the agar plates were seeded with 0.1 ml of the standardized inoculums of each test organism. The inoculums were spread evenly over plate with sterile glass spreader. A standard cork borer of 6 mm diameter was used to cut uniform wells on the surface of the agar and 150 µl of each medicinal plant extract (dissolved in Dimethyl sulfoxide, DMSO) was introduced in the well. Respective solvent was used as control. The inoculated plates were incubated at 37°C for 24 hours and 30°C for 4 days for bacterial and fungal tested organisms

respectively, and then the zone of inhibition was measured to the nearest millimeter.

Results

Isolation of bacteria: A total of 22 bacterial isolates were successfully collected from the outer body and inner surface of silkworm larvae. These isolates were classified into four phenotypes based on the colony shape and cellular characteristics, gram stain, spore- formation, capsules, oxygen requirement and motility. Bacterial isolates were given code number SW-AZ1, SW-AZ2, SW-AZ3, SW-AZ4. Morphological characterization of bacterial isolates are summarized in Table 1, SW-AZ1, SW-AZ3 and SW-AZ4 isolates were G+ bacteria while the isolate SW-AZ2 was G- bacteria. Only SW-AZ1 was cocci in clusters, SW-AZ2, SW-AZ3 and SW-AZ4 were rods under light microscope. SW-AZ1, SW-AZ2 and SW-AZ3 strains were facultative anaerobic while SW-AZ4 strain was microaerophilic. The isolates SW-AZ2, SW-AZ3 and SW-AZ4 were motile, while isolate SW-AZ1 was not. SW-AZ3 and SW-AZ4 were spore-forming isolate, while isolate SW-AZ1 and SW-AZ2 were non spore-forming isolates. All tested strains were non-capsulated (Table 1). Biochemical and physiological characterization of bacterial isolates are summarized in Table 2. All isolates were similar in ability to fermented sugar (glucose, lactose and sucrose), except SW-AZ4 strain had not lactose fermented. All isolates produced catalase, nitrate reduction and Haemlysis. The isolates were found to diverse and differ in other biochemical and physiological studies such as production Oxidase, Urease, Coagulate, Idol test, Methyl red test, Voges-Proskauer test, Citrate utilization, Pigment production, Casein hydrolysis and Propionate utilization (Table 2). According to morphological, biochemical and physiological and characteristics of the bacterial isolates illustrated in Tables 1 and 2, four bacterial species were identified as follows: - *S. aureus* SW-AZ1 (n=4), *E. coli* SW-AZ2 (n=3), *B. licheniformis* SW-AZ3 (n=9) and *B. thuringiensis* SW-AZ4 (n=6).

Table 1: Morphological characterization of some bacterial isolated from *Bombyx mori.*

Morphological characteristics	SW-AZ1	SW-AZ2	SW-AZ3	SW-AZ4
Gram staining	Positive cocci in clusters	Negative short rod	Positive, short rod	Positive rod
Colony characteristics	Black and shiny with narrow white margins and surrounded by clear zone	Greenish, metallic sheen in reflected light with blue black center	Large flat creamy, wide spreading and glistening surfaced colonies	Yellowish, round
Motility	-	+	+	+
Spore formation	-	-	+	+
Oxygen requirements	Facultative anaerobic	Facultative anaerobic	Facultative anaerobic	Microaerophilic
Capsule	Non-Capsulated	Non-Capsulated	Non-Capsulated	Non-Capsulated
No. of isolates	4	3	9	6

+: Positive, -: Negative, AZ= Al-Azhar, SW= Silkworm.

Table 2: Physiological and biochemical characteristics of some bacterial isolated from *Bombyx mori*.

Physiological and biochemical characteristics	SW-AZ1	SW-AZ2	SW-AZ3	SW-AZ4
Coagulate test	+	-	-	-
Catalase test	+	+	+	+
Oxidase test	-	-	+	+
Indol test	-	+	-	+
Methyl red test	+	+	-	+
Voges-Proskauer test	+	-	-	+
Citrate utilization	+	-	+	+
Pigment production	+	-	+	-
Nitrate reduction	+	+	+	+
Casein hydrolysis	-	+	+	+
Propionate utilization	+	+	-	+
Urease test	+	-	+	+
Haemlysis test	++	+	+	+
Sugar fermentation acid				
Glucose	+	+	+	+
Lactose	+	+	+	-
Sucrose	+	+	+	+
Suspected organism	*S. aureus*	*E. coli*	*B. licheniformis*	*B. thuringiensis*

+: Positive, -: Negative, AZ= Al-Azhar, SW= Silkworm.

Isolation of fungi: Depending on morphological and culture characteristics of all fungal isolates illustrated in Tables 3, six fungal species were identified as follows: *Aspergillus niger* (n=2), *A. flavus* (n=1), *A. terrus* (n=1), *A. fumigatus* (n=2), *Pencillium citrinum* (n=1) and *Fusariu. oxysporum* (n=2) (Table 3).

Antimicrobial activity of some medicinal plant extracts against bacterial and fungal pathogens: From results given in Tables 4 and 5, the highest activity among plant extracts tested against all pathogenic bacteria and fungi isolated and identified in our study (Table 4 and 5 respectively) was recorded by *C. zeylanicum* in series gram-negative bacteria *S. aureus* (44 mm), *B. licheniformis* (32 mm) and *B. thuringiensis* (30mm), gram-negative bacteria *E. coli* (25.5) and in series fungi against *Spergillus* spp., (*A. niger, A. flavus, A. terrus* and *A. fumigates*) were recorded (37.5, 25.3, 30.5 and 33.3 mm respectively), *P. citrinum* (29.5mm) *and F. oxysporum* (22.5mm), while *A. sativum* and *Z. officinalis* have activity against only grame positive bacteria and *S. aureus* (26.5 and 17.8mm), *B. licheniformis* (21.5 and 13.5 mm) and *B. thuringiensis* (19.5 and 0.0mm) respectively and *A. niger* (22 and 23.3mm), *A. flavus* (19.5 and 0.0 mm), *A. terrus* (17 and 29 mm), *P. citrinum* (0.0 and 20 mm) *and F. oxysporum* (21 and 0.0 mm) respectively. The lowest activity showed by propolis against only *S. aureus* (16.5 mm), *A. terrus (*22.5mm*)* and *P. citrinum* (16 mm). *C. longa and F. vulgare* showed no activity against tested pathogenic microorganisms.

Table 3: Cultural and morphological characteristics of fungi isolated from silkworm larvae and their identification.

Cultural characteristics	Microscopic characteristics	Identification
Dark- brown colony, without colour reverse side	Dark-brown conidia, conidiophores are long, globose vesicles that are completely and Biseriate Phialides	*A. niger*
Yellow/grayish green and colorless to yellow reverse side	Branched septate hyohae, Yellow/greyish green conidia and Biseriate Phialides	*A. flavus*
Pinkish cinnamon to deeper with age and Pale to bright yellow to deep brown reverse side	Small, round, hyaline conidia attached to the vegetative Branched septate hyohae and Biseriate Phialides	*A. terrus*
Greenish grey colony and colorless to yellow reverse side	conidia were rod shaped and colorless mycelia and uniseriate Phialides	*A. fumigates*
Dark green colony and Pale yellow reverse side	Globose to subglobose, smooth conidia with septate hyohae	*P. citrinum*
Pale, dark to peach-violet	Branched uniseriate Phialides and no septate, oval-ellipsoidal, straight to curved microconidia	*F. oxysporum*

Discussion

Diagnosis of silkworm diseases is mainly based on the morphological symptoms or isolation and observation of infection causing agents under light microscope (Prudhomme & Couble, 2002). The silkworm having been domesticated over a number of years has become very delicate and susceptible to the infection by a number of pathogens (Anitha Redddy et al., 2014). In the present study, four bacterial species were isolated from mulberry silkworm and identified as follow, *S. aureus* SW-AZ1, *E. coli* SW-AZ2, *B. licheniformis* SW-AZ3 and *B. thuringiensis* SW-AZ4. Various other studies have also demonstrated gram positive and negative from mulberry silkworm (Abou El-Ela et al., 2015; Sakthivel et al., 2012; Anitha et al., 1994). Depending on morphological and culture characteristics of all fungal isolates illustrate in our study, six fungal species were identified as follow: *A. niger, A. flavus*, *A. terrus*, *A. fumigatus*, *P. citrinum* and *F. oxysporium*. Many *Aspergillus* species have been reported to infect silkworm (Aoki, 1971).

Table 4: Bioactivity of some medicinal plant extracts on pathogenic bacteria isolated from mulberry silkworm.

Plant extracts	*Mean diameter of inhibition zone (mm)			
	S. areas	*E. coli*	*B. licheniformis*	*B. thuringiensis*
Control	0.0	0.0	0.0	0.0
C. zeylanicum	44.0±0.0	25.5±1.0	32.0±0.0	30.0±0.0
Z. officinalis	17.8±1.0	0.0	13.0±0.0	0.0
C. longa	0.0	0.0	0.0	0.0
F. vulgare	0.0	0.0	0.0	0.0
Propolis	16.5±1.0	0.0	0.0	0.0
A. sativum	26.5±1.7	0.0	21.5±0.0	19.5±0.0

* = Mean values of triplicates determination were calculated. Control= DMSO

Table 5: Bioactivity of some medicinal plant extracts on pathogenic fungi isolated from mulberry silkworm.

Plant extracts	*Mean diameter of inhibition zone (mm)					
	A. niger	*A. flavus*	*A. terrus*	*A. fumigates*	*P. citrinum*	*F. oxysporum*
Control	0.0	0.0	0.0	0.0	0.0	0.0
C.zeylanicum	37.5±1.9	25.3±0.5	30.5±2.5	33.3±0.5	29.5±1.7	22.5±0.6
Z. officinalis	23.3±0.5	0.0	29.0±0.0	0.0	20.0±1.2	0.0
C. longa	0.0	0.0	0.0	0.0	0.0	0.0
F. vulgare	0.0	0.0	0.0	0.0	0.0	0.0
Propolis	0.0	0.0	22.5±0.6	0.0	16.0±0.0	0.0
A. sativum	22.0±0.0	19.5±0.0	17.0±0.0	0.0	0.0	21.0±0.8

* = Mean values of triplicates determination were calculated. Control= DMSO

More than 10 species of *Aspergillus* were reported from Thailand, Indonesia, Srilanka and India (Govindan & Devaiah, 1995**)** as pathogenic to silkworm, such as *A. flavus, A. tamari, A. oryzae, A. niger, A. ochraceus, A. sojae, A. fumigatus, A. nidulans, A. flavipes, A. clavatus, A. terreus, A. melleus, A. elegans, A. parasiticus, A. flavus* and *A. tamari* are most common in India. *A. bombycis* is described species known only from domesticated silkworm *B. mori* culture in Indonesia and Japan (Peterson et al., 2001). In our finding, the highest activity among medicinal plant extracts against all pathogenic bacterial and fungal isolates was *C. zeylanicum* in series gram-negative bacteria *S. aureus, B. licheniformis* and *B. thuringiensis,* gram-negative bacteria *E. coli,* and in series fungi against *Spergillus spp, P. citrinum and F. oxysporum*, while *A. sativum* and *Z. officinalis* have activity against only grame positive bacteria and *S. aureus*, *B. licheniformis* and *B. thuringiensis* respectively and *A. niger, A. flavus, A. terrus, P. citrinum and F. oxysporum* respectively. The lowest activity showed by propolis against only *S. aureus*, *A. terrus* and *P. citrinum*. *C. longa* and *F. vulgare* showed no activity against tested pathogenic microorganisms. Similar investigations were performed a few years back by several research groups that studied the antibacterial and antifungal activity of cinnamon and other medicinal plant extracts (Eralp et al., 2016; Uzma et al., 2013; Chen et al., 2013; Karuppiah & Rajaram, 2012). In the present study we detected the highest activity of *C. zeylanicum* among medicinal plant extracts against all bacterial and fungal pathogen isolated from the mulberry silkworm, *B. mori* followed by *A. sativum, Z. officinali, C. longa and F. vulgare* showed no activity against tested pathogenic microorganisms. These results recommend that further studies should be performed on the toxicity of *C. zeylanicum* prior to its clinical use; studies on the mechanism of the antibacterial and antifungal effects of its extracts and essential oils; on the separation, purification and identification of the most effective antibacterial constituents of cinnamon and for control of mulberry silkworm larval pathogens.

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