paved way for the entry of the pathogens into haemocoel. In the present study, the haemolymph of diseased larvae showed maximum number of pathogens \((19.8 \times 10^5 \pm 1.75 \text{ cfu/gm}^{-1})\) when compared to the healthy larvae \((4.97 \times 10^5 \pm 1.66 \text{ cfu/gm}^{-1})\). This finding strengthens the earlier reports of Govindan and Devaiah (1995), whom reported the mechanisms involved in immune suppression in haemolymph and disease development. The high proliferation of bacterial count in the haemocoel is because of the breakdown in the cellular and humoral immunity producing agents in the haemolymph. Circulating haemocytes involved in phagocytosis and nodule formation and humoral defense due to the synthesis of several antimicrobial peptides in haemolymph were affected.

Lu Yup-Lian and Liu-Fuan, (1991) revealed that different pathogens enter into the body of the silk worm by means of parasites, crowded trays, accumulation of faeces in the trays and rough manipulation during the transport. The silkgland also become an ideal ground for the growth of pathogenic bacteria. Bacteria in silkgland infect the silkworm larvae mostly through the mouth and digestive tract and less commonly through the egg, integument and trachea. In the present investigation the total number of bacteria present in the silkgland was \(5.97 \times 10^5 \pm 0.92 \text{ cfu/gm}^{-1}\). In the alimentary canal, the bacteria produce enzymes that damage the midgut epithelium and facilitate bacteria to enter the haemocoel. According to Cooksey (1971) there is a ballooning, exfoliation and break down of gut epithelium. In the present study, the total number of bacteria in the gut was 93.38 per cent when compared to control. Most of the insect pathogenic
bacteria occur in the families *Bacillaceae*, *Pseudomonadaceae*, *Enterobacteriaceae*, *Streptococcaceae*, and *Micrococcaceae* (Tanada and Kaya, 1993).

In the present study, out of nine species isolated, 33.3 per cent was identified as *Bacillus* sp., 22.2 per cent as *Staphylococcus* sp. and 11.1 per cent *E. coli*, *Pseudomonas* sp., *Serratia* sp. and *Aerobacter* sp., each. Manimegalai and Chandramohan (2006) had reported that among the bacterial isolates identified, 4.65 per cent were found to be *B. thuringiensis*. *Bacillus* had been identified as one of the most important pathogenic organisms involved in flacherie by many workers. Various species of *Bacillus* has been reported as *Bacillus cubonianus* (Cuboni and Garbini, 1890), *B. thuringiensis satto* (Ishiwata, 1902), *Bacillus megaterium* and *Bacillus ellenhachi* (Sawamura, 1906), *Bacillus bombysepticus* (Hartman, 1931), *Bacillus bombycoides*, a spore forming bacterium, isolated from pupae of silkworms (Paillot, 1942), *Bacillus mycoides* and *Bacillus laterosporus* (Steinhaus, 1949). Toumanoff and Vago (1951) isolated *B. cereus var. alesi* from silkworm larvae suffering from dysentery. Chitra et al. (1973) isolated *A. cloacae* and *A. delmarvae* from haemolymph. *P. boreopolis*, *P. ovalis*, *Escherichia freundi* and *S. albus* from the midgut of flaccid silkworm. In the present study nine types of bacteria were isolated viz., *B. subtilis*, *B. cereus*, *B. thuringiensis*, *S. aureus*, *P. aerogenosa*, *E. coli*, *S. marcescens*, *S. albus* and *A. aerogenes*. Nataraju et al. (1991) had isolated six spore forming *Bacillus* from silkworm litter samples and found two of them to be pathogenic. Chisti et al. (1991) had isolated *Bacilli* group of bacteria which were gram
positive and spore forming rods. Rajakumari (2005) had identified presence of 43 types of bacterial species in the flacherie infected silk worms. Priyadharshini et al. (2008) had isolated six bacterial species. Of these, three of them are Bacillus sp. Enomoto et al. (1987) reported that S. faecalis is more infective than S. faecium, Serratia is more pathogenic than Pseudomonas and Proteus. Members of the family Bacillaceae produce endospores and most of the insect pathogen of this family includes the genera Bacillus. S. marcescens is a facultative pathogen, remain beneficial inside the gut and not pathogenic when present in digestive tract in small numbers (Sikorowski, 1985). The gut pH and environment inhibits its pathogenicity. On damage to the gut wall, this bacteria S.marcescens invade the haemocoel and cause many health problems.

The spore forming B. thuringiensis produce one or more parasporal bodies or crystals in the sporangial cell, which are proteinous by the proteolytic enzymes at an alkaline pH in the gut form smaller toxic peptides, the γ-endotoxins (Tanada and Kaya, 1993). In the present investigation, B. thuringiensis, B. subtilis, B. cereus were reported from different organs. Krishnan et al. (2002) had studied B.subtilis, cause superoxide dismutase activity in haemocytes and haemolymph of B. mori following bacterial infection. Choudhury et al. (2002) reported that B. thuringiensis was found to be the most pathogenic and produced 100 per cent mortality at 72 hours. Bacillus remains the major pathogenic bacteria isolated in majority of area. Krishnan et al. (2002), Priyadarshini et al. (2008) and Ravikumar et al. (2009) reported B. subtilis to cause flacherie in B. mori. Hossain et al. (2006)
revealed the activity of the endotoxin limited to the digestive tract. The bacterial exotoxins kill silkworms during infection. The intensity of bacterial flacherie, depends on the bacterial species. Kaito and Sekimizu (2007) had injected *S. aureus* into the haemolymph of silkworm larvae and all the larvae were killed within two days. The present study, alerts the sericulture farmers to adopt proper bed cleaning, hygienic methods of handling the larvae, rearing room, personal hygiene of the labourers providing clean disease and pest free mulberry leaves and also proper ventilation. The domestication of silkworms and their rearing in artificial environment had every chance to inhibit their innate and acquired immunity (Govindan and Devaiah, 1995).
CHAPTER - II

INTRODUCTION

Since the discovery and exploitation of antibiotic agents in the 20\textsuperscript{th} century, the targeted selective toxicity of such agents have ensured their wide spread and largely effective use to compact infection, however it has paradoxically resulted in the emergence and dissemination of multidrug resistant pathogens. Bacterial resistance is a growing problem world wide (WHO, 2000 and Cohen, 2002).

One of the problems in the development of resistance of haemotherapeutic agent was due to abuse of drugs (Reuter, 2005; Al-Bakri and Afifi, 2007; Abere \textit{et al.}, 2007 and Neogi \textit{et al.}, 2007). Therefore there was a need to develop alternative antimicrobial drugs for the treatment of infectious diseases from medicinal plants (Clarck, 1996; Cowan, 1999; Cordell, 2000 and Ekwency and Elegalam, 2005). Certain plants are good sources of therapeutic agents due to their higher antimicrobial properties. When compared to others, botanicals are available at reduced cost, relative lower incidence and of reactions compared to modern conventional chemical pharmaceuticals (Essawi and Srour, 2000; Planta \textit{et al.}, 2000 and Karachi, 2006).

Indian sericulturists, often experienced a total cocoon crop failure owing to the out break of diseases in silkworm. Chemical disinfectants, bed
disinfectants, antibiotics and botanicals are being used for disease management in an integrated approach. The most commonly used method for preventing disease is the use of chemicals as disinfectants, though many of them had proved effective in checking the spread of diseases in silkworm crops, they have raised concern due to their slow biodegradability and accumulation of residual toxicity in the environment (Sasidharan et al., 1999 and Shubha et al., 2010).

The etiology of viral and fungal diseases had been investigated thoroughly, whereas the etiology of bacterial diseases is not fully understood because of the multiplicity of factors involved in bacterial infections. Other than preventive measures, no effective remedial measures have been developed to check the infection and further spread of the disease (Choudhury et al., 2002). Among the various methods of disease management, use of plant products is appropriate for the current scenario because of their cost effectiveness and ecofriendly nature. The efficacy of aqueous extract of botanicals, viz., *Psoralea corylifolia* L. and *Plectranthus amboinicus* L. against grasserie disease of *B. mori* have been reported (Manimegalai and Chandramohan, 2006 and Manimegalai, 2009).

The occurrence of substances strongly inhibitory to the growth of pathogens and acts mainly as protectors of the plant against diseases (Gaumann, 1950 and Walker and Stahmann, 1955). *Abies balsamea* plants having the inhibitory effect against *Bacillus* sp. (Kushner and Harvey, 1960) and their role in insect resistance to disease was also reported (Kushner and Harvey, 1962). Onion extracts or onion oils suppress the growth of
intestinal worms, fungi and bacteria both in vivo and in vitro (Didry et al., 1987 and Kim, 1997). In vitro evidence of the antimicrobial activity of fresh and freeze dried Allium extracts against many bacteria (Cavallito and Bailey, 1944 and Rees et al., 1993) were studied. Different extracts of seeds of Helianthus annus (Asteraceae), leaves of Acalypha indica L.(Meliaceae), bulbs of A. cepa (Liliaceae) were screened for antimicrobial activity against two gram positive bacteria (B. subtilis and S. aureus) and four gram negative bacteria (E. coli, P. vulgaris, P. aeruginosa and S. typhi ) and the methanol extracts of four different members of Allium namely, A. cepa (variety-1), Allium sativum L., A. cepa (variety-2), A. ascalonicum had antimicrobial properties against Klebsiella and Micrococcus (Chaithradhyuthi et al.,2009). Onion extracts have both antifungal and antibacterial properties against S. typhi, E. coli and S. aureus (Noureddine et al.,2005).

An aqueous extract of garlic and onion were effective against gram positive and gram negative organisms and fungi was studied (Shelef, 1983; Nabuji, 1994; Billing and Sherman, 1998; Yani et al., 2006 and Shan et al., 2007). Antibacterial enhancement (synergistic effects) of A. sativum with C. longa against a number of infections for generations was reported (Neogi et al., 2007). Garlic and other Allium vegetables shows antibacterial effect against Aerobacter, Aeromonas, Bacillus, Citrella, Citrobacter, Clostridium, Enterobacter, Escherichia, Klebsiella, Lactobacillus, Mycobacterium, Proteus, Pseudomonas, and Staphylococcus was reported (Uchidha et al., 1975 and Reuter et al.,1996).
Antibacterial activity of an aqueous extract of dried garlic against 
*S.aureus* (Shakrazadeh and Ebadi, 2006) and against, *E.coli, S.typi and B.cereus* (Gomaa and Hashish , 2003) were studied. Preparation of fresh garlic and vacuum dried powdered garlic preparation were effective against *S. aureus, K. pneumoniae, P. vulgaris, E. coli, B.subtilis, B.thuringiensis and S.marcescens* (Adetumbi and Lau, 1983; Khan et al., 1985; Hughes and Lawson, 1991; Ahsan and Islam, 1996; Tsao et al., 2003 and Zhou, 2003).

Fresh *A.sativum* (garlic extract ) was most effective against the silkworm pathogen, *P.aureginosa* (Choudhury et al., 2002) and *A. sativum* extracts can also prevent the formation of *Staphylococcus* enterotoxins A, B and C and also thermonuclear was reported (Gonzalez Fandos et al., 1994).

Bacterial cultures, resistant to commonly used antibiotic chloromphenical appear sensitive to garlic (Jezpwa et al., 1966). The microbial activity of the eight plant extracts against 34 microorganisms was described by Panzaru et al. (2009). Garlic extracts had a strong antifungal effect and inhibit the formation of mycotoxins like the aflatoxin of *A. parasiticus* (Lawson, 1996).

45 extracts belonging to different groups of marine organisms including mangroves, seaweeds, seagrasses and cyanobacteria explored novel antibacterial and antifungal compounds against silkworm pathogens (Ravikumar et al., 2009) *Pseudoranthemum atropurpureum* L. and *Bougainvilla spectabilis* L. extracts acts against viruses (Verma et al., 1985).

Some plants like *A. indicum, B. diffusa, P. coryleifolia, T. terrestris* and *C. nucifera* were studied for the suppression of grasserie disease in silkworm *B. mori* (Sivaprakasam et al., 1999). *In vitro* and *In vivo* studies were
conducted for the identification of effective botanicals for the management of bacterial flacherie of silkworm, B. mori against B. thuringiensis (Manimegalai and Chandramohan, 2005). *In vivo* studies conducted with botanicals revealed that the plant *basil* and *asparagus* were effective against *Staphylococcus* sp. and the plant amla and *Boerhaevia diffusa* L were effective against *Bacillus* sp. in silkworm (Priyadharshini, 2006). Some medicinal plants like *Aadhatoda vasica* L., *Phyllanthus niruri* L., *P. coryleifolia* L., *Tribulus terrestris* L. and *Withania somnifera* showed an antiviral activity against silkworm pathogen BmNPV (Shubha *et al.*, 2010).
MATERIALS AND METHODS

Test bacteria – *B. subtilis* (Gram positive).

The pure culture of the test bacteria were isolated from the fourth instar infected silkworm, *B. mori* (race: PM x CSR2) was obtained from Model Sericulture Development Centre and also from sericulture farms in and around Konam village, Nagercoil. The culture collection was deposited in the research lab. The pure culture of bacteria were maintained in a nutrient agar slants. The cultures were maintained in refrigerator for use and regularly checked for contamination. Periodic transfers were made aseptically. Mortality rate of *B. mori* was recorded against *B. subtilis*.

Collection of plants

For the preparation of extracts *A. cepa*, *A. sativum* were brought from local market (Vadachery). *S. trilobaliu*m plants were collected from botanical garden of Scott Christian College, Nagercoil.

Extraction

For the aqueous extract preparation, fresh produce were peeled, finely chopped and stomached at ambient temperature, employing a mixer grinder for 10 minutes. Prior to recovery of supernatant, which was subsequently filter sterilized through muslin cloth and stored in the refrigerator at 4°C until use.
For the ethanolic and methanolic extract preparation, ten g of each plant were homogenized with mortar and pestle and was extracted with 100 ml of 70% ethanol / methanol in conical flasks, sealed with foil and allowed to stand for 72h, they were filtered to obtain crude ethanolic extracts and stored at 4°C when not in use (Choudhury et al., 2002 and http://www.microbiocare.com/ and http://www.iadvaita.com).

**Preparation of bacterial inoculums**

Nutrient broth was prepared, dispensed (about 5ml ) into a clean test tubes, plugged with cotton and sterilized. After sterilization and cooling the pure cultures of bacteria maintained on slants were transferred to the nutrient broth taken in test tubes asceptically and incubated at 37° C for 24 hours. The antibacterial activity was assessed using the simple disc diffusion and well diffusion method, where the drug impregnated filter paper disc were placed on nutrient media inoculated with the test bacteria so as to get a lawn culture on incubation. The drug diffuses into the medium and inhibits bacterial growth round the disc if it is effective. This indicates the antibacterial activity. If the drug is not effective or is not having antibacterial activity then the zone of inhibition will not be observed.

**Screening for antibacterial activity**

The antibacterial activities of plants were evaluated by agar disc diffusion method. Using nutrient agar (Hi-media) for the assay the bacteria were activated by inoculating a loopful of strain in the nutrient broth (25ml) and incubated at 37° C for 24h. 0.1ml of inoculum was introduced into the
pre solidified agar plates and spread plate technique was applied using L shaped spreader. Sterile filler paper disc (the Whatman No.1) were impregnated with different crude extracts and dried in a hot air oven at 60°C for 5 min. Then it was placed aseptically above the seeded agar and pressed a little to facilitate proper diffusion and incubated at 37 ± 1°C for 24 h. The diameter of the inhibition was measured by using graduated scale (http://www.microbiocare.com/ and http://www.iadvaita.com)

**Methodology for HPLC analysis**

For HPLC analysis, 1g of dried and powdered plant material was extracted with water (10ml) for 4 hours at room temperature. The plant extract was subjected to qualitative and quantitative analysis by using a 1090 liquid chromatograph with diode array detection, Hewlett-packard, Palo alto, USA, HPLC system, using column: 250 x 10mm. Sample amount taken was 125 µg. Stationary phase: Spherisorb ODDS-1, 5µm particle size. Mobile phase: Water - a cetonitrile (1:1 V/V) acidified with 0.00625% formic acid; ISO critic elution. At the flow rate of 1.5ml/min. Column over - 40°C. The results were detected in UV at 195 nm.
RESULTS

Mortality rate of *B. mori* was measured through serial dilution technique. 50 per cent mortality was obtained at $1 \times 10^{-5}$ µg / ml concentration of *B.subtilis* (Table 2.1). The three plant extracts such as *A. cepa*, *A. sativum* and *S. trilobatum* possessed antimicrobial activity against the tested silkworm pathogen of *B. subtilis* (Table 2.2). The maximum antibacterial activity was observed in *A. cepa* aqueous extract (Plate 2.1a) with 22.3 mm diameter of inhibition zone (DIZ), and the lowest antibacterial activity was observed in ethanolic extract of *A. cepa* with the inhibition zone of 8.0 mm (Plate 2.1a). The extract prepared from *A. sativum* had antibacterial property against *B. subtilis*. The maximum effects was observed at aqueous extract (DIZ 15.6 mm) (Plate 2.1b) and the lowest antibacterial activity was observed in methanol extract (8.2 mm) (Plate 2.1b). The extract prepared from leaves of *S.trilobatum* had low antimicrobial property when compared to the other two plant extracts. The highest antibacterial activity against *B.subtilis* was observed in aequous extract (12.5 mm) and the least inhibition was shown in methanol extract (7.1 mm) (Plate 2.1c).

Induction of antimicrobial activity

Antibacterial activity was induced in the haemolymph of silkworm larvae by the infection of live *B. subtilis* through mulberry leaves along with the plant extracts. Haemolymph of treated groups shows antibacterial activity. The highest activity was observed when the silkworm larvae was treated with *A. cepa* at 5% concentration. The zone of inhibition against
B. subtilis was 23.3mm and the minimum zone of activity was observed at 10% of A. cepa (16.1mm) (Table 2.3 and Plate 2.2a). A. sativum shows zone of inhibiton of antimicrobial activity 16.6mm at 10% (Table 2.3c and Plate 2.2b) and S. trilobatum shows zone of inhibition at 1% was 13mm (Table 2.3c and Plate 2.2c). Application of A. cepa extracts on silkworm, have registered significant results on antimicrobial activity. Maximum inhibitory activity with 5% was shown at 12 h of exposure (25.1 mm) and minimum at 1 hr of exposure (8.2 mm) at 1% (Table 2.4 and Plate 2.3 a).

HPLC analysis was used to determine Seleno group compounds, representing the major flavonoids in onion peel. Based on the peak value it was searched in the HPLC chromatogram analyzing software- chemical database management software (Fig. 2.1)

The compounds obtained were Selenic acid, Selenate, SeO$_4^{2-}$ (Na$_2$SeO$_4$); Se-Methylselenocysteine, CH$_3$SeCH$_2$CH(COOH)NH$_2$; Selenomethionine, CH$_3$SeCH$_2$CH$_2$CH(COOH)NH$_2$ and g-Glutamyl-Se-methyl selenocysteine, CH$_3$SeCH$_2$CH(COOH)NHC(O)CH$_2$CH$_2$CH(COOH)NH$_2$. 
DISCUSSION

The effects of plant extracts on bacteria, had been studied by a very large number of researchers in different parts of the world (Citarasu et al., 2003; Nair et al., 2005; Adomi, 2006; Latha and Kannabiran, 2006; Chehregani et al., 2007; Neogi et al., 2007; Bhaskar et al., 2008; Adomi, 2008; Chaithradhyuthi et al., 2009 Shubha et al., 2010., and Maribashetty et al., 2010).

In the present study antimicrobial activity of 3 plant extracts viz., *A. cepa*, *A. sativum* and *S. trilobatum* against *B. subtilis* were analysed. Maximum antibacterial activity was observed with *A. cepa* (22.3 mm). This was in corroboration with Garg and Jain (1998) and Mahesha et al. (1999). The antibacterial effect of essential oil from rhizomes of *Curcuma caesia* Roxb. Rich in curcumene, ionome and tumeron was demonstrated by Garg and Jain (1998). Aqueous extract of *Parthenium hysterophorus* L. was more effective in increasing the rate of survival in silkworm races NB18 (85.16 %) and PM x NB 18 (89.0 %) breeds compared to *Tridax procumbens* L. (83.0 % in PM x NB 18) and water control (72.66 % in NB18 and 76.16 % in PM x NB18) was demonstrated by Mahesha et al. (1999).

Manimegalai et al. (2009) reported that among the various methods of disease management, use of plant molecules is appropriate for the current scenario because of their cost effectiveness and ecofriendly nature. Panzaru et al. (2009) had reported that, plant material might exert a physiological effect through an alternative modality to antimicrobial infection.
Giese (1994); Brull and Coote (1999) and Juven et al. (1994) had demonstrated the possible mechanism of antimicrobial action of onion and garlic. According to them hydrophobic and hydrogen bonding of phenolic compounds of plants to membrane proteins followed by partition in a lipid bilayer. Cox et al. (2000) had studied the perturbation of membrane permeability consequent to its expansion and increase fluidity causing the inhibition of membrane embedded enzymes, involved in antimicrobial activity.

Panzaru et al. (2009) studied the antimicrobial activity of bulbs of *A. cepa* against *B. subtilis*. In the present study, the maximum antibacterial activity (22.8 mm zone of inhibition) against *B. subtilis* was resulted due to *A. cepa*. It was in agreement with Priyadharshini (2006) who attributed the inhibition zones of 7.0 mm and 9.0 mm by Amla at a dose of 20,000 and 30,000 ppm against *B. subtilis* while inhibition zones of 6.0 mm and 7.0 mm were observed due to *Asparagus* against *B. subtilis*. Manoharan (1996) had studied the effect of aqueous extract of *A. cepa* on grasserie in silkworm larvae. Watt and Merrill (1963) analysed that fresh bulbs of *A. cepa* consist mainly of water (88%), saccharides (6%) and proteins (1.5%). However, the particular composition depends on a larger number of factors, such as growing conditions, time of harvest and length and conditions of storage. According to Steinegger et al. (1999) and Bianchini and Vainio (2001), the most predominant sulphur containing compounds are the aminoacids, cysteine and methionine, the S-alk(en)yl substituted cysteine sulphoxides and the γ-glutamyl peptides.
In the present study, on HPLC analysis, the highly active compound present in *A. cepa* was Selenic acid, Se-methyl Selenocystene and Selenomethronine. This finding was in agreement with Suzuki (1962); Virtanen and Matikkala (1976); Block (1985); Steinegger *et al.* (1999) and Bianchini and Vainio (2001). Block (1985) had reported that onions mainly contain S-propenyl cysteine sulfoxide, but also other sulfoxides, including S-propylcysteine sulfoxide and S-methylcysteine sulfoxide. Steinegger *et al.* (1999) and Bianchini and Vainio (2001) had reported that sulfur compounds generated from the highly reactive sulphenic acids were responsible for the typical smell, taste and pharmacological actions of onion extracts. According to Lines and Ono (2006); Jalali *et al.* (2006); Kanter *et al.* (2007) and Murota *et al.* (2007), *A. cepa* contains flavonoids such as quercetine. Bruneton (1995) had reported that a fresh *A. cepa* bulb contains fructants with a low degree of polymerization and sulphur containing compounds. The presence of these compounds might explain the antimicrobial activity of this plant.

Pasteur (1858) and Lehmann (1930) proved the use of garlic extract as a first modern scientific evidence for medicinal and antibacterial use of garlic extract. Reuter and Sendil (1995); Harris *et al.* (2001); Marbidoni *et al.* (2001); Ichikawa *et al.* (2003); Stephen (2005); Ariga and Seki, (2006) and Nies *et al.* (2006) had reviewed the pharmacology and medicinal application of garlic isolated compounds for the treatment of number of diseases. The present study revealed the antimicrobial activity of three different extracts of *A. sativum* _viz_, aqueous, methanol and ethanol respectively, among that,