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Synergistic Activity of *Andrographis paniculata* Nees with *Bacilus thuringiensis* var *israelensis* Against Malarial Vector, *Anopheles stephensi* Liston (Diptera: Culicidae)

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ABSTRACT

Integrated vector control is an effective and essential part of any successful vector control program. The synergistic activity between the current effective pesticides and phytochemicals is one such strategy and powerful tool for developing vector control strategies. *Bacillus thuringiensis* var *israelensis* (Bti) and ethanolic extracts (Ee) of *Andrographis paniculata* were both observed for their larvicidal as well as pupicidal activity against *Anopheles stephensi* Liston. Ee extract with lethal concentrations (LC_{50} and LC_{90}) values of 36.56 and 53.04 p. p. m. after 24 h and LC_{50} 22.28 and LC_{90} 51.26 p. p. m. after 48h for fourth instar larvae, respectively. LC_{50} and LC_{90} for *Bti* were 0.0967 and 0.1003 p.p.m after 24h and 0.2944 and 0.4158 p. p. m. after 48 h of exposure for fourth instar larvae, respectively. Combined formulations were evaluated for synergistic activity and a 1:4 ratio of Bti and Ee was observed to be more effective than 1:2 and 1:1 ratios. However the larvicicdal activity is minimal when the mixed formulations contained an equal amount of both constituents (i. e a 1: 1 ratio). A ratio of 1: 4 of Bti and Ee of *A. paniculata* was 51.6 fold more toxic at the LC_{90} for larvicidal activity, than Bti alone and this high level of activity resulted from synergism between the Bti and Ee of *A. paniculata* Nees.

Key words: *Andrographis paniculata*, *Anopheles stephensi*, *Bacillus thuringiensis*, larvicidal activity, synergistic activity.

INTRODUCTION

Integrated vector control is an effective and essential part of any successful vector control program. Chemical control, although effective, is often used only as a temporary solution to disease outbreaks. The over use of chemical control often leads to resistance to these chemicals, resulting in a rebounding vector population and disease potential. Selected botanicals have been shown to be effective larvicides and adulticides and in some cases are more eco-friendly against non-target animals (Prakash and Rao., 1997). Natural plant extracts provide potential for the development of botanical pesticides and synthetic analogs (e.g. pyrithrin) and have been successfully tested for various biocontrol programs (Jeyabalan *et al*., 2003; Sharma *et al*., 2006). Ethanolic extract of *Andrographis paniculata* showed oviposition deterrent, ovicidal and mortality effects against gravid and oviposited females of *Anopheles stephensi* with oviposition

activity index values of -0.28, -0.45, -0.49 and -0.59 for extract concentrations of 29, 35, 41 and 46 p. p. m., respectively. High degrees of gravid mortality were also observed with various concentrations: 1.12 (control) to 11.70 for gravid females, and 0.65 (control) to 10.25 for oviposited females and suggesting possible contact toxicity of the extract (Kuppusamy *et al.,* 2008). Further the ethanol and methanol extracts of *Andrographis paniculata* were also showed remarkable effects on growth, development and reproduction of malarial vector *Anopheles stephensi,* after 8 days of treatment, 88.60 and 85.25% of the larvae treated at 35 p. p. m. failed to emerge in ethanol and methanol extracts respectively. In addition, the duration of larval instars and the total development time were prolonged, while female longevity and fecundity were markedly decreased (Kuppusamy *et al.,* 2010).

Identifying insecticides that are efficient, as well as being suitable and adaptive to ecological conditions, is imperative for continued effective vector control management. Synergistic activity between current effective pesticides and phytochemicals is a powerful tool for developing insect control strategies (Bernard and Philogene., 1993). Most studies on the synergistic and additive toxic effects of binary mixtures involving phytochemicals have been conducted on agricultural rather than vectors of diseases. Therefore, the objective of the present study was to assess the synergistic action of ethanolic extracts (Ee) of *Andrographis paniculata* Nees with *Bacillus thuringiensis* var *israelensis* (Bti) against the *Anopheles stephensi* Liston. This combined approach would, on one hand, reduce the amount of the Bti formulation needed (thereby reducing costs, risk to non-target organisms and the risks of resistance), and on the other, significantly decrease farmer dependence on synthetic pesticides. In the larger context, this would translate into reduced pesticide contamination of the environment.

MATERIAL AND METHODS

Colonization of *Anopheles stephensi* Liston

Anopheles stephensi Liston were maintained in the laboratory from the lines obtained from National Institute of Communicable Diseases-Field station, Cunnoor. The colonies of mosquitoes were maintained at conditions 27± 2^0 C and 80% ± 5 relative humidity under 12 L: 12 D cycle. The larvae were reared in enamel trays and fed finely ground dog biscuits and Yeast at 60: 40 ratio. Water in rearing containers was refreshed every 2 days, pupae were transferred from the trays to a cup filled with dechlorinated tap water and placed in screened cages where adults emerged. The adult mosquitoes were maintained in a net cage (90×90×90 cms) and were continuously 10% sucrose solution provided in a jar with a cotton wick. For continuous culture selected numbers of Mosquitoes were allowed to feed chicken blood and every third day, thereafter moist filter paper was kept in beaker in the cages for mosquitoes to lay their eggs on, eggs laid on the filter paper were immersed in larval basins containing water for the maintenance of the colony.

Phytoextract preparation

The leaves and roots of *Andrographis paniculata* Nees were collected locally from the foothills of the Western Ghats area adjacent to Bharathiar University, Coimbatore, Tamil Nadu, India. The whole plants were washed with double distilled water and were shade dried at room temperature. The dried parts were chopped into small pieces of approximately 1 cm size by a falcon stem cutter (Biocraft Scientific India, Uttar Pradesh, India) and powdered with the help of an electric blender. The dried powder was subjected to ethanol in a Soxhlet apparatus (Borasil, Mumbai, India) for 72 h (Saxena *et al.,*1994). After removing the solvents from the plant extracts in a vacuum rotary evaporator, stock solution of 1% was prepared with 200 mg residue in 20 mL ethanol and was kept in a screw-cap vial with aluminum foil over its mouth. The stock solution was then serially diluted ten-fold in ethanol (2 mL solution to 18 mL solvent) and test concentrations were obtained by adding 0.1-1.0 mL of the appropriate dilution to 100 mL distilled water (WHO 2005).

For combination studies, keeping Bti as the standard, its stock was mixed with the stock of most efficient phytoextract in ratios of 1: 1, 1: 2 and 1: 4. Test concentrations for each of the mixed formulation ratios were prepared by further diluting the combination mixture in distilled water. Efficacy for each formulation was observed as above and a lethal concentration (LC_{50} as well as LC_{90}) was determined.

Bacterial products

Aqueous formulations of *Bacillus thuringiensis* var *israelensis* SPIC BIO-AS, Tuticorin Alkali chemicals and fertilizer limited, Chennai, India were used.

Larvicidal bioassay (WHO 2005).

The larvicidal bioassay was assessed by using standard WHO Protocols (WHO., 2005) For experimental treatment, one ml of desired concentration obtained from stock solution of methanolic extracts of *A.paniculata* (alone or in combination with Bti) was added to 100 ml of distilled water in a 250 ml of enamel bowel which was shaken lightly to ensure a homogenous test solution, then 25 1^{st}, 2^{nd}, 3^{rd}, and 4^{th} instar larvae of *Anopheles stephensi* were transferred by means of strainers to that bowel, each experiment was performed in 4 replicates with a final total of 100 larvae for each concentration and the equal number of controls were set up simultaneously with distilled water to which 1 ml of ethanol was added, experiments were conducted at 27+1^{o}C, 85% RH with photoperiod of 12L: 12D. Symptoms of the treated larvae were observed and recorded immediately and at timed intervals and no food was offered to the larvae, mortality and survival was registered after 24 and 48 h of the exposure period. The moribund and dead larvae in four replicates were combined and expressed as a percentage of larval mortality of each concentration, dead larvae were identified when they failed to move after probing with a needle in the siphon or cervical region, moribund larvae were those incapable of rising to the surface (within a reasonable period of time) or showing the characteristic diving reaction when the

water was disturbed. The larvae showed discoloration, unnatural positions, tremors, unco-ordination or rigor were also counted.

In case of experiment for determining pupicidal activity, the mouth of each bowl containing pupae was covered with muslin cloth to prevent the escape of any emerged adult mosquitoes. Mortality in larvae and pupae was recorded at 24, and 48 hrs.

DATA ANALYSIS

Mortality data produced for phytoextracts and Bti bioassays alone and for mixed formulations, were analyzed by probit analysis (Finney., 1971). The corrected percent mortality was calculated by Abbot's formula (Abbot 1925).

Corrected % mortality = [(T-C) / (100-C)] ×100

Where T is the percent mortality in the test concentration and

C is the percent mortality in the control.

Regression equations, LC_{50} and LC_{90} were obtained along with standard error and fiducial limits at 95% confidence level. A co-toxicity coefficient (Sarup *et al.,*1980) and a synergistic factor (Kalayanasundaram and Das, 1985) for mixed formulation experiments were calculated after calculating LC_{50} and LC_{90} for each combination as effectiveness of the components of the mixture in terms of their potency relative to one of the components of the mixture.

Co-toxicity coefficient (CTC) = [toxicity of insecticide (alone) /

Toxicity of insecticide with plant extract] ×100

Synergistic factor (SF) = toxicity of insecticide (alone) /

Toxicity of insecticide with plant extract

A value of SF > 1 indicates synergism and SF < 1 indicates antagonism.

RESULTS

The data were presented in Fig. 1. and Table 1. provides the larvicidal and pupicidal activity of whole plant ethanol extract of *A. paniculata* Nees on different larval instars and pupae of *An. stephensi*. The results showed that most of the larval and pupal mortalities occurred after 24 hours, and then increased slightly and gradually till 48 hours. Mortality was concentration dependent. The lower concentrations (29ppm) showed mortality percentages ranging from 33.4, 33.7, 29.3, 27.9, and 34.8 within 24 hrs, then mortality increased to reach 78.5, 70.3, 66.9, 63.5 and 60.2% at 48 hrs (35ppm) for 1^{st}, 2^{nd}, 3^{rd}, 4^{th} instars of larvae and pupae, respectively. On the other hand the higher concentrations (53ppm) showed mortality percentages ranging from 96.4, 94.3, 89.9, 86.9, and 78.3 within 24 hrs. After 24 hrs, the mortality increased to reach 100.0, 98.5, 94.2, 92.1, and 84.6 for 1^{st}, 2^{nd}, 3^{rd}, 4^{th} instars of larvae and pupae respectively.

The LC_{50} and LC_{90} values of whole plant ethanol extracts of *A. paniculata* for 1^{st} instars larvae were 31.98, 45.69 ppm; 33.97, 48.11 ppm for 2^{nd} instars larvae; 35.72,

51.44 ppm for 3rd instars larvae; 36.59,53.04 ppm for 4th instars larvae; and 38.32, 56.81ppm for pupae respectively at 24 hrs, which is further decreased at 48 hrs as for 1st instars larvae 14.58, 38.59ppm; 19.98,42.51ppm for 2nd instars larvae; 21.12, 47.49 ppm for 3rd instars larvae; 22.28, 51.26 ppm for 4th instars larvae and 22.97, 56.48 for ppm for pupae respectively. Among different larval stages, the 1st instars (Younger) larvae were more susceptible than the other instars of (older) larvae. The regression and chi-square values are also given in Table 1.

The Fig. 2 provides the larval and pupal mortality of *An.stephensi* after the treatment of Bti. The Bti treatments were effective against larvae of all instars and pupae and mortality was concentration dependent. The lower concentrations (0.02ppm) showed mortality percentages of larvae and pupae ranging from 42.5, 37.5, 32.2, 27.5 and 23.3 within 24hrs, increased to 58.5, 55.2, 53.0, 49.0, and 47.5 at 48 hours for 1st , 2nd, 3rd, 4th instars of larvae and pupae respectively. On the other hand, the higher concentration (0.32 p. p. m.) showed mortality percentages ranging from 94.6, 91.2, 82.2, 77.5 and 74.4 within 24 hrs. After 24 hrs, the mortality increased to reach 100, 98.8, 94.8, 90.2, and 84.7 for 1st, 2nd, 3rd, 4th instars of larvae and pupae respectively. The LC_{50} and LC_{90}, regression and chi-square values are also presented in Table 2.

The Fig. 3 provides the cumulative mortality of various instars of *An.stephensi* treated in combination of Bti with ethanol extract of *A. paniculata* treated at 24 and 48 hrs in different ratios. The resulting toxicity was much greater than that observed for Bti alone and also as the age increases the susceptibility also decreases i.e. second to third instar. As stated above, the LC_{50} and LC_{90} values were 0.1202, 0.2252 ppm for 1st instars; 0.1587, 0.2740 ppm for 2nd instars; 0.2378, 0.3734 ppm for 3rd instars; 0.2944, 0.4158 ppm for 4th instars; and 0.3663, 0.4372 ppm for pupae, respectively.

In the presence of 1:1 ratio of Bti to ethanol extract of *A. paniculata*, the LC_{50} and LC_{90} were 0.0006, 0.0029 for 1st instars; 0.0010, 0.0041ppm for second instars; 0.0020, 0.0059ppm for 3rd instars; 0.0031, 0.0072ppm for 4th instars; and 0.0034, 0.0091 ppm for pupae, respectively (Table 3.). When the proportion of Bti in the combinations reduced, the mixture continued to be highly toxic with LC_{50} and LC_{90} values of 0.0003, 0.0028ppm for 1st instars; 0.0004, 0.0033ppm for 2nd instars; 0.0015, 0.0049ppm for 3rd instars; 0.0024, 0.0060ppm for 4th instars; and 0.0022, 0.0076ppm for pupae at1:2 ratios (Table 4.); and 0.0002, 0.0018ppm for 1st instars; 0.0005, 0.0030ppm for 2nd instars; 0.0012, 0.0047ppm for 3rd instars; 0.0019, 0.0057ppm for 4th instars; and 0.0025, 0.0069ppm for pupae at 1: 4 ratios, respectively.

The concentration of Bti in the latter 2 ratios was sub lethal, where as for the 1:1 ratio; Bti could have contributed between 28 and 53% of the observed mortality. The 1:4 ratios of Bti and ethanol were the most active combination tested. Although toxicity was slightly lower at 1: 1, 1:2 ratios, these combinations were still highly toxic.

The Bti and ethanol extract mixtures had an additional effect on toxicity. In contrast to Bti, which normally requires 48h for maximum toxicity, high toxicity was observed within 24h after exposure to the combination of Bti and ethanol extract. In general, concentrations did not increase significantly between 24 and 48h of exposure to these

combinations. However when Bti present at a lower proportion in the mixture 1: 2, 1:4 ratios, a significant increase in mortality after 48h was noted.

Calculation of the synergism factors from the 24h to 48hrs data for the various combinations indicated that all of the mixtures were synergistic. Synergism factors ranged from 65.0 to 86.5 and 47.91 to 66.77 for 1st instars; 30.42 to 39.4 and 48.92 to 52.9 for 2nd instars; 44.26 to 35.75 and 48.49 to 50.59 for 3rd instars; 41.79 to 50.89 and 46.2 to 51.64 for 4th instars; and 42.58 to 46.8 and 44.61 to 53.05 for pupae at LC_{50} and LC_{90} for 1:4 ratio respectively. Similar synergism factors with high activity were observed for 1:1, 1:2 ratios (Table 3., 4.). The synergism factors in agreement with the toxicity levels were greatest with the 1:4 ratios of Bti to ethanol extract of *A. paniculata*.

DISCUSSION

Effect of *A. paniculata* extracts on larval and pupal mortality against An. *stephensi*

The results indicated that ethanol extract had the most effective larvicidal and pupicidal activity. The higher activity of the ethanol extract indicated that the active compounds might be more soluble in ethanol than the other solvent extractions (Heisey., 1990). The mortality of the larvae increases as the doses of the sample were increased. The same trends were also observed in cause time elapse mortality. The fourth instars larvae were less susceptible to the samples than 1st instars larvae. The LC_{50} and LC_{90} values were also age dependent. This may clearly support the ideas of others that insect age plays an important role in influencing the susceptibility. (Kumar *et al*., 1987). In general, after exposure to the different extract of the *A. paniculata*, the treated larvae exhibited slower movements in the water, biting its respiratory siphons vigorously with peculiar coiling and sluggish movements, its inability to move to the surface and rested at the bottom of the bowel and finally died. These paralyses like symptoms seem to suggest some neural and muscular disturbance caused by the presence of cytotoxic diterpenoids viz. Deoxy andrograholide,-19-Dglucosidel (Techadamrongsin *et al*.,1999), four lactones (schri.,1973, Deng *et al*.,1982, Tang *et al*., 1992) and flavonoids (Zhu and Liu., 1984., Kuroyanagi *et al*., 1987). The results are favorably supported by the larvicidal and pupicidal activity of neem limonoids against *An.stephensi* (Sengottayan senthil Nathan *et al*., 2005). Ethyl acetate fraction from leaf extract of *Calophyllum inophyllum* (Pushpalatha *et al*., 1999), ethanolic leaf extracts of *Cannabis sativa* (Jalees *et al*., 1993) and steam distillation of different parts of *Cedrus deodara* (Kumar *et al*., 1987), which caused excellent larval and pupal mortality against *An. stephensi*.

The presence of variety of active compounds in *A. paniculata* which are effective in reducing gut movements (Sawasdimongkol *et al*., 1990) and disrupting peritropic membrane activity in insects and intestinal brush border membrane bound hydrolases in animals. It accumulates in insect brain and other nervous tissues causing

neuro-endocraine disruptions. The main compound found *A. paniculata* is diterpene lactones called Andrographolide-B and Neo andrographolides. These compounds at low concentrations promote effective intercellular communications and cellular metabolism, but at high concentrations were known to cause serious disruptions to the normal timings of events in the cell cycle (Sinha *et al*., 2000). Since these compounds at high concentrations black cell cycle at G_0-G_1 phase, 52% decrease in the number of cells entering into the G_2-M phase and 30% in the S phase of the cell cycle, indicating that better cell cycle arrest was evident, moreover, after prolonged exposure to the diterpenoids the cells were dying after 48hrs treatment with andrographolides an increased population of the cells were found in the sub G_1 phase of the cell cycle and showed cytotoxicity and antiproliferative effects which have been shown to induce cell death at high concentrations (Chitrakala *et al*., 2004). Hence in the present study also reveals that larval and pupal mortality may be due to cell death and cellular disruptions which affected the development and survival and inflicted considerable larval and pupal mortality.

The larvicidal effects of *Bacillus thuringiensis* var *israelensis*

Studying on environmentally safer measures of mosquito control have been ongoing in India for a long time now. Biological control has been the dominant approach for the control of mosquito vectors because growing environmental concerns over the use of organo phosphorous larvicides has led to an increased use of bacterial larvicides such as Bti considered to be more environmentally sound. Bacterial larvicides were shown to be highly effective against *An.stephensi* and were extremely sensitive indicated by LC_{90} values. The mortality of the larvae increases as the doses of the samples were increased. The same trends were also observed in cause time elapse mortality. The fourth instars were less susceptible to the samples than 1st instars larvae. The mortality of the larvae was due to Cry proteins after ingestion are dissolved and activated under the alkaline action of the midgut and intestinal proteinases. The released toxins bind to receptors on the epithelial midgut cells, leading to swelling and lysis of the affected cells and eventually cause larval death (Georgiou and Wirth, 1997, Regis *et al*., 2001).

The results are favorably supported by the finding of Ana *et al*., (2007) evaluated the Bti against *Ae.aegypti* and results showed a LC_{50} of 0.26mg/L and a potency of 750 ITU/mg, and killed 98-100% of the larvae and the mortality remained high for six months in the shade. While Mulla *et al*.,(2001) reported the reduction in larvae was 87-98% for 7 weeks after first treatment at 200mg/m^2 resulting in a reduction of 24 to 73% (2 and 7 days post treatment)and 87 to98% (2-6 weeks) in the adults.

Synergistic activity of *Bacillus thuringiensis* var ***israelensis*** **with extracts of *A. paniculata* in different ratios.**

The entomopathogenic bacterium *Bacillus thuringiensis* var *israelensis* is one of the most commonly used biological control agent proving its efficacy against many mosquito species with no adverse effects on beneficial species. El-Hussini (1981), Burgerjon and Mastouret (1971) demonstrated that the susceptibility to Bti infection is

dependent upon two main factors; firstly the factors related to the insect species such as degree of the PH in both foregut and midgut and action of proteolytic enzymes. Secondly, the factors associated with the bacterial strains. Other effects include interference in RNA transcription, disturbance in ATP production and disequilibrium in membrane permeability. The use of Bti can be and should be advocated within an integrated vector management in combining with a botanical pesticide.

A combined approach would, on one hand, reduce the amount of the Bti formulation needed, thereby reducing costs, risk to non- target organisms, and the risks of resistance. And on the other, significantly decrease dependence on synthetic pesticides. In the larger context, this would translate into reduced pesticide contamination of the environment.

The results revealed that an apparent increase in efficacy of the combination between Bti and methanolic extracts of *A. paniculata* was evident from that of Bti and methanolic extracts of *A. paniculata* when each was used alone. The combination of Bti with methanolic extracts was greatly enhanced at different ratios. However, the larvicidal activity is minimal when the mixed formulations contained an equal amount of both constituents (i.e. a 1:1 ratio). The co-toxicity co-efficient and synergistic factors for ratio 1:4 were higher than for the other ratios of the mixed formulations tested. The results indicated that combinations of Bti with Ee extract had the most active larvicidal and pupicidal activity. A ratio of 1:4 of Bti and Ee of *A. paniculata* was 51.6 fold more toxic at the LC_{90} for larvicidal activity, than was Bti alone and this high level of activity resulted from synergism between the Bti and Ee extracts of *A. paniculata*. In this ratio, a possible explanations may be that the Bti crystal proteins paralyses the gut, and prevents further feeding and the neuro-toxic effect of plant compounds increases the toxicity combined with Bti crystals and caused abnormal symptoms such as sluggishness and circular movement observed in treated larvae indicated that increased toxic effect of combined treatments on the neuromuscular system. The present study is favorably supported by the findings of Schmutterer (1990) the combined application of neem derivatives and Bti caused increased larval and pupal mortality with significant synergistic factors. Ludlum *et al.,* (1991) have reported that aromatic compounds and plant allelochemicals increase Bti activity and caused swelling of the gut epithelial cells (Nasiruddin and Mordue, 1993).

Murugan (2006) demonstrated that *Glycosmis pantaphylla*, *Ocimum basilicum* and *Albizzia amara* significant synergism was noted with Bti; the synergistic factors was 1.5, 1.2 and 1.0 respectively. The results obtained herein were similar to those reported by Chiu shin-Foon (1990) demonstrated the efficacy of Bt with Ee of Derris roots on *Crocidolomia binotalis*, while Ramaprasad *et al.,* (1982) recorded the efficiency of Bt with aqueous extracts of *Pongamia glabla* on *Spodoptera litura*, while neem enhanced the action of Bt against *Spodoptera frugiperda* (Hellap and Zebitz 1986), furthermore, Murugan *et al.,* (2002) demonstrated that combination of neem oil and Pongamia oil with Bti resulted in a synergistic effect on larvae of *Cx.quinquefasciatus* and caused swelling of mitochondrial cristae and endoplasmic reticulum followed by enlargements of vacuoles; and condensations of the mitochondrial matrix.

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Table 1. Lethal concentration values of whole plant ethanolic extract of *Andrographis paniculata* Nees against different larval instars and pupae of *Anopheles stephensi* Liston treated with 24 and 48 hours.

Instars	Exposure hours	Regression equation	X^2	LC_{50} values (Fiducial limits) (ppm)	LC_{90} values (Fiducial limits) (ppm)
First instars	24	X=0.0935 Y=-2.9912	1.122	31.9876 (29.9437-33.6006)	45.6923 (43.8123-48.9693)
	48	X=0.0533 Y=-0.7784	4.038	14.5848 (9.4697-21.4221)	38.5964 (35.5760-42.1561)
Second instars	24	X=0.0906 Y=-3.0793	1.957	33.9761 (32.1541-35.4848)	48.1164 (46.6777-51.6051)
	48	X=0.0569 Y=-1.1374	2.462	19.9895 (10.8585-24.8649)	42.5121 (39.8690-46.3612)
Third instars	24	X=0.0815 Y=-2.9116	4.460	35.7216 (33.8914-37.2767)	51.4442 (49.0688-54.7235)
	48	X=0.0486 Y=-1.0264	2.245	21.1201 (11.6105-26.1279)	47.4901 (35.5283-53.1116)
Fourth instars	24	X=0.0778 Y=-2.8495	5.598	36.5909 (31.7136-40.0265)	53.0472 (48.0948-63.9925)
	48	X=0.0442 Y=-1.0026	0.961	22.2849 (12.5608-27.3063)	51.2668 (42.6081-50.6974)
Pupae	24	X=0.0963 Y=-2.6559	1.307.	38.3284 (29.2289-44.1906)	56.8161 (49.0699-88.9885)
	48	X=0.0382 Y =-0.8785	2.958	22.9713 (12.5608-27.3063)	56.4810 (51.0813-67.6172)

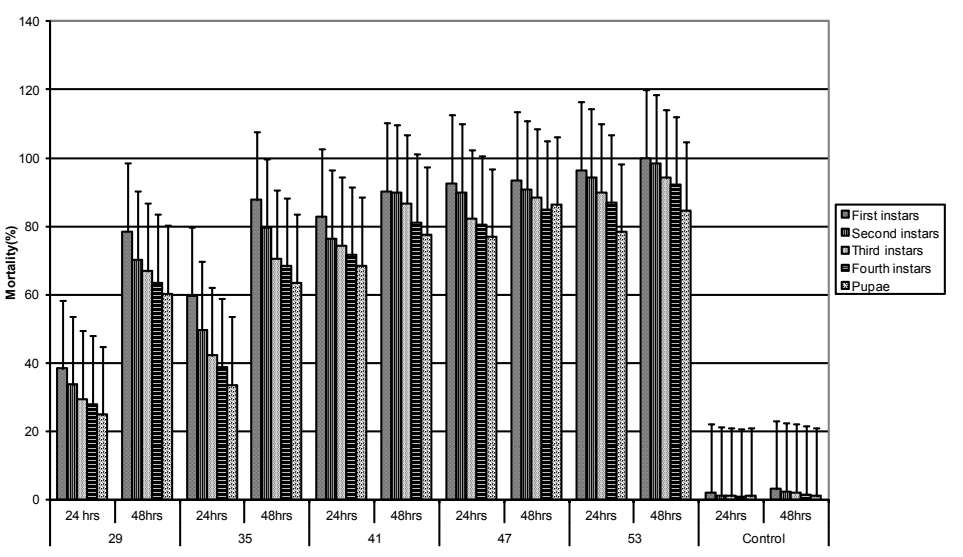

Fig.1. Mortality effects of whole plant ethanolic extract of *Andrographis paniculata* Nees against different larval instars and pupae of *Anopheles stephensi* Liston treated with 24 and 48 hours.

Table 2. Lethal concentration values of *Bacillus thuringiensis* var *israelensis* against different larval instars and pupae of*Anopheles stephensi* Liston treated with 24 and 48 hours.

Instars	Exposure hours	Regression equation	X^2	LC_{50} values (Fiducial limits) (ppm)	LC_{90} values (Fiducial limits) (ppm)
First instars	24	X= 5.5158Y= -0.3890	12.127	0.0173 (0.0105-0.0210)	0.0195 (0.0180-0.0215)
	48	X=9.1404 Y= -0.1824	4.668	0.1202 (0.0999-0.1544)	0.2252 (0.1369-1.0267)
Second instars	24	X=5.0831 Y= -0.1114	10.245	0.0197 (0.0119-0.0219)	0.0213 (0.0167-0.0243)
	48	X=6.9497 Y= -0.1783	4.391	0.1587 (0.1322-0.2021)	0.2740 (0.1787-1.0298)
Third instars	24	X= 4.1751Y= -0.2774	7.833	0.0429 (0.0297-0.0567)	0.0664 (0.0515-0.0802)
	48	X= 4.5793Y= -0.1924	4.872	0.2378 (0.1954-0.3119)	0.3734 (0.2499-1.0605)
Fourth instars	24	X=4.0014Y= -0.4074	8.457	0.0967 (0.0792-0.0153)	0.1003 (0.0893-0.01082)
	48	X= 4.1587Y= -0.0571	3.135	0.2944 (0.2422-0.3868)	0.4158 (0.2751-1.0893)
Pupae	24	X=4.0846 Y= -0.5045	10.259	0.1016 (0.1005-0.1070)	0.1235 (0.1112-0.0114)
	48	X=3.3209 Y= -0.0656	4.148	0.3661 (0.2922-0.5132)	0.4372 (0.2821-1.0979)

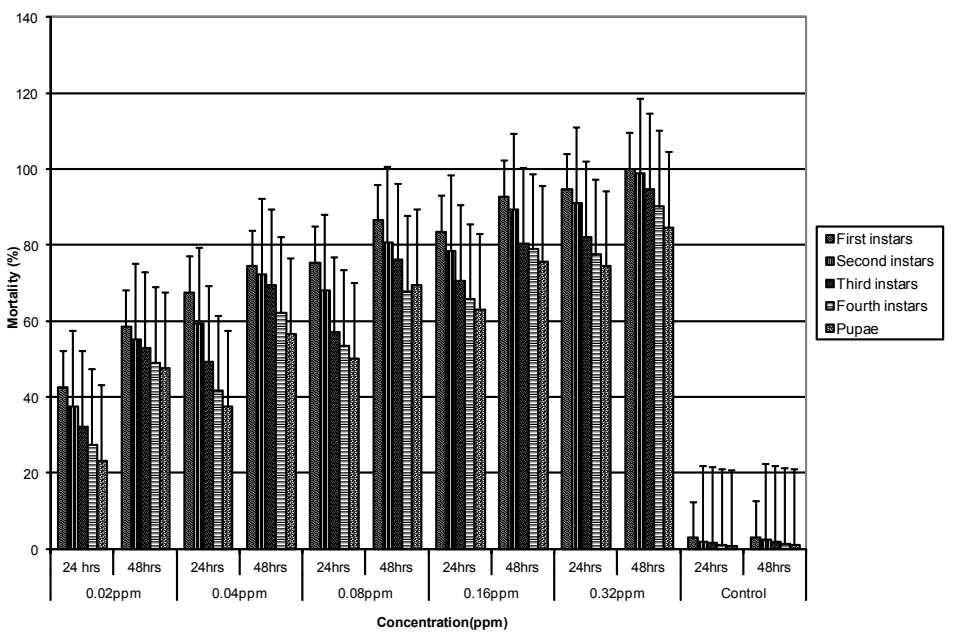

Fig. 2. Mortality effect of *Bacillus thuringiensis* var *israelensis* against different larval instars and pupae of *Anopheles stephensi* Liston treated with 24 and 48 hours.

Table 3. Lethal concentration and synergistic values of *Bacillus thuringiensis* var *israelensis* in combination with ethanolic extracts of *Andrographis paniculata* Ness against different larval instars and pupae of *Anopheles stephensi* Liston treated with 24 and 48 hours at 1:1 ratios.

Instar	Exposure hour	Regression equation	LC_{50} (Fiducial Limits)	CTC	Synergistic Factor	Type of action	X^2	LC_{90} (Fiducial Limits)	CTC	Synergistic Factor	Type of action
1st	24	Y=24.64481X=-0.20294	0.0008 (0.0002-0.0021)	2437.5	24.37	Synergistic	5.519	0.0060 (0.0046-0.0094)	3753.33	37.53	Synergistic
	48	Y=19.25931X=-0.71071	0.0006 (0.0001-0.0020)	2883.33	28.83	Synergistic	3.441	0.0029 (0.0020-0.0039)	4144.82	41.44	Synergistic
2nd	24	Y=20.5112X=-0.24742	0.0012 (0.0007-0.0027)	1775.00	17.75	Synergistic	6.693	0.0074 (0.0055-0.0138)	3702.70	37.02	Synergistic
	48	Y=16.73903X=-0.59019	0.0010 (0.0005-0.0024)	1970.0	19.7	Synergistic	0.589	0.0041 (0.0032-0.0053)	3870.73	38.70	Synergistic
3rd	24	Y=16.87384X=-0.38485	0.0022 (0.0012-0.0030)	3018.18	30.18	Synergistic	3.988	0.0098 (0.0084-0.0123)	3810.20	38.10	Synergistic
	48	Y=14.52749X=-0.42397	0.0020 (0.0017-0.0028)	2145.00	21.45	Synergistic	2.071	0.0059 (0.0048-0.0078)	4030.50	40.30	Synergistic
4th	24	Y=15.90116X=-0.56724	0.0035 (0.0027-0.0042)	2865.71	28.65	Synergistic	2.309	0.0116 (0.0098-0.0147)	3584.48	35.84	Synergistic
	48	Y=13.66047X=-0.29097	0.0031 (0.0024-0.0039)	3119.35	31.19	Synergistic	1.653	0.0072 (0.0059-0.0097)	4088.88	40.88	Synergistic
5th	24	Y=16.14309X=-0.63787	0.0039 (0.0032-0.0046)	3166.66	31.66	Synergistic	3.023	0.0118 (0.0100-0.0150)	3705.08	37.05	Synergistic
	48	Y=11.48896X=-0.22664	0.0034 (0.0027-0.0041)	3411.76	34.11	Synergistic	1.107	0.0091 (0.0073-0.0134)	4023.07	40.23	Synergistic

Table 4. Lethal concentration and synergistic values of *Bacillus thuringiensis* var *israelensis* in combination with ethanolic extracts of *Andrographis paniculata* Ness against different larval instars and pupae of Anopheles stephensi Liston treated with 24 and 48 hours at 1:2 ratios.

Instar	Exposure hour	Regression equation	LC_{50} (Fiducial Limits)	CTC	Synergistic Factor	Type of action	X^2	LC_{90} (Fiducial Limits)	CTC	Synergistic Factor	Type of action
1st	24	Y=24.64481X=-0.20294	0.0008 (0.0002-0.0021)	2437.5	24.37	Synergistic	5.519	0.0060 (0.0046-0.0094)	3753.33	37.53	Synergistic
	48	Y=19.25931X=-0.71071	0.0006 (0.0001-0.0020)	2883.33	28.83	Synergistic	3.441	0.0029 (0.0020-0.0039)	4144.82	41.44	Synergistic
2nd	24	Y=20.5112X=-0.24742	0.0012 (0.0007-0.0027)	1775.00	17.75	Synergistic	6.693	0.0074 (0.0055-0.0138)	3702.70	37.02	Synergistic
	48	Y=16.73903X=-0.59019	0.0010 (0.0005-0.0024)	1970.0	19.7	Synergistic	0.589	0.0041 (0.0032-0.0053)	3870.73	38.70	Synergistic
3rd	24	Y=16.87384X=-0.38485	0.0022 (0.0012-0.0030)	3018.18	30.18	Synergistic	3.988	0.0098 (0.0084-0.0123)	3810.20	38.10	Synergistic
	48	Y=14.52749X=-0.42397	0.0020 (0.0017-0.0028)	2145.00	21.45	Synergistic	2.071	0.0059 (0.0048-0.0078)	4030.50	40.30	Synergistic
4th	24	Y=15.90116X=-0.56724	0.0035 (0.0027-0.0042)	2865.71	28.65	Synergistic	2.309	0.0116 (0.0098-0.0147)	3584.48	35.84	Synergistic
	48	Y=13.66047X=-0.29097	0.0031 (0.0024-0.0039)	3119.35	31.19	Synergistic	1.653	0.0072 (0.0059-0.0097)	4088.88	40.88	Synergistic
5th	24	Y=16.14309X=-0.63787	0.0039 (0.0032-0.0046)	3166.66	31.66	Synergistic	3.023	0.0118 (0.0100-0.0150)	3705.08	37.05	Synergistic
	48	Y=11.48896X=-0.22664	0.0034 (0.0027-0.0041)	3411.76	34.11	Synergistic	1.107	0.0091 (0.0073-0.0134)	4023.07	40.23	Synergistic

Table 5. Lethal concentration and synergistic values of *Bacillus thuringiensis* var *israelensis* in combination with ethanolic extracts of *Andrographis paniculata* Ness against different larval instars and pupae of Anopheles stephensi Liston treated with 24 and 48 hours at 1:4 ratios.

Instar	Exposure hour	Regression equation	LC_{50} (Fiducial Limits)	CTC	Synergistic Factor	Type of action	X^2	LC_{90} (Fiducial Limits)	CTC	Synergistic Factor	Type of action
1st	24	Y=28.64646X=-0.09105	0.0003 (0.0001-0.0008)	6500.00	65.0	Synergistic	6.382	0.0047 (0.0035-0.0079)	4791.48	47.91	Synergistic
	48	Y=16.26645X=-0.21234	0.0002 (0.0001-0.0005)	8650.0	86.5	Synergistic	5.026	0.0018 (0.0012-0.0081)	6677.77	66.77	Synergistic
2nd	24	Y=25.82291X=-0.18088	0.0007 (0.0004-0.0010)	3042.85	30.42	Synergistic	6.105	0.0056 (0.0043-0.0091)	4892.85	48.92	Synergistic
	48	Y=25.87690X=-0.73527	0.0005 (0.0002-0.0015)	3940.0	39.4	Synergistic	0.620	0.0030 (0.0026-0.0085)	5290.0	52.9	Synergistic
3rd	24	Y=20.03347X=-0.27126	0.0015 (0.0010-0.0031)	4426.66	44.26	Synergistic	5.187	0.0077 (0.0067-0.0093)	4849.35	48.49	Synergistic
	48	Y=25.53522X=-0.45835	0.0012 (0.0009-0.0029)	3572.0	35.75	Synergistic	1.134	0.0047 (0.0042-0.0106)	5059.57	50.59	Synergistic
4th	24	Y=19.53661X=-0.48610	0.0024 (0.0016-0.0037)	4179.16	41.79	Synergistic	2.867	0.0090 (0.0078-0.0108)	4620.0	46.2	Synergistic
	48	Y=19.76839X=-0.37526	0.0019 (0.0014-0.0035)	5089.47	50.89	Synergistic	0.754	0.0057 (0.0050-0.0110)	5164.91	51.64	Synergistic
5th	24	Y=17.88561X=-0.47809	0.0029 (0.0019-0.0043)	4258.00	42.58	Synergistic	2.916	0.0098 (0.0084-0.0120)	4461.22	44.61	Synergistic
	48	Y=17.82586X=-0.33911	0.0025 (0.0016-0.0041)	4640.0	46.4	Synergistic	1.927	0.0069 (0.0051-0.0122)	5305.79	53.05	Synergistic

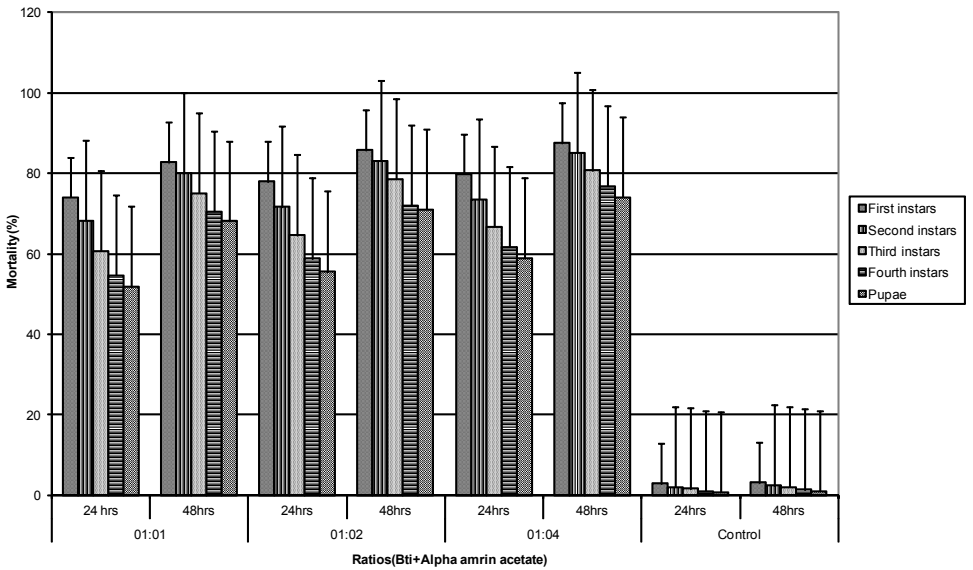

Fig. 3. Cumulative mean mortality of various instars of *Anopheles stephensi* Liston treated in combination of *Bacillus thuringiensis* var *israelensis* and ethanolic extract of *A. paniculata* in different ratios.

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