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Integrated control and prevention of malaria (*Anopheles stephensi*) and dengue (*Aedes aegypti*) vectors with plant extracts through ether, insecticides and BTI

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Abstract

Mosquitoes act as life threatening disease vectors. Due to non-availability of vaccine and treatment for most of these diseases, the only solution is to control the mosquitoes. The continuous application of synthetic insecticides causes development of resistance (in vector species), biological magnification (of toxic substances through the food chain) and adverse effects (on environmental quality and non-target organisms including human health). So, under the Integrated Mosquito Management (IMM), emphasis is given on the application of alternative strategies in mosquito control such as use of insecticides, plant extracts and *Bti*. Mosquito larvae were collected from different habitats and brought for identification. After identification, *Anopheles* and *Aedes* mosquitoes were reared separately and treated with different plant extracts, growth regulators and *Bti*. Plant extracts through ether and insecticides and *Bti* were tested in combination to test their efficacy against *Anopheles* and *Aedes* larvae. Again mortality data was collected and subjected to probit analysis to calculate LC_{50}. The least value of LC_{50} (1.3-40 ppm) observed with solution of ether extracts, *Bti* and insecticides for *Anopheles* and *Aedes* larvae. By adopting these techniques we should able to manage the populations of *Anopheles* and *Aedes* in the environment.

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Introduction

Insect vectors, especially mosquitoes are responsible for spreading serious human diseases like malaria, Japanese encephalitis, yellow fever, dengue and filariasis (Ghosh *et al*., 2012). There are different kinds of mosquitoes in the world but medically three genera are important, i.e., *Anopheles*, *Aedes* and *Culex* (Rathy *et al*., 2015). *Anopheles* mosquitoes are responsible for malarial disease in human beings that is at the top in vector borne diseases (Govindarajan and Sivakumar, 2011). Malaria is endemic in 91 countries, with about 40% of the world population at risk. In 2013, an estimated 198 million cases of malaria occurred worldwide with 500,000 deaths, mostly children in the African region (Anonymous, 2014). *Culex* is responsible for malaria in animals and West Nile virus in human beings. *Aedes* mosquitoes are important vectors of dengue fever, lymphatic filariasis, chikungunya, yellow fever and Zika virus. There are more than 50 million cases of dengue infection worldwide each year (Sinha *et al*., 2004). Dengue hemorrhagic fever affects about 500,000 patients worldwide (WHO, 2011). As these diseases are mostly viral, so, there is neither a proper vaccine nor treatment is available for most of these diseases. So, the only solution is to manage the mosquito population(Abbas *et al*., 2013).Various synthetic products and devices designed to combat such vectors are not successful because of the increased resistance developed by various mosquito species. Moreover, most of the mosquito repellent formulations available in the market are mainly prepared with active ingredients of synthetic origin.

The continuous application of synthetic insecticides causes development of resistance in vector species, biological magnification of toxic substances through the food chain and adverse effects on environmental quality and non-target organisms including human health. Due to the effect of these substances, the human body immune system becomes weak against the diseases. Long term exposure of new born babies and children to parathyroid based mosquito repellents is known to cause clinical, biochemical and neurological changes (Sinha *et al*., 2004). Therefore, alternative methods of control are needed. For this purpose, a new strategy IMM (Integrated Mosquito Management) is introduced for the management of mosquitoes. Under the Integrated Mosquito Management (IMM), emphasis is given on the application of alternative strategies in mosquito control such as use of selective chemicals, plant extracts and *Bti*. Sensible control strategies involve reducing breeding sites which provides long term control for mosquito populations by controlling mosquito populations during aquatic stages. Larviciding allows control measures to be conducted over the smallest possible area when mosquitoes are concentrated in breeding pools, before adults spread throughout the community. Larval and adult mosquitoes have primarily been controlled with a variety of chemical pesticides.

The emergence of resistance in insect populations to chemical pesticides has led to increased interest in biological control agents, including some naturally occurring entomopathogenic bacteria, such as *Bacillus thuringiensis israelensis* (*Bti*) and plant extracts. These two biological insecticides, due to their environmental safety and specificity to nematoceran Diptera (especially mosquitoes), have become mosquito control agents of choice almost throughout the world (Becker, 1998; Fillinger *et al*.,2003). Along with the, use of growth regulators and selective chemicals is safe method (Ghosh *et al*., 2012).

In Punjab Province, mosquitoes belonging to genera *Culex, Anopheles* and *Aedes* are commonly found. Large populations of these mosquitoes occur almost year round. Among these, *Anopheles* spp. not only causes a biting nuisance primarily in summer months but is also a serious threat to public health due to their potential for malaria transmission. About ¼th of Punjab population is at risk of malaria (Qasim *et al*., 2014). Punjab is the populous province of Pakistan. Since 2010, dengue out breaks occurs every year in Punjab especially during rainy season when the conditions are conducive for mosquito breeding. Dengue fever was first reported during 1994 and a

huge epidemic occurred in Punjab province during 2011 with 100 confirmed cases daily (Shakoor *et al.*, 2012). Punjab is an agricultural province and different types of trees, herbs, and shrubs are present here. So, this study is proposed to evaluate our local weeds, herbs and shrubs separately and in combination with *Bti* and growth regulators.

Materials and methods

The present study was carried out to assess the larvicidal activity of oil extracts of plants parts, commonly used commercial insecticides and *Bti* in combination. The study was carried out in the following steps:

Mosquito larvae collection and rearing

Mosquito larvae were collected with dipper from different sites such as industrial, non-industrial, sewerage, ponds, agriculture fields and fish ponds were brought to the entomology laboratory in the Department of Zoology, Government College University Faisalabad for rearing (Nasir *et al.*, 2015).

After rearing in glass cages, the adults were identified using standard manual (Qasim *et al.*, 2014) up to genus level. *Anopheles* (*An. Stephensi* &*An. culicifacies*) and *Aedes* (*Ae. aegypti*&*Ae. albopictus*) genera were reared separately. Male mosquitoes were fed 10% sugar solution while female with rat blood (Nasir *et al.*, 2017). After egg lying, hatched larvae (2^{nd}& 3^{rd}) were used for this study. Twenty larvae of each group were introduced into beakers separately containing 250 ml of their water along with fish diet.

Collection of plant samples and handling

Plant samples were selected based on their local availability and insecticidal properties. The plant parts were collected from healthy plants, free from dust, dirt and other impurities. These experimental materials were brought to the entomology laboratory in the Department of Zoology, Government College University Faisalabad for subsequent processing.After washing, the material was dried under shade and then oven dried (Alkan *et al.*, 2015). Dried Plant material was transferred into powder form by using a grinder. Then this powder was used for oil solution extraction.

Oil Extraction

Essential oils were extracted from the selected plant parts with the help of Soxhlet apparatus (Cheng *et al.*, 2009). Fifteen grams powder of each plant material with 200 ml of solvent (petroleum ether) was used for 8 to 24 hrs to extract oil through Soxhlet apparatus.

After extraction, vacuum evaporator was used to evaporate solvent to attain filtrate in dehydrated form, which was then stored in eppendorf for further use. Different concentrations (10, 1.0, 0.1, 0.01, 0.001 & 0.0001%) were formulated in the respective solvent petroleum ether (Nasir *et al.*, 2017).

Synthetic insecticides

Deltamethrin, Mortein liquid, and *Bti* were used for this study.

Mixing Treatments

In this study, plant extracts, *Bti* and significant insecticides were used in combination.

Again 6 concentrations of each combination with 3 replications of each were used along with control group. Mortality data was calculated to check knock down effect after 2, 4, 8, 16, 32, 64, 128 hrs.

Data collection

Data of mortality was collected with carefully. All the reading was noted after the interval of 2, 4, 8, 16, 32, 64, 128 hrs for mixing trials.

Statistical analysis

The mortality observed was corrected using Abbott's formula during the observation of the larvicidal potentiality of the plant extracts. After recording mortality data, it was subjected to ANOVA for determining the significant plants and insecticides.

Statistical analysis of the experimental data was performed with MS Excel 2007 to find the Mean and Standard deviation, LC_{50} using probit analysis with Minitab.

Results

This information is critical for the discovery of new safe techniques to control the mosquito population. This study highlights the potential of plant extracts, *Bti* and chemicals for dengue and malarial vector control and underlines several important entomological parameters that should be quantified in a proof of concept clinical trials in order to effectively determine the impact of plant extracts, *Bti* and selected insecticides.

Experiments were conducted on larvae of mosquito reared in the entomology laboratory of Zoology Department at the GovernmentCollege University Faisalabad. Seven sets of beakers (One for control and six for treatments) were taken and placed on a laboratory shelves.

Control set was applied for environmental effects and no check set was taken because the solutions were prepared in petroleum ether. The plant extracts were applied on beakers containing 20 larvae and different concentration (10%, 1.0%, 0.1%, 0.01%, 0.001% and 0.0001%) with 3 replications. The beaker contains 50 ml water from habitat and 199 ml of tab water and 1 ml of prepared solution for the trial.

Table 1. LC_{50} (Mean±SE) at different time intervals for significant plant extracts through ether with significant insecticides and *Bti* against *Anopheles* larvae.

Sig. Plants Vs Sig. Insecticides, *Bti*	2 hrs	4 hrs	8 hrs	16 hrs	32 hrs	64 hrs
Kor tumma *(Ether)* - sig. inse. *Bti,*	175.50±2.00	142.60±1.70	112.79±1.55	79.40±1.33	49.46±0.79	24.414±0.138
Datura leaf (Ether) - sig. inse. *Bti,*	177.40±2.10	149.10±1.62	119.90±1.21	82.46±0.66	52.37±.44	27.080±0.034
Charita (Ether) - sig. inse. *Bti,*	197.70±4.05	170.70±2.41	140.99±1.93	103.47±1.01	71.15±0.56	41.070±0.030
Neem leaf (Ether) - sig. inse. *Bti,*	182.20±3.00	162.40±2.06	130.97±1.46	99.41±1.03	66.15±1.80	36.109±0.042
Kor tumma, datura leaf (Ether) - sig. inse. *Bti,*	128.10±2.00	102.30±1.67	70.97±1.26	48.90±1.04	29.21±0.79	4.350±0.108
Kor tumma, charita (Ether) - sig. inse. *Bti,*	193.60±1.20	161.60±0.75	131.70±0.50	98.75±0.31	71.50±0.26	40.251±0.088
Kor tumma, neem leaf (Ether) - sig. inse. *Bti,*	182.30±2.20	162.50±1.81	132.61±1.59	103.97±1.11	81.79±0.85	50.134±0.049
Datura leaf, charita (Ether) - sig. inse. *Bti,*	185.20±3.10	164.10±2.06	137.44±1.74	106.53±1.35	83.95±0.91	52.082±0.033
Datura leaf, neem leaf (Ether) - sig. inse. *Bti,*	181.50±3.20	159.20±2.74	129.78±1.69	105.02±1.30	82.80±1.05	50.130±0.055
Charita, neem leaf (Ether) - sig. inse. *Bti,*	192.40±1.00	172.80±0.63	130.05±0.49	94.34±0.26	72.99±0.17	40.273±0.121
Kor tumma, datura leaf, charita (Ether) - sig. inse. *Bti,*	172.10±2.00	152.35±1.64	122.56±1.20	88.24±0.92	64.38±0.75	30.586±0.205
Datura leaf, charita, neem leaf (Ether) -, sig. inse. *Bti*	180.20±1.00	155.51±0.66	129.83±0.43	98.79±0.24	68.18±0.16	34.540±0.207
Kor tumma, datura leaf, charita, neem leaf (Ether) - sig. inse. *Bti,*	122.00±0.90	100.44±0.63	62.83±0.44	32.13±0.25	8.21±0.18	1.317±0.482

LC_{50} of significant plant extracts through ether with significant insecticides and *Bti* against *Anopheles* larvae.

Mixing trials for the larvae of mosquito were conducted in the Entomology laboratory of Zoology Department at the Govt College University Faisalabad and Ayub Agriculture research institute, Faisalabad. For the mixing trials, different plant extracts with petroleum ether, *Bti* and selected Insecticides were used.

The LC_{50} was observed of *Anopheles* and *Aedes* with different concentrations of plant extracts, *Bti* and Insecticides at different time intervals. During the mixing trials, the LC_{50} of different 2nd and 3rd instar larvae of*Anopheles* and *Aedes*in different concentrations of plant oil extracts at different treatmenttime intervals were noted.

Table (1) showed that the LC_{50} values for with kor tumma, significant insecticides and *Bti* against *Anopheles* larvae was (175.50±2.00) ppm after 2 hours that decreased after the interval of 64 hours to (24.414 ± 0.138) ppm. The LC_{50} for datura leaf, significant insecticides and *Bti* was (177.40±2.10) ppm after 2 hours that also decreased after 64 hours to (27.080±0.034) ppm while this value for charita, significant insecticides and *Bti* was (197.70±4.05) ppm after 2 hours that decreased after 64 hours to (41.070±0.030) ppm. The LC_{50} value for neem leaf, significant insecticides and *Bti* was (182.20±3.00)

ppm after 2 hours that also decreased after 64 hours to (36.109±0.042) ppm while the LC_{50} value for kor tumma, datura leaf, significant insecticides and *Bti* was (128.10±2.00) ppm after 2 hours that also decreased after 64 hours to (4.350±0.108) ppm. The LC_{50} value for kor tumma, charita, significant insecticides and *Bti* was (193.60±1.20) ppm after 2 hours that also decreased after 64 hours to (40.251±0.088) ppm. The LC_{50} value for kor tumma, neem leaf, significant insecticides and *Bti* was (182.30±2.20) ppm after 2 hours that also decreased after 64 hours to (50.134±0.049) ppm. The LC_{50} value for datura leaf, charita, significant insecticides and *Bti* was (185.20±3.10) ppm after 2 hours that also decreased after 64 hours to (52.082±0.033) ppm. The LC_{50} value for datura leaf, neem leaf, significant insecticides and *Bti* was (181.50±3.20) ppm after 2 hours that also decreased after 64 hours to (50.130±0.055) ppm. The LC_{50} value for charita, neem leaf, significant insecticides and *Bti* was (192.40±1.00) ppm after 2 hour that also decreased after 64 hours to (40.273±0.121) ppm. The LC_{50} value for kor tumma, datura leaf, charita, significant insecticides and *Bti* was (172.10±2.00) ppm after 2 hours that also decreased after 64 hours to (30.586±0.205) ppm.

Table 2. LC_{50} (Mean±SE) at different time intervals for significant plant extracts through ether with significant insecticides and *Bti* against *Aedes* larvae.

Sig. Plants Vs Sig. Insecticides, *Bti*	2 hrs	4 hrs	8 hrs	16 hrs	32 hrs	64 hrs
Kor tumma *(Ether)* - sig. inse. *Bti,*	192.40±2.50	162.60±1.70	132.79±1.65	89.40±1.36	57.46± 0.84	29.424± 0.148
Datura leaf (Ether) - sig. inse. *Bti,*	187.80±2.00	165.10±1.62	132.90±1.21	92.46±0.66	67.37±.44	37.080±0.034
Charita (Ether) - sig. inse. *Bti,*	217.50±3.00	188.60±2.41	155.69±1.93	113.57±1.00	82.15±0.56	51.070±0.030
Neem leaf (Ether) - sig. inse. *Bti,*	202.40±3.20	182.40±2.09	145.87±1.46	109.31±1.03	77.15±1.80	41.109±0.042
Kor tumma, datura leaf (Ether) - sig. inse. *Bti,*	140.10±2.50	112.40±1.77	77.77±1.36	58.90±1.02	35.21±0.79	6.430±0.108
Kor tumma, charita (Ether) - sig. inse. *Bti,*	213.40±1.20	174.60±0.75	143.50±0.50	106.55±0.31	78.50±0.26	45.151±0.068
Kor tumma, neem leaf (Ether) - sig. inse. *Bti,*	200.30±2.30	162.50±1.81	132.61±1.59	99.97±1.21	69.79±0.75	38.124±0.052
Datura leaf, charita (Ether) - sig. inse. *Bti,*	198.20±3.10	156.10±2.26	129.44±1.74	96.53±1.35	64.85±0.71	36.082±0.033
Datura leaf, neem leaf (Ether) - sig. inse. *Bti,*	199.80±3.40	172.20±2.64	142.78±1.89	115.02±1.40	79.80±1.35	44.130±0.055
Charita, neem leaf (Ether) - sig. inse. *Bti,*	220.20±1.30	189.60±0.83	143.55±0.69	104.34±0.46	79.99±0.27	43.173±0.131
Kor tumma, datura leaf, charita (Ether) - sig. inse. *Bti,*	189.20±2.40	165.35±1.84	134.56±1.00	96.24±0.82	71.28±0.65	33.586±0.205
Datura leaf, charita, neem leaf (Ether) - sig. inse. *Bti,*	195.70±1.80	170.81±0.96	141.73±0.63	106.59±0.34	74.48±0.26	37.540±0.237
Kor tumma, datura leaf, charita, neem leaf (Ether) - sig. inse. *Bti,*	134.10±1.10	110.44±0.63	68.83±0.54	35.13±0.25	8.81±0.18	1.817±0.482

LC_{50} of significant plant extracts through ether with significant insecticides and *Bti* against *Aedes* larvae.

The LC_{50} value for datura leaf, charita, neem leaf, significant insecticides and *Bti* was (180.20±1.00) ppm after 2 hours that also decreased after 64 hours to (34.540±0.207) ppm. The LC_{50} value for kor tumma, datura leaf, charita, neem leaf, significant insecticides and *Bti* was (122.00±0.90) ppm after 2 hours that also decreased after 64 hours to (1.317±0.482) ppm.From this table we can note that the solution having mixture of kor tumma, datura leaf, charita, neem leaf, significant insecticides and *Bti* had less LC_{50} value that means this mixture of kor tumma, datura leaf, charita, neem leaf, significant insecticides and *Bti* was more potent and played a significance role in mortality as shown in the table.

Table (2) showed that the LC_{50} values for with kor tumma, significant insecticides and *Bti* against *Aedes* larvae was (192.40±2.50) ppm after 2 hours that decreased after the interval of 64 hours to (29.424 ± 0.148) ppm. The LC_{50} for datura leaf, significant insecticides and *Bti* was (187.80±2.00) ppm after 2 hours that also decreased after 64 hours to (37.080±0.034) ppm while this value for charita, significant insecticides and *Bti* was (217.50±3.00) ppm after 2 hours that decreased after 64 hours to (51.070±0.030) ppm. The LC_{50} value for neem leaf, significant insecticides and *Bti* was (202.40±3.20) ppm after 2 hours that also decreased after 64 hours to (41.109±0.042) ppm while the LC_{50} value for kor tumma, datura leaf, significant insecticides and *Bti* was (140.10±2.50) ppm after 2 hours that also decreased after 64 hours to (6.430±0.108) ppm. The LC_{50} value for kor tumma, charita, significant

insecticides and *Bti* was (213.40±1.20) ppm after 2 hours that also decreased after 64 hours to (45.151±0.068) ppm. The LC_{50} value for kor tumma, neem leaf, significant insecticides and *Bti* was (200.30±2.30) ppm after 2 hours that also decreased after 64 hours to (38.124±0.052) ppm. The LC_{50} value for datura leaf, charita, significant insecticides and *Bti* was (198.20±3.10) ppm after 2 hours that also decreased after 64 hours to (36.082±0.033) ppm. The LC_{50} value for datura leaf, neem leaf, significant insecticides and *Bti* was (199.80±3.40) ppm after 2 hours that also decreased after 64 hours to (44.130±0.055) ppm.

The LC_{50} value for charita, neem leaf, significant insecticides and *Bti* was (220.20±1.30) ppm after 2 hour that also decreased after 64 hours to (43.173±0.131) ppm. The LC_{50} value for kor tumma, datura leaf, charita, significant insecticides and *Bti* was (189.20±2.40) ppm after 2 hours that also decreased after 64 hours to (33.586±0.205) ppm. The LC_{50} value for datura leaf, charita, neem leaf, significant insecticides and *Bti* was (195.70±1.80) ppm after 2 hours that also decreased after 64 hours to (37.540±0.237) ppm. The LC_{50} value for kor tumma, datura leaf, charita, neem leaf, significant insecticides and *Bti* was (134.10±1.10) ppm after 2 hours that also decreased after 64 hours to (1.817±0.482) ppm.

From this table we can note that the solution having mixture of kor tumma, datura leaf, charita, neem leaf, significant insecticides and *Bti* had less LC_{50} value that means this solution of kor tumma, datura leaf, charita, neem leaf, significant insecticides.

Discussion

This study introduced the comparison between the biological and chemical control against the mosquito with the use of plant extracts, significant insecticides and *Bti*. This information is critical for the discovery of new safe techniques to control and prevention of dengue and malaria vectors. This study highlighted the potential of plants, insecticides and *Bti* for mosquito control and hence, the prevention of dengue and malaria vectors. Thus this study underlined several important entomological parameters that should be quantified in order to effectively determine the impact of plants, insecticides and *Bti*.

Biochemical mechanisms allow a mosquito to survive insecticide exposure and thus threaten to dengue and malaria control. However, the threat of resistance increased day by day then need to new biological way to control the generation of mosquito. The aim of this research was therefore to identify important biological factors to control and prevent the dengue and malaria vectors.Plant extracts through petroleum ether with insecticides and *Bti* have played a major role for the effective control of dengue and malaria vectors.

The plant extracts through etherproved themselves as highly toxic to mosquito larvae (2^{nd}& 3^{rd} instars larvae) and this response was time and concentration dependent in all larval stages. After 64 hrs, the least LC_{50} value (1.3 & 1.8ppm) were observed that the mixture of plant extracts *C. colocynthis, D. stramonium*, *A. indica* and *Swertia chirayaita* through ether with *Bti* and significant insecticides for *Anopheles* and *Aedes* larvae respectively. These results are in line with the Chang *et al.* (2014) who observed that mixtures of *Bti* and oil major constituents (E)-anethole (AN), (E)-cinnamaldehyde (CA), and eugenol (EU; 1:1 ratio) CA, AN, or EU were significantly more toxic against *Ae. albopictus* larvae (0.0084, 0.0134, and 0.0237 mg/liter) and *An. stephensi* larvae (0.0159,0.0388, and 0.0541 mg/liter) than either *Bti* (1.7884 and 2.1681 mg/liter) or CA (11.46 and 18.56 mg/liter), AN (16.66 and 25.11 mg/liter), or EU (24.60 and 31.09 mg/liter) alone. These results are also in the agreement of Mansour *et al.*, (2012) who observed *Bacillus thuringiensis israelinsis* (*Bti*) and *Bacillus sphaericus* 2362 (*Bs*), either singly or in combination with plant oils and commercial insecticides, was tested against larval and adult stages of *Culex pipiens* mosquitoes under controlled laboratory conditions. In terms of LC_{50} values recorded after 24, 48, 72 and 96 hrs, the bacterial toxins showed high potency towards both

larvae and adults of mosquitoes in a dose-dependent manner. Generally, the *Bti* toxin seemed to be more potent than the *Bs* toxin. For example, the *Bti* toxin showed a 24 hrs LC_{50} of 8.2 ppm against mosquito larvae compared to 13.6 ppm for the *Bs* toxin. In the adult bioassay, the obtained 24 hrs LC_{50} values were 0.064 and 0.085 mg/cm2, respectively for the two bacterial toxins. These findings are in agreement with the Mansour *et al.* (2015) who also observed *Bacillus thuringiensis israelinsis* (*Bti*) and *Bacillus sphaericus* 2362 (*Bs*) were tested either singly or in combination with plant oils and chemical insecticides against the larvae and adults of *Musca domestica* in laboratory. Based on the 24hrs-LC_{50} values, the essential oil from Apium *graveolens* showed the highest toxicity against the house fly larvae (59.4 ppm), while *Petroselium crispum* oil was the highest toxic to the fly adults (0.62 ppm). The *Bti* toxin proved to be more toxic than the *Bs* one.

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