Invitro larvicidal investigations of various organic synergize based crude formulation of indigenous plant (*Melia azadrich*) against deadly Dengue vectors mosquitos.

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ABSTRACT: The Aedes genus of mosquitoes is comprised of major vectors for many human diseases such as dengue, dengue hemorrhagic fever, chikungunya, etc., causing millions of deaths every year. Currently, no effective vaccines are available globally in the medical field; therefore, entomologically, the whole reliance is on vector control. Chemical control is effective and is used in emergency outbreaks, but due to the dangers it poses to human health, the environment, and vector behavioural resistance, more emphasis is being placed on environmentally friendly control methods other than synthetic chemicals. As an alternative source for synthetic chemicals, the use of easily degradable plant-based compounds is considered one of the safest and cheapest approaches to insect pest control, particularly for dengue vectors. Therefore, research on the evaluation of indigenous plant-based biopesticides (*Melia azadrich*) against the deadly vector mosquitoes was carried out under the in vitro conditions of the medical entomology laboratory, plant protection division, and Nuclear Institute of Food and Agriculture (NIFA), Peshawar, during the year 2022. For this purpose, four formulations, viz., sodium bicarbonate (70% inner matter + 20% catalyst + 10% active ingredient), sand (70% sand + 20% catalyst + 10% active ingredient), mixed (35% sand + 35% soda + 20% catalyst + 10% active ingredient), and catalytic formulation (66% catalyst + 24% soda + 10% active ingredient) were tested under laboratory conditions against the wild vector population collected from the field. Temephos, an organophosphate commonly used as a larvicide by the KP public health department, was used as a control as a standard check. Thirty larvae were placed in each of the 248 mL soft drinks and 2 mL of the desired solution in disposable cups, with 4 replications for each set of bioassays. Each trail maintained a consistent larval diet for each replication and control. The percentage of mortality for each treatment of the formulation was recorded under standard laboratory conditions (272C0, 60% R.H.). The formulation of PF04 as inert matter combined with an increased synergist amount and $NAHCO_3$ was found to have the best term for required mortality (90–100%), followed by the mix and sand formulations, respectively. The knockdown times were classified in order of 24 h, 28 h, and 72 h, respectively, for all the formulations. These in vitro investigations were helpful in selecting the best carrier (NaHCO3) as inert matter in combination with an effective organic synergist and thus can be helpful in developing plant-based biopesticides.

Key words: Dengue, vectors, bio pesticides, environment friendly.

INTRODUCTION

Mosquitoes are the world's most dangerous creatures and are annoying pests as well as lethal vectors (Paul *et al.*, 2022; Khan *et al.*, 2022), and they are present in more than 128 states. Up to 400 million people worldwide contract dengue each year, and 22,000 die from the virus's severe forms (Sharif *et al.*, 2022; Ahmad *et al.*, 2017). According to World Health Organization estimates (WHO, 2017, 2018), there are an estimated 219 million instances of vector-borne diseases, with dengue cases exceeding 2.5 billion, particularly in metropolitan areas of temperate zones worldwide (Monisha *et al.*, 2020). Due to disease-carrying arthropods, the world's population is at risk and has increased in proportion (Khan *et al.*, 2022).

The main strategy for stopping the spread of dengue infections is mainly through the control of Aedes mosquitoes. Spraying of chemical pesticides both indoors and outdoors is an important part of controlling Aedes mosquitoes, while chemical (such as Temephos) or biological methods are used to control larvae (Zhou *et al.*, 2021). Various methods used for eradicating mosquitoes depend on the target species' egg, larval, pupal, and adult stages (Tiwary *et al.*, 2007). The mosquito’s population usually rises with rising temperatures, and perhaps more breeding sites will be available in the rainy season (Pusawanget *et al.*, 2022). The breeding of Aedes in individual houses is, however, greatly influenced by human activities involving water storage and the removal of potentially water-holding containers, which may result in the supply of breeding habitats all year round (Rattanarithikul *et al.,* 2016). The amount of sunshine exposure, the frequency of water replenishment, and the availability of food for the larvae are important variables that may affect Aedes productivity in various container types. (Choochote *et al*., 2013) and WHO (2016) have therefore placed emphasis on the container coverings. These vectors' adaptability to urban residential settings has allowed them to use a variety of artificial containers, and they are also able to take advantage of possible breeding water that is either indoors or outside (Sriwichai, 2017; Aikpon, 2013).

Source reduction is frequently used as the first line of defense against *Aedes* mosquitoes; however, when holders can't be taken out or purged, larvicides (synthetic compounds) are utilized. The commonly used pesticides, spinosad and deltamethrine, are applied routinely (Marcombe, 2014). The insecticide sprays assume a significant role in controlling dengue (Kayshik, 2019). Controlling the rearing site of juvenile hatchlings is a compelling strategy for crisis management that leaves no room for resistance development. However, the use of synthetic larvicide imposes threats not only to human health but also to the ecosystem because when they are applied to the environment directly or indirectly, the public is ultimately affected (Mathivanan *et al.,* 2010). The discovery of synthetic insecticides such as dichlorodiphenyltrichloroethane (DDT) in 1939 sidetracked the application of phytochemicals in mosquito control programs (Ghosh, 2012). DDT, probably the best known and most useful insecticide in the world, has damaged wildlife and had negative effects on human health (Turusov *et al.,* 2002). Sadly, after using the same poisons for more than 40 years, mosquitoes and other insect pests have developed resistance to various groups of pesticides (Grisales *et al.,* 2013; Vatandoost *et al.,* 2021). Populations of *A. aegypti* have been found to be resistant to permethrin and/or deltamethrin across Thailand (Splernsub *et al.,* 2016). Aedes species have also developed resistance in Ghana (Owusu-Asenso2022) by using the same insecticides and species (Jirakanjanakit, *et al.,* 2014). Similar to this, DDT was investigated and shown to have a status of resistance to lethal vectors (Suzuki *et al.,* 2016). Synthetic chemicals have thus been ineffective in controlling the Aedes species. Different insect species have various pesticide protection mechanisms (Felix *et al.,* 2021). Insecticides used in the public health sector have been discovered to be resistant to them in earlier studies, and they also negatively affect environmental factors (Manzoor *et al.,* 2021).

These issues have made it more apparent that innovative management methods for mosquito larvae are required. Our focus is to develop environment friendly strategies to evaluate biopesticides as an alternative to chemical pesticides for mosquito control at breeding sites to save other useful ecosystem organisms. For this purpose, we selected the effective wild plant (*Melia azadrich*) based on our previous research studies (Khan *et al.*, 2014). The development of a friendly environmental strategy through the exploitation of indigenous plant-based means is a good option for the control of deadly vector mosquitoes at their larval stage. However, the effective formulation following larvicidal properties was a major issue, which was addressed in the current study in order to find a suitable inert matter combined with an effective synergist for an effective formulation to be eventually applied in the field. We hope that this technique, in the form of a biopesticide formulation, will be safe for other fauna in the aquatic ecosystem of mosquitoes and will effectively suppress the deadly mosquitoes.

MATERIALS AND METHOD

The qualified larvicide Temephos chemical were checked in comparison with synergizes based various formulations of *Melia azadrich* plant against the *Aedes agypti* larvae. Invitro experiments were conducted in relative to both standard and control. Each experiment was replications four times for the confirmation of the results and maintenance of accuracy.

Mosquitoes rearing

The *A. aegypti* L. larvae were collected from the dengue hot sport region of district Peshawar during indoor spray campaign and identified culture were established in the medical entomology laboratory of NIFA. The larvae were feed with NIFA larval diet; pupae were isolated with small plastic pipette and transferred into adults rearing cages. The adults were feed with 10% sugar solution and *albino* mouse for blood feeding. Ovi-cups were exposed in the cages after the blood meal to collect the eggs. The ovi-cups with eggs were kept in the hatching jars and after hatching the first instars larvae were poured in to larval trays. The larval trays were maintained at ($27\pm2C^0$) with relative humidity of 65 %. The subsequent 3^{rd} and $4^{th\ instars}$ larvae were used for the bioassay trials of the various formulation of the plant based biopesticide.

Preparation of Formulations

The inner matter (1, 2, 3, and 4) under examination was obtained from a nearby departmental store, and the organic synergize was supplied by Rayon Pest Control firm at a nearby branch. The local plant bunches were gathered, and the seeds were then separated. The leaves were kept for drying in the shade and pulverized in an electrical stainless-steel blender before being sieved to obtain a fine powder from which the extract was made. Four distinct formulations were created using varied catalyst concentrations and the same amount of dried powder as the active ingredient. For testing the inner matter test with different ratios of inner matter and catalyst, 10% plant powder was combined as an active ingredient in each formulation. Bioassays were carried out according to WHO, 2005 guidelines and instructions.

Inner matter tests

For making an effective formulation in the form of granules, dunk etc. various amount of synergize and inner matter in making the formulation were kept for drying. The time interval for drying and molding into granules or dunk shape was recorded. Four formulations were transformed—three into dried particles and one into moist cylindrical granules. Each formulation was stored in a covered container for protection. The stock product was tested at the appropriate concentrations of 3, 1.5, 1, and 0.5 grams, and the fourth concentration was tried again based on how well the previous three performed. The following concentrations; 0.4, 0.3, 0.25, 0.2, 0.15, 0.1, and 0.05 in grams were tested in the bioassay's trials.

Mosquito's larvicidal trials

The larvicidal experimental trials were carried out using a method that was somewhat modified from that recommended by the World Health Organization (WHO 2005). Various concentrations of the prepared product were tested. The four testing units; 0.5, 1, 1.5, 3 grams of the inner matter tests were carried out initially. The larvicidal efficacy of the selective formula was then tested at diluted levels of 0.5, 0.4, 0.3, 0.25, 0.2, 0.15, and 0.1. Aedes agypti mosquito larvae of the 3rd instar were selected from a laboratory for mosquito breeding and put into two 248 ml + 2 ml of the desired solution. Along with prevention and normal chemical testing, a mosquito larvae feed was introduced to each set of the experiment. Temephos was used as standard check in the experiment. Larvae that had died were counted in 3 knockdown timings of 24, 48 and 72 hours after exposure of each respective treatment. The average of four replications was used to calculate the percent fatality. The test samples that had a hazardous potential equal to Temephos (the standard check) at the end of 24, 48 and 72 hours, were chosen as the desired formulation for filed subsequent field application. Using Polo Plus software with LC50/LC90 as the reference and Stratix 8.1, for the comparative toxicity of plant crude based biopesticides against 3rd–4th-instar of *Aedes agypti* for various exposure periods was estimated.

RESULTS

The effect of different formulations of Melia based biopesticides on the larvae of mosquitos are presented in Table1. Initially, for the purpose to work out the effective inert matter, laboratory tests of four formulations were carried out in a bioassay against the larval stage. The data on mortality parameters was statistically analyzed by using two-sided Dunnett's Multiple Comparisons with natural Control. Significant variation was observed in the larval mortality and was affected by different formulations. Mortality was maximum (99.383% & 99.280) in case of standard check, Temephos and Melia Formulation (MF04) respectively as shown in the Table-1, while in case of formulations; MF01, MF02, MF03, the low mortality percentage as 43.967%, 44.407% and 52.170%. Respectively was found significantly low and not desirable. The natural control mortality (6.130%) was used for the calculation of increase over the natural control and this parameter was again found significantly higher in the MF04 as 93.15%. The at par result of MF04 as compared to the standard and commonly used larvicide (Temephos), MF04 formulation was selected for the various lab tests and further evaluations.

Table 1. Effect of various formulations of Melia based biopesticides on percent reduction in 3^{rd} & 4^{th} instars larvae and overall increase over the natural control check.

Formulations	% Mean reduction	Lower bound	Upper bound	% Increase over control
MF01	34.967	-3.737	61.411	28.837
MF02	44.407	5.703	70.851	38.277*
MF03	52.170	13.466	78.614	46.040*
MF04	99.280	60.576	125.72	93.150*
Temephos Std.Check	99.383	60.679	125.83	93.253*

Natural Check	6.130			

MF: Melia formulation, ∗: Significantly different

The credibility of the MF04 was further confirmed by calculating the Median Lethal Concentrations (LC50) of all the 4 tested formulations. The results on LC50 of the various formulations, 95 % Critical limits and Slope values for 24 hours exposure of 3rd instar larvae of Aedes are shown in Table-2. The LC 50 was calculated on the basis mortality (%) in the natural control and corrected mortality calculations by following the Abbott's formula. Results showed that the biopesticides had a significant effect on the LC50. The lowest LC50 value (1.196 %) was found for Temephos standard larvicide with the highest toxicity. Similarly, the MF04 formulation was also with closer LC50 value (1.969 %) and with high toxicity with that of standard check Temephos. However, the value for LC50 were found higher in the formulations; MF01 (3.473 %) but with low toxicity. The LC50 value was in negatively correlated with mortality, the higher the value, the lower the mortality and vice versa. Overall MF04 formulation showed the lowest value close to the Temephos with great killing ability.

Table 2: Calculations of LC50, LC90, lower and upper confidence limits, slope and chi square ($\chi 2$) values of four formulations for 24 hrs. exposure of 3rd instar larvae of Aedes.

Treatment	**LC_{50}**	**95% CL**	**Slope**
MF01	3.473	2.98-3.41	6.301±0.992
MF02	2.672	2.03-3.56	4.535±0.969
MF03	2.854	2.50-3.77	5.085±0.917
MF04	1.989	1.41-2.28	3.021±0.883
Temephos (std.)	1.196	0.81-1.56	2.741±0.421

Table 3 shows the results of laboratory tests using various concentrations of the selected MF04 formulation and the selective formula based on the comparative lab efficacy tests. Results regarding the effect of different biopesticides concentrations on the larvae displayed that the mosquito's larval mortality was affected by different concentrations of biopesticides of formulation MF04. The mortality was high (100%) similarly in both the cases of 0.5 g/L (500 ppm) and 0.4 g/L (400ppm). However, the mortality factor declined sparingly with the decrease in concentrations like 0.3 g/L (300ppm), 0.25 g/L (250ppm), 0.2 g/l (200ppm) and 0.15 g/L (150ppm), the observed mortalities were 99.67, 97.33, 94.33, 93.00 and 80.33 percent respectively.

Table 3: Bioassays on different concentrations of selected MF04 bio-pesticides formulation for the standardization of the effective dose.

Concentrations	**Mean Percent mortality**
0.5 g/L (500 ppm)	100.00 A
0.4 g/L (400 ppm)	100.00 A
0.3 g/L (300ppm),	99.667A
0.25 g/L (250ppm),	97.333AB
0.2 g/l (200ppm)	94.333BC
0.15 g/L (150ppm)	93.000C
0.10 g/L (100ppm)	80.333D

Table foot note: Alpha 0.05, Standard Error for Comparison: 1.9650, Critical Value for Comparison: 4.1656, Critical T Value: 2.120. The means are presented in 4 groups (A, B, C, D) and are less distinguishable from one another

DISCUSSION

The high level of laboratory-based killing ability demonstrated by our results on the efficacy of crude form of Melia based plant in comparison to standard pesticides led us to search for a better formulation as larvicide that could offer more secure and long-lasting control against deadly vector mosquitoes. Other scientists and researchers used Melia azadrich to combat *Aedes aegypti* L and *Anopheles stephensi* using its larvacidal, pubicidal, and anti-oviposition properties (Carolina *et al.,* 2008; Carolina *et al.,* 2004; Nathan *et al.,* 2006). *Panonychus citri* and *Aleurocanthus spiniferus* also had good killing ability from Melia azadrich-based biopesticide. Our research is leading to the development of biopesticides from potent plant extracts for public consumption. The invasive *Melia azadrich* plant in our situation was discovered to be less successful in comparison to formulations made from the leaf of the *Melia azadrich* in the same way at the laboratory level and another formulation. Our three formulations (PF01, PF02, and PF03) have similar toxicity, but formulation PF04 has significant better results. At 63 ppm, the seaweed extract was used against the same pest and method, resulting in 80-100% mortality (Ali *et al.*, 2013). Our results from the PF04 formulation also have similar potential against the deadly mosquitoes. Researchers found toxic effects in 65 different plant extracts from the Colombian Caribbean region, and they separated the 16 most active ones at a concentration of 200 ppm (0.2 gram); (Diaz *et al.*, 2022). The extracts obtained from *P. hysterophorus* leaves were at a level of 1,000 ppm, and the same extract was tested at a dose of 2, 3 grams that resulted in 100% mortality were reported by (Kumar *et al.,* 2011; Amir *et al.,* 2017; Khan *et al.,* 2014). However, our results at the rate of 10% active ingredient are much better than the simple crude extract of 100% active ingredient. An earlier study discovered that the *Melia azadrich* extract is used as a pesticide against highly infectious and pest-causing organisms, and the tabulation of leaf, fruit, and seeds revealed that the leaf extract contained 77.77% of the extract, with the fruit and seeds containing the remaining substances (Rubae *et al.*, 2009). Based on their successful results, we developed a Melia leaf powder formulation that is not only better than *Melia azadrich* simple powders but also has the proper carrying agent (inner matter and catalyst), a small amount of the active ingredient (only 10%), the right granule shape, is safe, has good solubility properties, and is easy to use. Simple dry-powered plant being light puts applicators at risk of many health issues and hazards including skin allergies. Our research findings demonstrated that we can potentially produce it in huge quantities and use it in the public sector to cure the dengue mosquito larval habitat. Through these means the idea to encourage the use of risk-free pesticides and permit registration of or support the market's demand for biopesticides is fully supported through our findings. The plant based biopesticide do not harm the environment or nontarget creatures, cheaper and more cost-effective than chemicals, and the main problem of resistance does not develop in pests. Market preference is although negative (Dannon *et al.,* 2020) but efforts can be made to popularize the plant-based products against insect pests including mosquitoes. Kumar *et al.* (2021), have also hinted that *Melia azadrich* formulations can be used as an alternative to other pesticides for the management of vector-borne diseases because they are relatively less toxic, environmentally friendly, and do not let insects acquire resistance. Our findings further need the field studies trials for effective pest management of the target pests without impacting nontarget insects for presenting and popularizing the use of safe plant-based biopesticides in the public health sector.

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