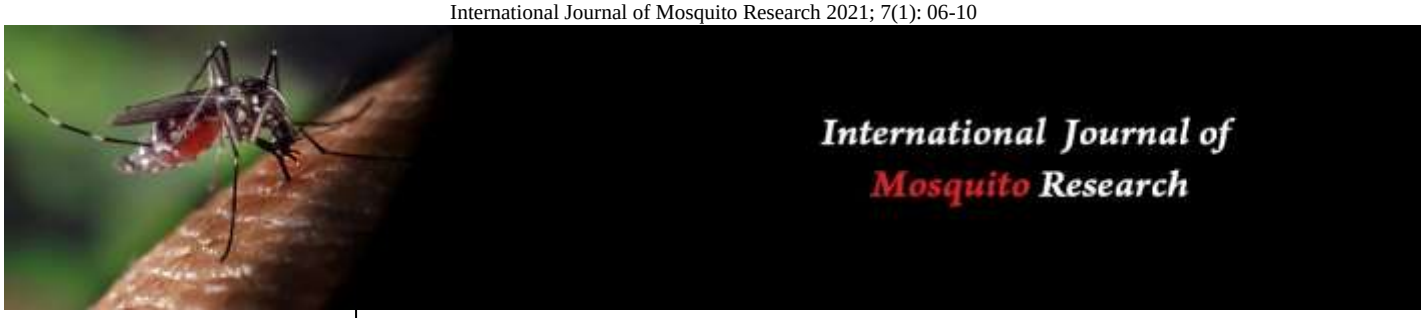

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Molecular identification and control of *Culex* mosquito by *Citrus limon* in West Bengal, India

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Abstract
Proper identification is a key factor to control the mosquito borne diseases. Genetic marker *ITS2* can successfully be used in characterization of *Culex* mosquito. Application of eco-friendly alternatives to control vectors has become the central focus of the control programme in lieu of the chemical insecticides. The present study has been undertaken to evaluate the larvicidal potential of a locally available botanical (Lemon Peel i.e. *Citrus limon*) against the 3rd instar *Culex* larvae in our laboratory which has been followed by GC-MS study. Our result based on GC-MS study showed that Griseoviridin is the potential active larvicidal component present in crude ethanolic lemon extract.

Keywords: *Culex*, *ITS2*, Phytochemical screening, *Citrus limon*, active component

Introduction
Mosquitoes are major causes of several diseases. Female mosquitoes nourish their ovaries by taking blood meal from host and transmit diseases [1]. More than half of the global human population is exposed to the risk of infection spread by mosquitoes. As different species show Accurate identification of a species transmitting a pathogen is essential for a proper perception of the mechanisms that rule any living system. Identification is mostly achieved by morphological features like wings, body stripes etc. But the problem arises in identification of sibling species and species with overlapping morphological characters or damaged samples collected from field. Hence, the use of molecular markers (like ITS2, COI etc.) has become an indispensable tool for this purpose. Again, plant parts, their products and secondary metabolites of floral origin have been utilized in pest control since ancient periods. During the pre-DDT period, decrease of different disease causing mosquitoes mainly depended on the control of their breeding sites. DDT and other synthetic organ chlorides and organophosphates were indiscriminately utilized as mosquito ides to reduce the transmission of mosquito borne diseases from early1950s. During the mid-1970s, the mosquito borne diseases showed resurgence and the vector species developed insecticide resistance. Therefore, importance was provided on the application of alternative techniques in the regulation of the population load of mosquito and emphasis was given on the system of Integrated Mosquito Management (IMM) [2]. Several plant extracts and isolated compounds from different plant families have been evaluated for their promising larvicidal activities [3]. About 2000 species of terrestrial plants have been reported for their insecticidal properties [4]. The mosquito borne parasites are continually developing resistance to available insecticides [5]. One of the extreme efficient alternative strategies under the biological control programme is to use the floral biodiversity and enter the arena of utilizing safer pesticides of plant origin as a simple and sustainable tool of controlling vector mosquito. An increasing number of researchers are reconsidering botanicals containing active phytochemical in their efforts to address some of these problems [6]. Plants possess some chemicals known as Phytochemicals which may not be nutritionally valuable, but they have some diseases preventive features. As for example, lemon is considered to be rich in phytochemicals [7]. The Environmental Protection Act (1969) has framed a number of rules and regulations to check the application of chemical control agents in nature. The search for safe and eco-friendly pest control options has led to exploration of pesticidal plant for potential alternative [8].

It has prompted the researchers to look for alternative approaches that would be environmental friendly, cost effective, biodegradable and target specific insecticides against mosquito species. Many scientists [9, 10, 11, 12, and 13] pointed out that plants contain a wide range of potential larvicidal phytochemicals (Tannis, terpens, saponins, isoflavanoids etc.) which are target specific ecofriendly, less toxic Several groups of phytochemicals likely alkaloids, steroids, terpenoids etc. from different flora have been reported earlier for their insecticidal properties. Insecticidal effects of plant extracts vary not only according to plant species, mosquito species, geographical varieties and parts used, but also due to extraction methodology adopted and the polarity of the solvents used during extraction. Thus, use of phytochemicals for mosquito vector control is widely accepted. Profound work on the use of *Citrus limon* peel extracts on *Culex* has not been carried out previously in our locality, therefore a preliminary work has been undertaken to evaluate the larvicidal potential of this locally available plant (*Citrus limon*) against the 3rd instar *Culex* larvae in our laboratory.

Materials and methods

Study area

- *Culex* larvae were collected from Serampore and Sheoraphuli of Hooghly District, West Bengal and taken to the laboratory of Serampore College (22.7505° N, 88.3406° E).
- Experiment was carried out in the Vector Molecular Genetics Research Unit of Serampore College.

Identification of mosquito species

Collection of mosquitoes

Larvae of *Culex* mosquito were collected from Serampore and Sheoraphuli areas of Hooghly district and have been cultured in our laboratory. Collection was made from different biotopes like drains, small drains near cattle sheds and human dwellings. Some adult mosquitoes were collected by manual aspirator also.

DNA isolation and amplification

DNA has been isolated from individual adult mosquitoes by phenol chloroform extraction by following protocols [14, 15, 16] and standardized in the laboratory [17]. The ITS2 region of r DNA has been amplified using the specific forward and reverse primer consisting of 20 - 21 base oligomers having the sequence 5' TGTGAACTGCAGGACACA CAT 3' (CODE 46JB) and 5' TGTGCTTAAATTCAGGGGGT 3' (CODE 47JB) respectively. A PCR Master Mix is prepared by mixing 10x PCR buffer, DNTP mix (100mM each), mgcl2, TAQ polymerase (3 unit/ml) double distilled water and template DNA. The thermal cycling condition is.... initial denaturation at 95°C for 5 min followed by 40 cycles of denaturation at 95°C for 30 sec / 1 min, annealing at 50°-60°C for 1 min, extension at 72° C for 2- 5 min and final extension at 72°C for 10 min. The PCR product and standard DNA ladder has been electrophoresed in 2% agarose and visualized with Ethidium Bromide. Genomic DNA was extracted from the mosquito provided by the customer by conventional method and ITS region amplification was carried out with ITS primer sequences. The capillary sequencing was done by ABI 3130XL Genetic Analyzer machine as per manufacture's information.

Amplification strategy

Sample: 1µl (10 times dilution of genomic DNA)
Primer (10pmol/µl): 0.25/0.25µl (ITS1/ITS2)
TaqMaster mix (G9 TAQ): 12.5µl
Distilled water: 11µl
Total volume: 25µl
PCR CYCLE:
95.0°C- 5min
95.0°C- 30sec
55.0°C- 30sec 30 cycles

Preparation of plant extract

i. *Citrus limon* (peels) were collected and air dried at room temperature. After 10 days the materials were powdered and stored.
ii. 3 grams of powdered plant material was added in 50ml of Ethanol (Ethanol is a polar solvent) in a brown bottle and kept for 3 days at room temperature.
iii. After 3days the mixture was filtered through Whatman no. 1 filter paper. Then the yield % and the concentration of the stock solution were measured.
iv. The remaining stock solution was refrigerated at 4°C until the subsequent larvicidal bioassay.

To determine the yield % of the stock solution the remaining dried material of sample dust in filter paper after percolation were weighing in a Petridish taking the weight of Petridish and concentration of stock solution was measured by firstly weighting a blank clean watch glass and secondly weighting that watch glass and precipitated 1 ml of stock solution in it. The 1 ml of stock solution was precipitated inside the watch glass by incubating it at 30°C for few minute.
In present study the calculation of yield % is as follow
For *Citrus limon* = (15gm - 12.303) GM / 15 × 100 = 17.98%
In present study the calculation of concentration of stock solution is as follows
For *Citrus limon* (14.187- 14.172) GM/ ml
= 0.015 GM/ ml
= 15 mg / ml

Evaluation of larvicidal efficacy

i. Larvae were placed in a tray and the temperature was and relative humidity was
ii. 40ml of working solution was prepared from stock solution by the following formula-$V_1S_1=V_2S_2$

Following the above formula 40ML of working solution was distributed into 3 petri dish in the following way:

1. Paper glass containing 40ml of working solution in which 38mL distilled water & 2mL stock solution (plant extract) for 1000ppm strength.
2. Paper glass containing 40mL of working solution in which 39mL distilled water & 1mL stock solution for 500ppm strength.
3. Petridish as control containing only 40mL of distilled water.

3rd instar larvae were collected to perform this bioassay. Each experimental dish contains 25 larvae and a control (Ethanol) was also included. Dead larvae were discarded and kept the experiment for 24hrs.
The percentage of larval mortality was recorded after 24hrs of larvicide exposure and the percentage of larval mortality was

corrected using Abbott's formula (Abbott, 1925)

Purification and identification of active biomolecules

Gas chromatography mass spectrometry is an analytical method that combines the feature of gas chromatography and mass spectrometry to identify different substances within a test sample. This technique involves the separation of volatile components in a test sample using suitable capillary column coated with polar and non-polar or intermediate polar chemicals. In this experimental study GC-MS method has been used to identify the presence of active compound in *Citrus* (peels).

Results

Molecular identification of vector mosquito

ITS2 study has revealed that most of the collected samples were *Culex pipiens* as the *ITS2* of collected samples shows highest similarity with this species of accession number

Table1: ITS 2 sequence of collected sample

AGTTCATACTAGGTTGAGTTGTTTGTGTTCGTTCTCTACAGAGAGAGCGAGCGAGTTCAA
AGAAAAAACACTGTTTTATCGGTGGGTCTCCGGGGGTAAAGAAGACCAATCCTACCCCT
GCCTGAATGCACCGACCACCCCCGTACCGGTGGTAGCGCTGCGTGCATGTAGTATACGCG
GACATAGGACGAACCTGCCGCACACGCTTGCGCTCGGTACCGCGATCTAAACTGGCCAG
CCCACCCCGGGGCCGTTCGGTCGGTCGGTCTCTCTCGTTCTCTTGTTCCTTTTGTTCCCCTG
CCCCTTCCGTTTGCGCACCGAACACAAACACGCACCTTGTGTGTGATCACCGGGGAGGGG
GTCCGGAGTGGACTGGTGATAACTGGGGTACACTGGGGGATTACGTGTGTGTTGTGTTCA
GCTTGCACAAACCTGCGACATTTGAAAGCGGAGAAGCGCCGAGAGACTATCGCCTATAG
ACTTTCCTTCCTCCTCTACTTTCCCTCACAGGAACACATTACGTGTACGCTGTCATACTTC
GTGTCGTGGCTGTCATGGTATGTCAAAAGTATGTTACAGAAATGATCCTTCCACGGGTTC
CACCGGGGAGAAAATCTTACGGAACA

Larvicidal efficacy

Effect of ethanolic extract of lemon peel on 3rd instar larvae of *Culex* SP is represented in the table2 and the fig 1&2 reveals the comparative analysis of the effect of plant extract and only ethanol (solvent) as control on the *Culex* larvae. Both the 1000ppm and 500 ppm of plant extract shows 100 percent mortality KU056509. 1. The sequence is as follows:

Table2: Larvicidal efficacy of plant extract (*Citrus limon*) on 3rd instar larva of *Culex pipiens*

Mosquito	Plant Extract	Conc. of larvicide (ppm)	Total no. of larvae introduced	No. of dead larvae	Mortality (%)	Corrected mortality (%)
Culex sp.	*Citrus limon* (Peel)	1000 (38ml ethanol + 2ml ethanolic extract)	25	25	100	100
		500 (39ml ethanol + 1ml ethanolic extract)	25	25	100	100

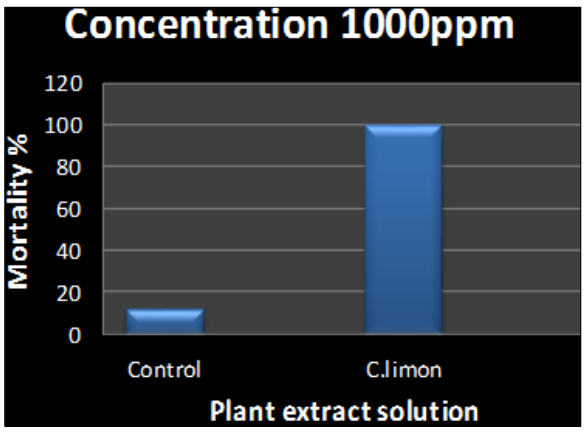

Fig 1: Comparative study of *Culex* larvae mortality at 1000 ppm concentration of plant extracts and control solution

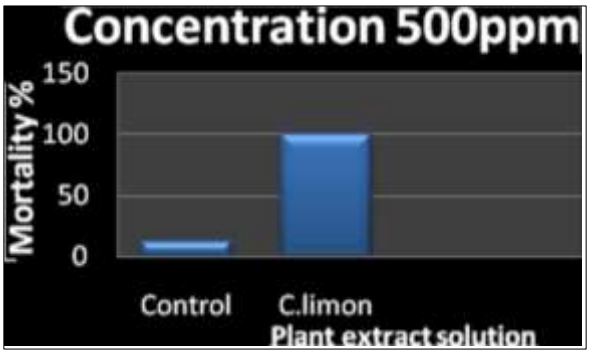

Fig 2: Comparative study of *Culex* larvae mortality at 500 ppm concentration of plant extracts and control solution

GC-MS result has indicated that the major compound (active principle) namely Griseoviridin, may harbour insecticidal potential in lemon peels as this component shows highest RT area%

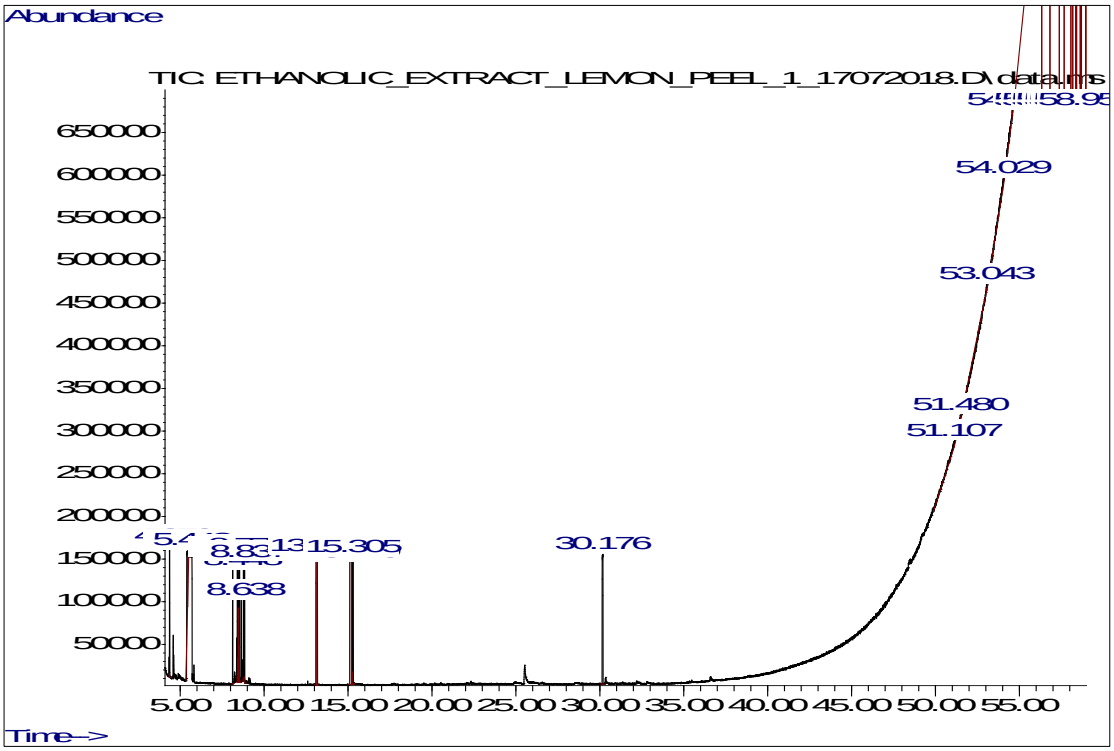

Fig 3: GC graph (Chromatogram) of ethanolic lemon peel extract by using GC-MS method showing the highest peak of active principle at RT 30.176

Discussion

As vectors of serious human diseases, mosquitoes are responsible for public health problems [18]. As different species show resistance to different mosquito ides, therefore, accurate identification of mosquito species is a key factor to control mosquito borne diseases. For this purpose molecular tool (like the use of ITS2 marker) is applied. It is known that, ITS2 rDNA is a non-coding DNA sequence. Therefore, it is subjected to a high degree of mutations, which makes it a good candidate to study phylogenetics of closely related species. Result based on ITS 2 sequence analysis (Table 1) indicates that the collected species from some parts of Hooghly district of West Bengal in this study is *Culex pipiens* which species is also responsible for transmission of virus of JEV complex [19]. Present experiment has revealed that lemon peels can be employed as a mosquito controlling agent. The ethanolic crude extract of lemon peels at both 1000 ppm and 500 ppm showing 100% mortality of 3rd instar (*Culex* sp.) larvae in contrast to less than 20% mortality (16% and 12% respectively) by the use of solvent only (Fig 1 and 2). After purification of the above mentioned crude extracts by applying GC-MS technique it has been identified that Griseoviridin is the probable active compound of Lemon peels (Fig 3). It is known that Griseoviridin has antibiotic property also [20]. So, beside its anti-microbial and anti-oxidant activities lemon is also important as mosquito larvicide. Previous experiment also revealed the larvicidal effect of methanolic extract of lemon peel on mosquito larvae [21].Hence, finally it can be mentioned from our study that crude ethanolic extracts of lemon peel have larvicidal efficacy that might also be used as an alternative strategy to control the *Culex* mosquito.

Conclusion

ITS2 is a conserved sequence i.e. this sequence is similar in case of all organisms of a species but different in closely related species. Similar instances also exist among *Culex* species [22]. By analysis of *ITS2* of collected *Culex* species, we have confirmed it as *Culex pipiens*. Phytochemicals have a broad spectrum insecticidal property and will certainly work as a new weapon and in future may act as suitable alternative substance to fight against mosquito borne diseases. Furthermore, botanic are eco-friendly, biodegradable and cost effective. Larvicidal efficacy of fruit peel extracts of *Citrus limon* has been reported where Griseoviridin has been identified as tentative active component.

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