Use of *Nepeta clarkei* extracts for controlling honey bee pathogenic bacteria and mosquito larvae

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ABSTRACT

Honeybee (*Apis mellifera*) population is going down across the globe due to honeybee pathogens. This greatly influences the bee-associated commercial food products production. Likewise, mosquitoes are an important vector responsible for spreading life-threatening human diseases, including malaria and dengue. The plant-based insecticides are a better substitute to the recent control practices of honeybee pathogenic bacteria and mosquito. Here, we performed an *in vitro* screening of *Nepeta clarkei* Hook. f. (Labiatae) aqueous extracts against three honey bee gut bacterial isolates including *Paenibacillus larvae* an infamous honeybee bacterial pathogen. The inhibitory zone was produced in the range of 6–14 mm diameters against three honey bee bacterial isolates. Likewise, fourth instars larvae of *Culex* (Diptera/Culicidae) were also subjected to check the possible larvicidal efficacy of *N. clarkei*. A normal media supplemented with *N. clarkei* in different concentrations (0.025% 0.05%, 0.1%, 0.15%, and 0.2%) affected the growth of larvae significantly. The lethal concentration at which 50% of larvae failed to become pupate was found to be 0.1% after 24 h of exposure. Considerable reductions in larval growth and pupal development of mosquito suggested that this plant should be utilized in mosquito control programmes.

1. Introduction

The genus *Nepeta* contains about 250 species, widely distributed mainly in Asia, North and South America and Europe. The *Nepeta* plant species have been used to treat kidney and liver diseases (Rather and Hassan 2011). In addition, several compounds isolated from *Nepeta grandiflora* and *Nepeta clarkei* showed repellency against various insects species *The Nepeta* spp. may prove potential against various insect pests but using a four-way olfactometer assay cereal aphid, *Sitobion avenae* were more sensitive to *N. clarkei* essential oils (Birkett et al. 2010).

Bee pathogen and parasitic mites play a major role in declining honeybee population (Di Prisco et al. 2011). *Paenibacillus larvae* affect honeybee broods and is being subjected to various antibiotics treatment to save bee population around the world (Antúnez et al. 2004). However, such antibiotics accumulation in bee products is the main drawback of their usage (Antúnez et al. 2008) and hence this not only reduces the value of honey and other bee by-products for human consumption but greatly affects the life span of the honeybee as well. In these circumstances, the substitution of conventional synthetic antibiotics with natural ones is very imperative.

Additionally, mosquitoes are vectors for a number of infections like malaria, dengue, yellow fever and other bacterial diseases (Ali et al. 2013). Lymphatic filariasis is the fastest spreading mosquito-borne disease affecting about 146 million people (Scholte et al. 2003). Similarly, malaria is another mosquito-borne disease infecting millions of people worldwide (Bagavan et al. 2009). It has been reported that 2 billion humans including approximately 700 million children are living in Japanese encephalitis endemic areas (Bagavan et al. 2009).

Therefore, the control of dipteran mosquito and other honeybee pathogen is an important health concern to stop the impending outbreaks in the world. For this purpose, an updated report by Khan et al. (2015) has documented the effectiveness of plant-derived compounds as insecticides to be an alternative for synthetic insecticides (Mathew et al. 2009) with minimum side effects risks (Khan et al. 2015). As the continuous use of synthetic insecticides has developed resistance in vectors, so searching for plant-derived natural compounds as a substitute for vector control is the need of modern approaches (Traboulsi et al. 2005; Kamaraj et al. 2008; Kweka et al. 2008; Mohanty et al. 2008). Though more than 2400 plant species have been tested to act as fungicidal, bactericidal, larvicides, insect growth regulators, repellents and oviposition attractants, but Lamiaceae family is extensively used for vector control practices around the world (Khan et al. 2015; Sakthivadivel et al. 2015; Pavela and Benelli 2016; Tisgratog et al. 2016). The plant *N. clarkei* is also a...
member of the Lamiaceae family which has not yet attracted much attention of the researchers, this lack of knowledge formed the basis of this study to investigate the efficacy of *N. clarkei* (Hook) extracts against honeybee brood pathogen (bacteria) and mosquitoes larvae.

### 2. Material and methods

#### 2.1. Bacterial culture from honey bees

Three bacterial strains were isolated from the honey bees (*Apis mellifera*) alimentary canals. The worker bees were collected from the North West regions (Bannu, Kohat and Karak) KPK Pakistan. Complete bees' gut were dissected in a sterile environment. The whole gut was macerated in 0.8% NaCl solution and different dilutions were made, i.e. 1/10, 1/100, 1/1000, etc. One hundred microlitre of each dilution was inoculated into LB Agar plates. The plates (named as master plates) were incubated for 24–48 h at 37°C (Figure 1(A)). After 24 h various types of colonies were observed. These bacterial colonies were streaked in new LB agar plates and incubated at 37°C for 24 h to obtain pure colonies (Figure 1(B)). The bacteria were preserved in glycerol stock at −80°C. The preserved stocks were re-cultured on BHI agar plates and well-isolated colonies were subjected for antibiotic susceptibility tests (Boonsai et al. 2014).

#### 2.2. Bacterial strain characterization

The cultured bacterial strains were identified by various bacteriological techniques as given in Bergey’s Manual and by following the technical procedures mentioned in the recently published literature (i.e. Hussain et al. 2013; Iqbal et al. 2014). Additional identification of selected bacteria was achieved by colony PCR and 16s rDNA gene sequencing. The sequence reads were scanned for quality check using Sequence Scanner Version: 1.0 (Applied Biosystem) (Howard et al. 2009). Comparative sequence analyses of the sequences against GenBank repository were performed using the BLAST (Altschul et al. 1990). The MEGA6 software was used for phylogenetics tree analysis (Kumar et al. 2014; O’Halloran et al. 2014).

#### 2.3. Antibiotic susceptibility test

For each bacterial culture, a suspension with a concentration of 0.5 McFarland was prepared following the protocol of Clinical and Laboratory Standards Institute (CLSI) standards. The suspension was spread onto a BHI agar plate using an autoclaved cotton swab and dried. The *N. clarkei* extracts (10 µl) with concentrations of 15, 20 and 25 mg/ml were placed on the plates uniformly. Sterile distilled water and Amikacin 30 µg discs were used as a negative control and positive control, respectively. The plates were incubated for 24 h at 37°C. After 18–24 h the diameter of the inhibitory zones was measured using a ruler and noted. The tests were repeated three times (Judaki et al. 2014). The crude aqueous extract of *N. clarkei* was prepared following the protocol of Khan et al. (2012).

#### 2.4. Insect rearing

*Culex* mosquitoes were collected from stagnant water from the outskirts of Kohat district. Adult mosquitoes were reared and larvae were kept in a plastic tub. The experiments were carried out at 27 ± 2°C and 75–85% relative humidity. The larvae were fed with a diet of dog biscuits and brewery yeast (Gerberg et al. 1994). Pupae were transferred to clean plastic bottles containing tap water and were maintained in laboratory till adults emerged (van Uitregt et al. 2012).

#### 2.5. Larvicidal and pupal bio assay

One gram of crude extract of *N. clarkei* was dissolved in 10 ml of respective solvent (stock solution), then several working standards with concentrations ranges of 0.025%, 0.05%, 0.1%, 0.15% and 2% were prepared. The solutions were poured in sterile glass dishes (9 cm diameter/150 ml capacity). Ten 4th instar larvae of *Culex pipiens* were introduced separately into Petri dishes containing graded concentrations according to the WHO protocol (World Health Organization 1981). After 24 h, the number of dead larvae in each dish was noticed by counting immobile larvae that were unable to reach the water surface (Kumar et al. 2014). Larval mortality in control dishes was also recorded after 24 h exposure. Controls were set up using deionized water along with each experiment and were kept in the same environmental condition. Each experiment was repeated five times and LC50 (lethal concentration) was determined. The percentage mortality values and pupal development were based on initial numbers of tested larvae.

### 3. Results and discussion

Identification of efficient and suitable plant-based insecticides with biodegradable and widespread insecticidal property will obviously work as a new weapon in future to fight against honey bee pathogenic bacteria and mosquito (Amer and Mehlhorn 2006). The preliminary screening is a good means for evaluating the potential biocidal/larvicidal activity of plants popularly used for the control of multiple pathogens. In the current study, we screened the *N. clarkei* extract bio-activity with respect to antibiotics and insecticidal activities using honey bee pathogenic bacteria species and mosquito larvae, respectively.

The bacterial strains isolated from the alimentary canal of honey bee (*A. mellifera*) were identified as *Bacillus subtilis*.
BN8D, *P. larvae* KK10 and *Staphylococcus hominis* BN1E using 16s rDNA sequencing, comparative sequence analysis and phylogenetics tree construction on the basis of sequence similarities (≥97%) with the known bacterial strains from the GenBank database. All the positions in multiple alignments containing gaps and missing data were first eliminated and the final data set comprising 503 positions was generated. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analysed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Tamura et al. 2013). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and Bio NJ algorithms to a matrix of pairwise distances as estimated using the Maximum Composite Likelihood (MCL) approach implemented in MEGA6 utility (Figures 2 and 3).

The susceptibility tests produced variable inhibitory zones that increase for each bacterial strain along with a gradual increase in plant extract concentration (Table 1). The discs soaked with 30 µl of distilled water as a negative control showed no antimicrobial activity against all tested isolates. A recent study has documented a significant antibacterial activity of crude propolis extract against *S. aureus*, *E. coli* and *P. larvae* with MIC of 5 and 6.25 mg/ml using both micro-broth dilution and agar well diffusion methods (Boonsai et al. 2014). They reported 1 cm zone of inhibition at 100 mg/ml of the extract. Additionally, aqueous extracts of 10 different plant species showed inhibitory effects on *P. larvae* growth. Among these, especially *Eucalyptus cinerea* and *Minthostachys verticillata* have been reported to have 100% antibacterial activity against *P. larvae* (González and Marioli 2010). The present findings also observed a noticeable result of bacterial isolates (*P. larvae*, *N. clarkei* and *P. larvae* isolates) in which the *P. larvae* (P. larvae) showed maximum sensitivity (14 mm) at 25 mg/ml (Table 1). This suggests that the *N. clarkei* based medicines and findings might be an alternative way for the control of American foul brood disease of the honeybee.

The activities of *N. clarkei* against *P. larvae* found in the current study (Table 1) correspond well with the reported antibacterial activities. Only 14 out of 42 species of the genus *Hypericum* were documented to be important for the control of *P. larvae*. Hence *N. clarkei* may prove to be an effective source for the control of *P. larvae*. However, studies are still required to screen the latent specific compounds of *N. clarkei* for these biological actions.

Additionally, larvicidal activities of different concentrations of crude extract of *N. clarkei* have been presented and it was found that *N. clarkei* extract showing the highest mortality rate (=100%) at very low concentrations (0.1%, 0.15% and 0.2%) (Table 2 and Figures 4 and 5). The observed time and concentration-dependent effectiveness of *Nepeta* extract varied from 0.1% to 0.2% against mosquito larvae and 100% pupal development was retarded at 0.2%. An important finding of the present study is the mortality of the larvae increases in a dose-dependent manner with *Nepeta* extract; and the pupa formation, on the other hand, is decreased/delayed (Table 2 and Figures 4 and 5).

We also found that *N. clarkei* is more effective against the fourth instar larva with 50% mortality on a very low (0.1%) concentration (Figure 6), as reported by Dua et al. (2009) about the efficacy of neem oil formulation against the late third and early fourth instar larvae of *Culex quinquefasciatus* with lethal concentration (LC50) at 1.8 ppm (Dua et al. 2009). Likewise, the larvicidal activity of *Atlantia monophylla* (Rutaceae) against early immature stages of the *C. quinquefasciatus*, *A. stephensi*, and *A. aegypti* was also assessed and the larvae of *C. quinquefasciatus* and pupae of *A. stephensi* were found to be more susceptible with LC50 at 0.14 and 0.05 mg/l, respectively. Moreover, the extract that has been used in this study was found to be safe to the aquatic mosquito predators (*Gambusia affinis*, *Poecilia reticulata*, and Diplonychus indicus) (Sivagnaname and Kalyanasundaram 2004). In our study, the different concentrations of *N. clarkei* crude extract against the fourth instar larvae of *Culex* mosquito exhibited significant larvicidal activity (Table 2 and Figures 4–6). The lethal concentration at which 50% of the mosquito larvae failed to become pupae was found to be 0.1% after 24 h of treatment which is congruent to the finding of Sivagnaname and Kalyanasundaram (2004) and Dua et al. (2009). The retardation in larval growth and mortality at earlier stages of mosquito larvae suggests that *N. clarkei* as a good source of larvicidal potential and therefore recommended for future mosquito control strategies.

Pushpanathan et al. (2008) provided the insecticidal and repellent activity of *Zingiber officinale* essential oils against the larvae and adults of *C. quinquefasciatus* mosquito (a primary vector of filariasis). The 50% mortality (LC50) after 24 h was observed at 50.78 ppm (Pushpanathan et al. 2008). We also found that low concentrations of *N. clarkei* crude extract retarded *C. pipiens* pupa formation (Table 2 and Figure 4). The time associated mode of action against mosquito reflects that components of *N. clarkei* may provide a long-term control, if

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### Table 1. Susceptibility of honeybee gut bacteria to *N. clarkei* Hook extracts.

| Extracts     | Zone of inhibition (mm)*a | Bacillus subtilis BN8D | Paenibacillus larvae KK10 | Staphylococcus hominis BN1E |
|--------------|---------------------------|------------------------|---------------------------|----------------------------|
| *Nepeta clarkei* |                           | 09 ± 1.03              | 10 ± 0.57                 | 06 ± 0.72                  |
| Hook.        |                           | 12 ± 0.57              | 13 ± 1.15                 | 08 ± 0.57                  |
| AK 30c 1     | 26 ± 1.15                 | 25 ± 0.55              | 24 ± 0.65                 |
| Negative control  |                       | 00 ± 1.15              | 00 ± 0.65                 |

Note: *a* Absence of inhibition zone.

*Numbers represent the average diameter (in mm) of the inhibition zone.
Sterile water used.
Amikacin 30 µg (CT0107B) discs served as positive control.

### Table 2. Efficacy of *Nepeta clarkei* Hook F (Labiatae) against the larvae of *Culex* mosquito after 24 and 48 h of treatment.

| SN | Per cent concentration | Mean % mortality 24 h | Mean % mortality 48 h |
|----|------------------------|-----------------------|-----------------------|
| 1  | 0.025                  | 33                    | 26                    |
| 2  | 0.05                   | 53                    | 42                    |
| 3  | 0.1                    | 57                    | 70                    |
| 4  | 0.15                   | 74                    | 72                    |
| 5  | 0.2                    | 86                    | 83                    |
| 6  | Control                | 00                    | 00                    |
Figure 2. Phylogenetics tree showing relationship of isolated strain (BN8D) with closely related taxa.

Figure 3. Molecular phylogenetics analysis by maximum likelihood method showing relationship of KK10 isolate with *P. larvae*.

Figure 4. Efficacy of extracts of *N. clarkei* concentration on development of mosquito pupae 24 h after exposure.
considered for pest control strategies. Moreover, in accordance with our results, Bagavan et al. (2009) observed a similar action of chloroform and ethyl acetate extract of *R. nasutus* against *A. subpictus*, *C. tritaeniorhynchus* and *A. gossypii*. They reported that the increase in concentration and time of exposure caused more pupal inhibition and more larval deaths. The difference in LC50 might be happening due to the difference in time of exposures of extract. Furthermore, the ethanolic extracts of *M. officinalis*, *M. longifolia* and *T. divericatum* exhibited complete (100%) larvicidal mortality at 200 ppm and the study results did not significantly differ in term of larval deaths (Cetin et al. 2006). However, more comprehensive studies about the chemical structures/class of the latent compounds along with their larval toxicity evaluating bioassay screening are still required.

4. Conclusion
The current study suggests that *N. clarkei* extract hold strong potential to produce new safe and environment-friendly insecticidal and bactericidal agents that may pose little risk to human health compare to synthetic compounds. Additionally, the pupal inhibition of mosquito larvae reflects the existence of ecdysone (pupa formation hormone) inhibiting compounds in the extract as reported by Khan et al. (2015).

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Disclosure statement
No potential conflict of interest was reported by the authors.

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