Extracellular metabolites of *Trichophyton ajelloi* against *Anopheles stephensi* and *Culex quinquefasciatus* larvae

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Keratinophilic deuteromycetous fungus *Trichophyton ajelloi* was isolated from the soil by feather-baiting technique. It has previously been tested as a larvicidal agent of *Anopheles stephensi* and *Culex quinquefasciatus*. The extracellular metabolites of *T. ajelloi* were collected from the Sabouraud dextrose broth by filtering through Whatman-1 filter paper after 21 days of growth. The bioassays were run at six different concentrations chosen to produce larval mortalities between 20 and 95% for calculating the LC_{50} and LC_{90} values by probit analysis. The larvae of *Cx. quinquefasciatus* were 0.25-fold more susceptible than the *An. stephensi*. The 1st instars of both the species were more susceptible than the 2nd and 3rd instars. These extracellular metabolites of *T. ajelloi* could be regarded as alternatives to synthetic insecticides. This indigenously developed larvicide needs to be explored further in different climatic zones of India.

MICROBES are used as alternatives to conventional broad-spectrum synthetic insecticides because of their selective toxicity and are safe for the environment. The insecticidal secondary metabolites produced by entomopathogenic fungi have become a focus of interest for insect pathologists. The secondary metabolites of entomopathogenic fungi *Metarrhizium*[1,2], *Beauveria*[3], *Tolypocladium*[4,5] and *Fusarium*[6] have potential insecticidal activity. Some are well established as true insecticides, such as tolypin[5], beauvericin[7], destruxin[8,9] and cyclosporin A and C[4]. In India, Vijayan and Balaraman[10] have screened the efficacy of extracellular fungal secondary metabolites against 3rd instar of three important vectors *Anopheles stephensi* Liston, *Culex quinquefasciatus* Say and *Aedes aegypti*.

An. stephensi and *Cx. quinquefasciatus* are two major human-biting mosquitoes in India. The former acts as a malaria vector whereas as the latter works as a vector of filariasis and St. Louis encephalitis[11]. *Trichophyton ajelloi* Vanbreuseghem, a deuteromycetous fungus has been tested as a biocontrol agent of *An. stephensi* and *Cx. quinquefasciatus*[12]. This fungus is an imperfect one and reproduces asexually. Like other deuteromycetes, it grows on Sabouraud dextrose agar. The present communication describes the larvicidal effect of extracellular metabolites of *T. ajelloi* against 1st, 2nd and 3rd instars of *An. stephensi* and *Cx. quinquefasciatus*.

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Ten soil samples were collected near our institute at Agra. The soil samples were excavated by a sterile scalpel and collected in clean sterile plastic bags. Fifty grams of soil sample from each source was placed in separate petri plates. Five replicates of each source were prepared in separate petri plates. The keratinophilic soil fungi were isolated with the help of feather-baiting technique. *T. ajelloi* (EPL-5) strain used in this study has been originally isolated from the soil by feather-baiting technique[12]. Feathers of Indian peacock (*Pavo cristatus* Linn) were used as bait. The feathers were sterilized in an autoclave and then placed on the surface of the soil and moistened (5 ml/petri plate) with sterile distilled water. Pure culture of the fungus has been maintained in the laboratory since 1998. Petri plates were placed at 24 ± 2°C in incubators. After 7 days fungal colonies on the feathers were visible with the naked eye. For the preparation of a pure culture, a sub-sample of all fungal colonies grown on the feather was individually transferred with an inoculation needle to a petri plate containing Sabouraud dextrose agar (Ranbaxy, SAS Nagar, India). The Sabouraud dextrose agar was supplemented with 50 µg/ml of chloramphenicol as a bacteriostatic agent. If more than one fungal colony grew on a petri plate, each fungus was recultured as needed to isolate a pure colony. *T. ajelloi* was identified according to Frey *et al.*[13]. The chitinolytic activity of *T. ajelloi* was studied by the method of Ismail and Abdel-Sater[14]. The *T. ajelloi* colony was grown on a medium containing partially purified chitin (0.3% w/v). The source of chitin was insect and the medium was solidified by 1.5% agar.

The Sabouraud dextrose broth was prepared by the method of Gardner and Pillai[15]. Six 250 ml conical flasks, each containing 100 ml Sabouraud dextrose broth (Dextrose 40 g, peptone 10 g, distilled water 1000 ml) were autoclaved at 20 psi for 20 min. The broth was supplemented with 50 ìg/ml chloramphenicol as a bacteriostatic agent. *T. ajelloi* colonies grown on the Sabouraud dextrose agar plates were transferred to each flask using the inoculation needle. The conical flasks inoculated with *T. ajelloi* were incubated at 24 ± 2°C for 21 days. The extracellular metabolites were obtained by filtering the broth through Whatman No. 1 filter paper after the incubation period.

Mosquito larvae were maintained in the laboratory at a temperature of 25 ± 2°C, relative humidity of 70 ± 5% and a photoperiod of 14 : 10 h (light : dark). Different instars of *An. stephensi* and *Cx. quinquefasciatus* were maintained in separate enamel containers (25 cm × 15 cm × 5 cm). All larvae were fed 0.4 ml/beaker of a 5% (w/v) autoclaved suspension of freeze-dried yeast in distilled water on day-1 and day-2. Larvae were reared in double distilled water at pH 7.0. To counteract evaporation, water was added daily.

Twenty 1st, 2nd and 3rd instars of *An. stephensi* and *Cx. quinquefasciatus* were placed separately in 750 ml

beakers containing 500 ml test medium. Larvae of each instar were exposed separately to serial dilutions of extracellular metabolites. Six dilutions of the extracellular metabolite concentrate were made. Each instar of both the species was run at six different concentrations chosen to produce larval mortalities between 20 and 95% for calculating the LC_{50} and LC_{90} values. Bioassays were run in triplicates and conducted five times on different days of a month. All dead larvae were counted and discarded from the test medium every 12 h. Moribund and dead larvae were examined individually under a microscope. Assays were terminated at 72 h and mortality counts were made based on the number of live larvae remaining in each beaker.

The control was run with the experiment simultaneously. In the control, twenty 1st, 2nd and 3rd instars of each species were put in separate beakers with 500 ml sterile double distilled water. The control beakers were supplemented with respective concentrations of filtrate culture media. The larvae were fed the same food which was given to the experimental larvae on day-1 and day-2. The LC_{50} and LC_{90} values with 95% fiducial limits and probit equations were calculated using probit analysis[16]. Calculations of probit equations and LC values were made with the help of a software developed by Raymond[17].

Keratinophilic fungi are distributed in the Indian soil and their specificity against chitin of mosquito larvae has so far not been explored. The deuteromycetous fungus *T. ajelloi* was previously tested against the larvae of *An. stephensi* and *Cx. quinquefasciatus* as a control agent[12]. The invasion of conidia of *T. ajelloi* into mosquito larval host is supported with a system of protease, chitinase and lipase enzymes and without primary participation of the metabolites. The secondary metabolites were first characterized as antibiotics as they have some supportive activity on bacteria and fungi[18]. Their insecticidal activity was reported during the nineties. Metabolites of *T. ajelloi* are proteinous in nature. The concentration of protein in the culture filtrate after 21 days of growth was 75 ± 6.1 µg/ml (Lowry assay). The colony of *T. ajelloi* had variable rates of growth on dextrose and chitin media. The colony grown on dextrose had a higher rate of growth (radius, 4.7 ± 0.4 cm) after seven days than the colony grown on chitin source (radius, 3.7 ± 0.2 cm). The growth rate of *T. ajelloi* on chitin indicates that it has a high level of chitinase in metabolites. The objective of the present investigation was to observe the efficacy of extracellular metabolites against mosquito larvae and to compare the effect among the species.

Results of the present study confirm that the extracellular metabolites are efficacious against the larvae of *An. stephensi* and *Cx. quinquefasciatus*. The LC_{50} and LC_{90} values of all the three instars of both the species are given Tables 1 and 2. According to the LC values, it is evident that larvae of *Cx. quinquefasciatus* were 0.25-fold more susceptible than the larvae of *An. stephensi*. A comparison of LC_{50} and LC_{90} values among different instars of *Cx. quinquefasciatus* showed the 1st instar was two-fold more susceptible than the 3rd instar and the mortality of the 2nd instar was between those of the 1st and 3rd instars (Table 1). The efficacy of *An. stephensi* was similar to that of *Cx. quinquefasciatus*, except in the case of LC_{90} of the 1st instar. The LC_{90} of the 1st instar larvae was higher than that of the 2nd instar larvae. *T. ajelloi* was used as a positive control and the LC_{50} and LC_{90} values were recorded within the fiducial limit[12].

In India, Vijayan and Balaraman[10] reported the metabolites of 17 fungi to be highly larvicidal and their LC_{50} values against the 3rd instar of *An. stephensi* and *Cx. quinquefasciatus* were in the range of 7–83 and 3–24 µl/ml respectively. In the present study, LC_{50} of *An. stephensi* (41 µl/ml) was in the middle of the range as observed by Vijayan and Balaraman[10] and LC_{50} of *Cx. quinquefasciatus* was higher than the range. However, in our study the 1st instar of both the species was more susceptible than the other instars. Therefore, the extracellular metabolites of *T. ajelloi* were better equipped against the 1st instar of *An. stephensi* and *Cx. quinquefasciatus*. Priyanka *et al.*[19] recorded the efficacies of *An. stephensi* against culture filtrate of *Chrysosporium tropicum* and observed that the 2nd instar was more susceptible than the other instars. The difference in mortality compared to the present investigation may be the mode of action of the metabolites. Zizka and Weiser[7] evaluated the effect of beauvericin against L4 larvae of *Cx. pipiens autogenicus* and reported that beauvericin caused 44% mortality at a concentration

Table 1. Probit equations and susceptibilities of instars of *Culex quinquefasciatus* against extracellular metabolites of *Trichophyton ajelloi*. (Protein concentration in µg/ml is given within parentheses; *x* is the log concentration of extracellular metabolites of *T. ajelloi* in µl/ml)

	Probit equation	LC_{50} (µl/ml)	LC_{90} (µl/ml)
First instar	$1.728 + 2.701x$	16.25 (1.22)	48.42 (3.63)
Fiducial limit (95%)		15.28 (1.15) – 18.37 (1.38)	43.72 (3.28) – 53.91 (4.04)
Second instar	$1.237 + 2.760x$	23.06 (1.73)	67.14 (5.03)
Fiducial limit (95%)		21.88 (1.64) – 25.63 (1.92)	65.30 (4.89) – 70.94 (5.32)
Third instar	$0.825 + 2.775x$	31.92 (2.39)	92.47 (6.93)
Fiducial limit (95%)		26.36 (1.98) – 34.26 (2.57)	88.69 (6.65) – 99.07 (7.43)

Table 2. Probit equations and susceptibilities of instars of *Anopheles stephensi* against extracellular metabolites of *T. ajelloi.* (Protein concentration in μg/ml is given within parentheses; *x* is the log concentration of extracellular metabolites of *T. ajelloi* in μl/ml)

	Probit equation	LC_{50} (μl/ml)	LC_{90} (μl/ml)
First instar	1.503 + 2.487*x*	25.474 (1.91)	83.404 (6.25)
Fiducial limit (95%)		23.37 (1.75) – 28.12 (2.10)	79.06 (5.92) – 86.80 (6.51)
Second instar	1.042 + 2.734*x*	28 (2.1)	82.54 (6.19)
Fiducial limit (95%)		26.73 (2.00) – 31.95 (2.39)	72.36 (5.42) – 85.32 (6.39)
Third instar	0.669 + 2.674*x*	41.62 (3.12)	125.53 (9.41)
Fiducial limit (95%)		37.95 (2.85) – 44.16 (3.31)	98.16 (7.36) – 138.02 (10.35)

of 100 μl/ml. Although beauvericin is a purified crystalline compound, it is less effective. However, a crude extract of tolypin caused 100% mortality in the larvae of *Cx. pipiens* and *An. maculipennis* at a concentration of 100 μl/ml. The efficacies of extracellular metabolites of *T. ajelloi* can be regarded as an alternative to the above agents. It can thus be concluded that extracellular metabolites of *T. ajelloi* are effective against the 1st instars of both the species. The use of floating agent and wetting agent may increase the mortality of *An. stephensi*. The major vectors of malaria in India are *An. culicifacies* and *An. fluviatilis*, where these new metabolites should be tested. Nevertheless, the trials presented here can further initiate work in this area. The objective is to explore indigenous larvicide product, which can be helpful in controlling major vector-borne diseases in India. Field trials in different geoclimatic zones of India and abroad can further enhance its candidature as a future larvicide. A major breakthrough, probably a more purified version could be the ultimate goal for which further work is in progress.

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