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Larvicidal activity of endophytic fungi isolated from selected medicinal plants on *Aedes aegypti*

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Abstract
Endophytic fungi are one of the untapped resources of therapeutic compounds for various biological activities. It has a potential source for low-cost chemicals, used for developing eco-friendly control agents against mosquito-vector borne diseases. The present study focused on the larvicidal activity of ethyl acetate extract of endophytic fungi isolated from leaves of *Ocimum sanctum*, *Vitex negundo*, *Andrographis paniculata*, *Mentha piperita*, *Tagetes erecta* and *Azadiracta indica* on *Aedes aegypti*. *Aspergillus*, *Pencillium* and *Fusarium* species were isolated from the leaves of *Ocimum sanctum*, *Vitex negundo*, *Andrographis paniculata*, *Mentha piperita*, *Tagetes erecta* and *Azadiarcta indica* in the present study. The entire larvae bioassay test with endophytic fungal extracts showed a significant increase in the mortality percentage with the increase of concentration. Among the endophytic fungal extracts, the highest larvicidal activity was observed in *Aspergillus* and *Fusarium* species from the leaves of *Vitex negundo* was found to be 90.47% and 85.71% at 20 ppm respectively. The continuous exposure (24 h) of fungal extract resulted with least mortality effects. Moreover, the outcome of study is providing strong scientific evidences for developing more selective, ideal and eco-friendly mosquito larvicidal agents.

Keywords: Ocimum sanctum, *Vitex negundo*, Andrographis paniculata, Mentha piperita, Tagetes erecta, Azadiarcta indica and *Aedes aegypti*

1. Introduction
Mosquito is the most indisputable medicinal significant arthropod vector of diseases. The vector-borne diseases caused by mosquito are one of the major health problems throughout the world. Mosquitoes transmit various disease including dengue, malaria, yellow fever, filariasis Japanese encephalitis and chikungunya [1]. In the past several decades, a number of chemical insecticides have been effectively used to control mosquitoes. But the continuous usage 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 [2]. These factors have resulted in an urge to look for environment friendly, cost-effective, biodegradable and target specific insecticides against mosquito species [3]. Plants may serve as a reservoir of large numbers of organisms such as bacteria, insects, nematodes, protozoa, or fungi that can live endophytically within plant tissues. Endophytic microorganisms are fungi which live inside plant tissues or organs, without causing them any harmful effects. Indeed, since many are also able to produce substances of biotechnological interest, they may protect the plant from insect attacks and diseases [4-5]. Endophytic fungi are one of the untapped resources of therapeutic compounds for various ailments [6]. Fungal endophytes typically colonize living interior tissues of plants without bringing about any conspicuous negative impacts or outer indications. They may offer significant advantage to their host plants by delivering secondary metabolites that give assurance and survival advantages to the plants, for instance by giving plant growth controllers, antimicrobials, antivirals and insecticides or notwithstanding interceding protection from a few kinds of abiotic stress [7].
The efficiency in killing larval instars of important vector species and the lack of effects on non-target organisms, as well as the biological stability of extracellular metabolites, make this practice a promising alternative to mycelium- and conidial based larvicides. These products could be considered fungal based natural larvicides for vector control [8]. The present study focused on the larvicidal activity of ethyl acetate extract of endophytic fungi isolated from leaves of *Ocimum sanctum*, *Vitex negundo*, *Andrographis paniculata*, *Mentha piperita*, *Tagetes erecta* and *Azadiarcta indica* on *Aedes aegypti*.

2. Materials and Methods

2.1 Collection of plant material

Ocimum sanctum, *Vitex negundo*, *Andrographis paniculata*, *Mentha piperita*, *Tagetes erecta* and *Azadiarcta indica* (Fig. 1 –6) leaves were collected from Pachaiyappa's college, Chennai, Tamil Nadu. Plant species were brought to the laboratory in sterile bags and processed immediately to reduce the risk of contamination.

Fig 1: *Ocimum sanctum*

Fig 2: *Vitex negundo*

Fig 3: *Andrographis paniculata*

Fig 4: *Mentha piperita*

Fig 5: *Tagtes erecta*

Fig 6: *Azadirachata indica*

2.2 Isolation and purification of the endophytic fungus

Isolation of endophytic fungi from selected mosquito repellent plants were carried out following the method of Strobel *et al.* [9] The leaves were washed under running tapwater for 15 minutes. Before sterilization then samples were cut in to small pieces with 1-2 cm long. Then the samples were sterilized with 70% ethanol for 1-2 mts and 1.0% sodium hypochlorite for 1 mts and the samples were cleaned in 3 set of sterile distilled water. The surface sterilized plants segment were placed on plate containing potato dextrose agar (PDA) supplemented with 200 mg/l concentrations of streptomycin to arrest the bacterial contamination. The plates were incubated in BOD incubator at 25 ± 2ºC till the fungal mycelia starts growing from the samples. The mixed fungal culture was made into pure culture and stored in 4º C for further investigation.

2.3 Identification of the endophytic fungus by cultural and morphological methods

The identification of the endophytic fungal cultures were studied by using both cultural and morphological approaches. Cultural characteristics, such as color and the nature of the growth of the colony, were determined by visual observation. Morphological characteristics of the fungus, like mycelia, conidiophores, and conidia, were studied microscopically.

Secondary metabolites extraction

Fermentation and extraction of secondary metabolite was carried out by Radji *et al.* [10]. Positive endophytic fungal isolates were inoculated into 250 ml Ehenmeyer flasks containing 100 ml potato dextrose broth and incubated at room temperature for 21 days under stationary conditions with intermittent shaking. The broth culture was filtered to the filtrate ethyl acetate was added and mixed well for 10 mts and kept for 5 mts till the two clear immiscible layers formed. The upper layer of ethyl acetate containing the extracted compound was separated using separating funnel. The culture filtrate extracts were evaporated to dryness in hot air oven. The extract residue was dissolved in dimethyl sulfoxide (DMSO) and stored at 4ºC to be used as stock solution for the further studies.

Larvicidal Assay

Mosquito larvae were collected from Entomology Research Institute (ERI), Loyola College, Chennai, Tamil Nadu, to start the colony and larvae were kept in plastic and enamel trays containing tap water. They were maintained and all the experiments were carried out at 27 ± 1°C and 75–85 % relative humidity under light and dark cycles. Larvae were fed a diet of Brewer's yeast, dog biscuits and algae collected from ponds in a ratio of 3:1:1, respectively as per the method of Kamaraj *et al.* [11].

=The larvicidal effect of endophytic fungi isolated from medicinal plants was determined by the standard procedure of World Health Organization [12]. The stock solutions were prepared by dissolving extracts in dimethyl sulphoxide (DMSO). Then the larvae were kept into 100 mL beakers separately, containing 50 mL of distilled water to which 1.25 ppm, 2.25 ppm, 5 ppm, 10 ppm and 20 ppm of endophytic fungal extracts were added using capillary micro-pipettes to get the desired test concentrations. Three replicates were made for each concentration and the experiment was performed under laboratory conditions. A control was

maintained, containing only larvae and natural growth medium.

Statistical analysis

The mortality data were then subjected to one way ANOVA using the computer software SPSS of 16 version. Results with P<0.05 were considered to be statistically significant.

3. Results and Discussion

The present investigation elucidated the larvicidal activity of endophytic fungi isolated from some medicinal plants *viz*., leaves of *Ocimum sanctum, Vitex negundo, Andrographis paniculata, Mentha piperita, Tagetes erecta* and *Azadiarcta indica* on *Aedes aegypti*. Based on the microscopic and macroscopic characteristics endophytic fungi isolated were identified in the present study. Among the isolate *Aspergillus* sp, *Pencillium* sp and *Fusarium* sp was commonly present in *Ocimum sanctum, Vitex negundo Andrographis paniculata and Tagetes erecta*. *Mentha piperita* showed the presence of *Pencillium* sp and *Fusarium* sp. Likewise, *Aspergillus* sp and *Fusarium* sp was observed in leaves of *Azadiarcta indica* (Table 1).

Mosquitoes and its associated microorganisms are responsible for various diseases in people living in tropical regions [13]. Anti mosquito repellents derived from natural products were well received by the people due to easy biodegradation and less side effects [14]. In our study, the endophytic extracts showed a significant level of larvicidal activity. The larvicidal activities of isolated endophtyes from leaves of *Ocimum sanctum, Vitex negundo, Andrographis paniculata, Mentha piperita, Tagetes erecta* and *Azadiarcta indica* against the *Aedes aegypti* after 24 exposure were presented in Table (2 - 7 and Fig. 7 - 12). The entire larvae bioassay test with endophytic fungal extracts showed a significant (P< 0.05) increase in the mortality percentage with the increase of concentration. Among the endophytic fungal extracts of *Ocimum sanctum, Vitex negundo, Andrographis paniculata, Mentha piperita, Tagetes erecta* and *Azadiarcta indica*, the highest larvicidal activity was observed in *Aspergillus* and *Fusarium* species from leaves of *Vitex negundo* was found to be 90.47 and 85.71% at 20 ppm respectively. Likewise *Fusarium* sp from leaves of *Azadiarcta indica* and Pencillium sp from the leaves of *Tagetes erecta* showed the highest mortality percentage was found to be 83.33 and 72.22 at 20 ppm respectively. *Aspergillus*, *Pencillium* and *Fusarium* sp isolated from leaves of *Ocimum sanctum* revealed the moderate mortality on *Aedes aegypti* and the value was found to be 55.55, 61.11 and 44.44% at 20 ppm. Similarly, endophytes from leaves of *Andrographis paniculata and Mentha piperita* showed the mimimal larvicidal activity on *Aedes aegypti* when compared to *Aspergillus*, *Pencillium* and *Fusarium* sp from *Vitex negundo, Ocimum sanctum* and *Tagetes erecta*. Inhibition was dose and time dependent (P< 0.05)

Table 1: Isolation of endophytic fungi from leaves of *Ocimum sanctum, Vitex negundo, Andrographis paniculata, Mentha piperita, Tagetes erecta* and *Azadiarcta indica*

S. No	Name of the plants	Isolated endophytic fungi
1	*Ocimum sanctum*	*Aspergillus* sp
		Pencillium sp
		Fusarium sp
2	*Vitex negundo*	*Aspergillus* sp
		Pencillium sp
		Fusarium sp
3	*Andrographis paniculata*	*Aspergillus* sp
		Pencillium sp
		Fusarium sp
4	*Mentha piperita*	*Pencillium* sp
		Fusarium sp
5	*Tagetes erecta*	*Aspergillus* sp
		Pencillium sp
		Fusarium sp
6	*Azadiarcta indica*	*Aspergillus* sp
		Fusarium sp

Table 2: Larvividal activity of endophytic fungi isolated from *Ocimum sacnctum* on *Aedes aegypti*

Concentrations	Control	*Aspergillus* species	*Pencillium* species	*Fusarium* species
1.25ppm	0	0	0	0
2.25ppm	0	0	5.5 ± 0.08	0
5.00ppm	0	11.11 ± 0.12	16.66 ± 0.14	11.11 ± 0.11
10ppm	0	27.77 ± 0.18	33.33 ± 0.16	26.66 ± 0.13
20ppm	0	55.55 ± 0.21	61.11 ± 0.53	44.44 ± 0.23

Table 3: Larvividal activity of endophytic fungi isolated from *Vitex negundo* on *Aedes aegypti*

Concentrations	Control	*Aspergillus* species	*Pencillium* species	*Fusarium* species
1.25ppm	0	0	0	0
2.25ppm	0	20 ±0.11	0	0
5.00ppm	0	36.84 ± 0.17	9.5± 0.10	19.04 ±0.26
10ppm	0	50 ± 0.21	33.33 ± 0.13	42.85 ± 0.18
20ppm	0	90.47 ± 0.72	66.66 ± 0.16	85.71 ± 0.57

Table 4: Larvividal activity of endophytic fungi isolated from *Andrographis paniculata* on *Aedes aegypti*

Concentrations	Control	*Aspergillus* species	*Pencillium* species	*Fusarium* species
1.25ppm	0	0	0	0
2.25ppm	0	0	0	0
5.00ppm	0	0	12.5 ± 0.36	0 ± 0.18
10ppm	0	11.11± 0.18	27.77 ± 0.51	27.77 ± 0.27
20ppm	0	38.88 ± 0.23	33.33 ± 0.32	47.61 ± 0.32

Table 5: Larvividal activity of endophytic fungi isolated from *Mentha piperita* on *Aedes aegypti*

Concentrations	Control	*Pencillium* species	*Fusarium* species
1.25ppm	0	0	0
2.25ppm	0	0	0
5.00ppm	0	8.3 ± 0.02	0
10ppm	0	33.33 ± 0.18	23.80 ± 0.13
20ppm	0	53.33 ± 0.35	47.61 ± 0.18

Table 6: Larvividal activity of endophytic fungi isolated from *Tagetes erecta* on *Aedes aegypti*

Concentrations	Control	*Aspergillus* species	*Pencillium* species	*Fusarium* species
1.25ppm	0	0	0	0
2.25ppm	0	0	0	0
5.00ppm	0	21.02 ±0.12	9.5 ± 0.11	11.11 ± 0.12
10ppm	0	38.09 ± 0.21	28.12 ± 0.18	33.33 ± 0.17
20ppm	0	61.90 ± 0.23	72.22 ± 0.62	55.55 ± 0.34

Table 7: Larvividal activity of endophytic fungi isolated from *Tagetes erecta* on *Aedes aegypti*

Concentrations	Control	*Aspergillus* species	*Fusarium* species
1.25ppm	0	0	0
2.25ppm	0	0	0
5.00ppm	0	22.22 ± 0.13	33.33 ± 0.18
10ppm	0	50.00 ± 0.21	52.38 ± 0.21
20ppm	0	66.66 ± 0.32	83.33 ± 0.34

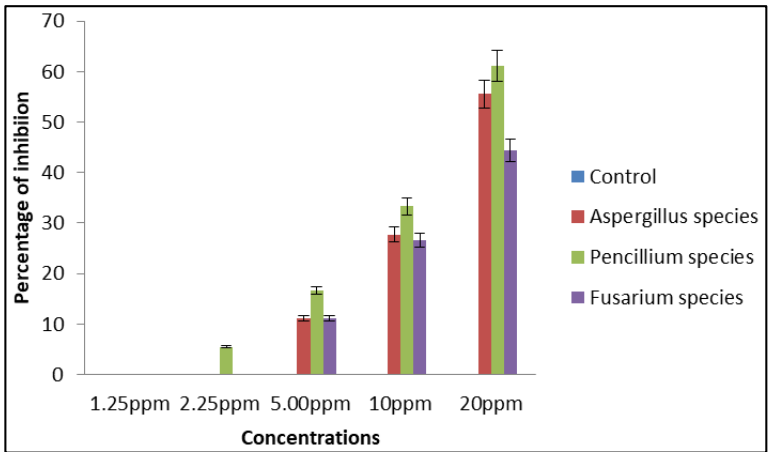

Fig 7: Larvividal activity of endophytic fungi isolated from *Ocimum sacnctum* on *Aedes aegypti* in 24 hour

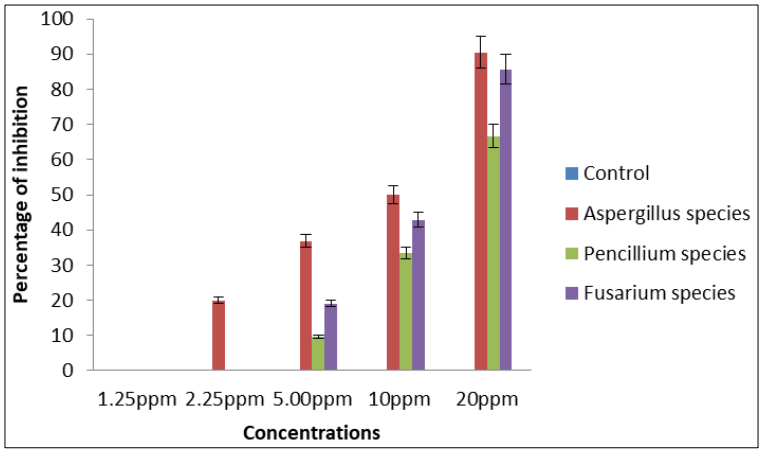

Fig 8: Larvividal activity of endophytic fungi isolated from *Vitex negundo* on *Aedes aegypti* in 24 hour

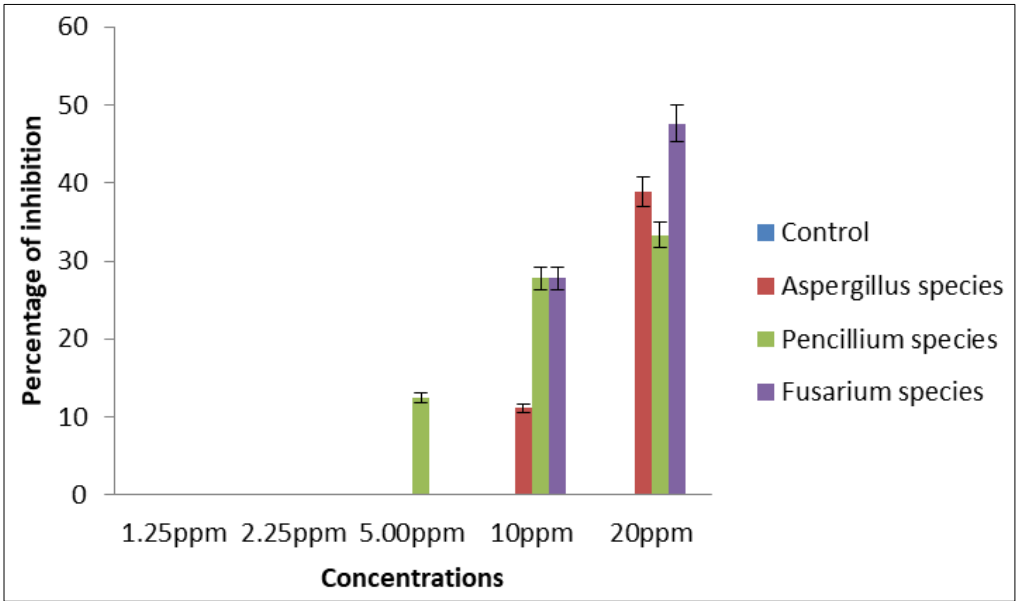

Fig 9: Larvividal activity of endophytic fungi isolated from *Andropgraphis paniculata* on *Aedes aegypti* in 24 hour

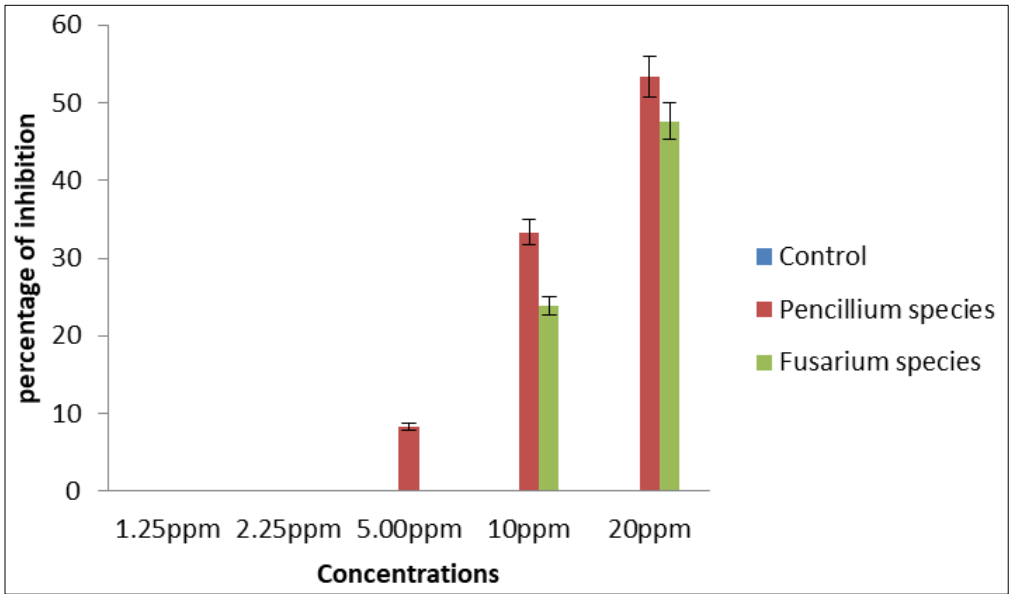

Fig 10: Larvividal activity of endophytic fungi isolated from *Mentha piperita* on *Aedes aegypti* in 24 hour

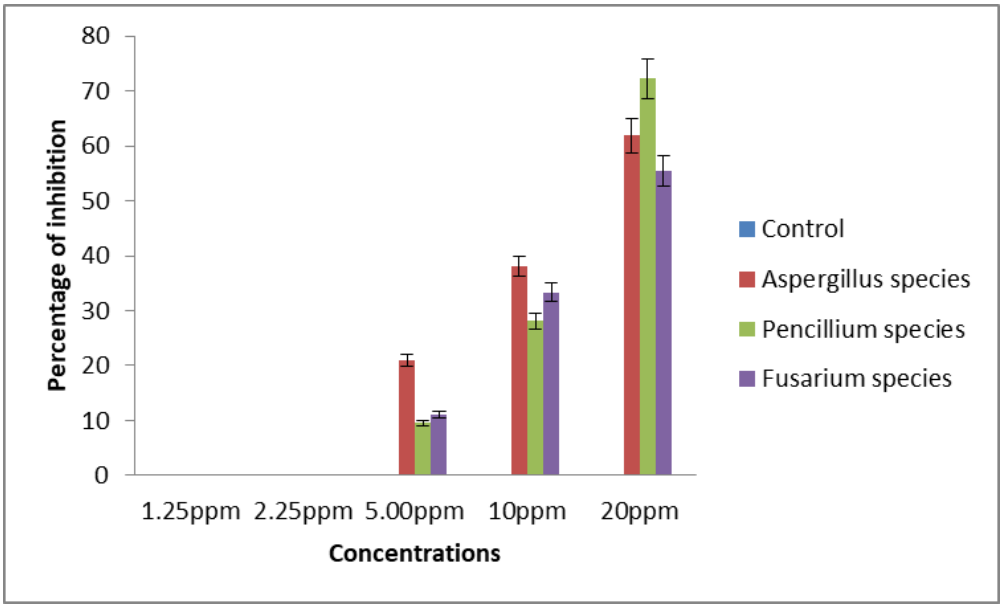

Fig 11: Larvividal activity of endophytic fungi isolated from *Tagetes erecta* on *Aedes aegypti* in 24 hour

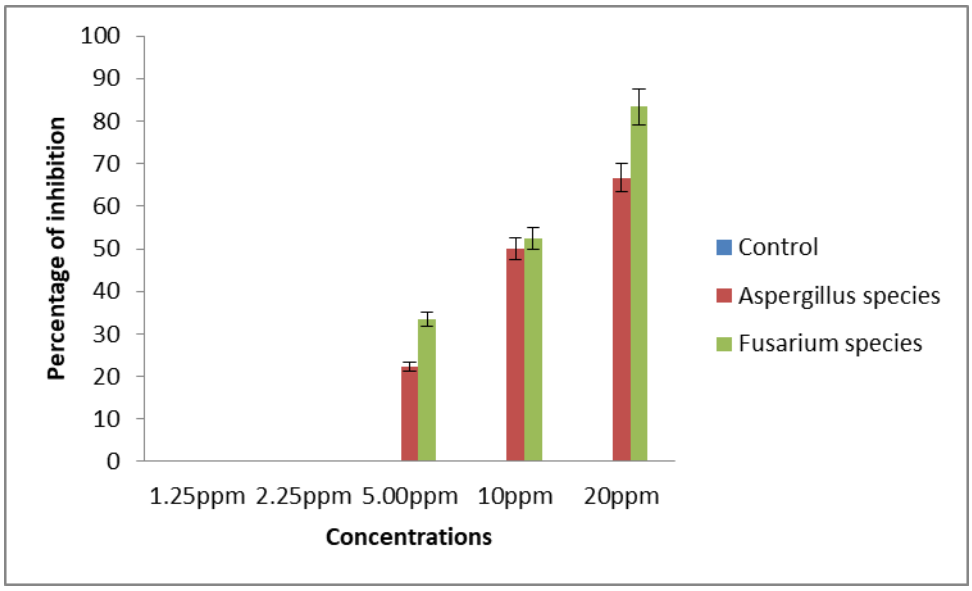

Fig 12: Larvividal activity of endophytic fungi isolated from *Azadirachta indica* on *Aedes aegypti* in 24 hour

Ananda Danagoudar [15] who reported the larvicidal activity of endophytic fungi isolated from *Tragia involucrata* on *Culex quinquefasciatus*. Vijayan and Balaraman [16] have previously reported the metabolites of 17 fungi are effective larvicidal agent against *Anopheles stephensi*. The larvicidal activity of endophytic fungi isolated from medicinal plants on mosquito species were reported by several investigators [17-20]. Our results were concurrence with Govindarajan *et al.* [1] who observed the effects of fungal species against the larvae of *Culex quinquefasciatus*.

Plants protect themselves from the pests and pathogens by symbiotically associating with the endophytes where plants provide the shelter to endophytes and in turn endophytes protect the plants against the pathogens. Endophytes with defensive secondary metabolites are selected by the host [21]. Rana *et al.* [22] and Yan *et al.* [23] opined that the endophyte fungi have been shown to protect their hosts against insect pests, pathogens, and even domestic herbivores. These include *Aspergillus flavus* and *Penicillium sublateritium* which live and feed on the host plant and in turn to produce functional metabolites that enhanced the fitness and resistance against stresses. A number of species, including selected *Fusarium* sp., are reported to act as antagonists against plant pathogens. Accordingly, fungi have commonly been used in practice as inocula to improve the growth of plants and suppress pathogens [24-26] Intriguingly, Bhattacharya and Chandra [13] studied the larvicidal activity of *T. involucrata* extract. However, the larvicidal potency of the endophytic extract was even better than the *T. involucrata extract*.

Conclusion

The endophytes isolated from leaves of *Ocimum sanctum*, *Vitex negundo*, *Andrographis paniculata*, *Mentha piperita*, *Tagetes erecta* and *Azadiarcta indica* showed varying degree of larvicidal activities. The highest larvicidal activity was observed in *Aspergillus* and *Fusarium* species from leaves of *Vitex negundo* on *A. ageypti* at maximum concentrations. From the results, we can conclude that *A. ageypti* was susceptible to the compounds in the fungus extracts. Such findings could be useful in promoting research aimed at the development of new mosquito control agents, based on bioactive chemical compounds from indigenous fungus sources as an alternative to chemical larvicides. Further studies on the isolation, characterization and understanding the mechanism of action of lead molecules will aid in the formulation of a novel as well as cost effective drugs against mosquito caused diseases.

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