Advances in Biotechnology & Microbiology
ISSN 2474-7637

Juniper Publishers key to the Researchers

Research Article
Volume 9 Issue 1 - April 2018
DOI: 10.19080/AIBM.2018.09.555752

Adv Biotech & Micro

Evaluation of Some Essential Oils against the Larvae of House Fly, Musca domestica by Using Residual Film Method

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Submission: December 24, 2018**; Published:** April 16, 2018

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Abstract

The housefly, *Musca domestica* L., is cosmopolitan insect, serves a role of vector for several etiological agents due to which it has attracted much attention for its control strategies. However, many tactics have been accomplished to combat its populations, among which use of essential oils seems to be the potential approach. Therefore in the present study, 3 different essential oils such as thyme (*Thymus vulgaris*), clove leaf (*Eugenia caryophyllus*), and basil (*Ocimum basilicum*) were tested against the 3rd instar larvae of *Musca domestica.* The attempts were made to assess the larvicidal activity by using residual film method and the effect on biochemical aspects like sugar, glycogen and protein contents in treated larvae were also determined. Thyme essential oil was found to be highly effective (LC_{50}, 2.82mg/ml) followed by clove (LC_{50}, 3.79mg/ml), and basil oils (LC_{50}, 5.99mg/ml). In biochemical assay, all essential oils under study showed potential influence on sugar, glycogen, and protein content of treated larvae. Basil oil significantly affects total sugar content (7.45±0.57µg/larva) whereas clove essential oil highly affects total glycogen (136.17±5.32µg/larva) and protein contents (246.06±7.12µg/larva) of *M. domestica*. These results reveal the significant efficacy of tested essential oils against *Musca domestica* which could be used to breakdown its populations.

Keywords: *M. domestica;* Essential oil; Residual film method; Larvae; Thyme; Efficacy

Introduction

Insects represent one of the largest groups of animals on earth, constitute over 1 million species and are still counting [1] serving many trophic roles like pests, pollinators, and vectors. The housefly Musca domestica, (Diptera: Muscidae) is an important medical and veterinary insect that causes irritation, spoils foods and acts as a vector for more than 100 species of pathogens, thereby causing serious threat to human health and livestock [2-4]. The control of this insect largely relies on synthetic insecticides which have led to many serious issues like resistance development [5], ecological imbalances, bioaccumulation and harm to non-target organisms, environmental contamination and incorporation in to food chains [6]. Therefore, considerable efforts have been made to develop the promising alternatives to these conventional insecticides.

Table 1: RFM: residual film method, FB: Fumigation bioassay

Sr. no.	Essential Oil	Organism	Method	References
1	Citronella sp.	*Ae. aegypti*	larvicidal assay	[14,15]
2	*Aniba rosaeodora*	*Ae. aegypti*	larvicidal assay	
3	*Lavandula angustifolia*	*Ae. aegypti*	larvicidal assay	
4	*Cinnamomum camphora*	*Ae. aegypti*	larvicidal assay	
5	*Pelargonium graveolens*	*Ae. aegypti*	larvicidal assay	
6	*Thymus serpyllum*	*Ae. aegypti*	larvicidal assay	
7	*Amyris balsamifera*	*Ae. aegypti*	larvicidal assay	
8	*Citrus limon*	*Ae. aegypti*	larvicidal assay	
9	*Juniperus virginiana*	*Ae. aegypti*	larvicidal assay	
10	*Boswellia carteri*	*Ae. aegypti*	larvicidal assay	

11	*Anethum graveolens*	*Ae.s aegypti*	larvicidal assay	
12	*Myrtus communis*	*Ae. aegypti*	larvicidal assay	
13	Bay, Clove leaf	*T. vaporariorum*	larvicidal assay	[16]
14	Cardamom	*C. maculatus*	RFM, FB	[17]
15	Cinnamon	*C. maculatus*	RFM, FB	
16	Clove	*C. maculatus*	RFM, FB	
17	Eucalyptus	*C. maculatus*	RFM, FB	
18	*Azadirachta sp.*	*C. maculatus*	RFM, FB	
19	Eucalyptus	herbivores	repellent/anti feedant	[18]
20	Thyme	*C. quinquefasciatus*	larvicidal	
21	*Mellisa officinalis, Nepeta cataria*	*M. domestica*	adulticidal	[19]
22	Clove	*C. pipiens*	repellent	[20]
25	*Amyris balsamifera*	*C. quinquefasciatus*	larvicidal	[21]
26	*Anthemis nobilis*	*C. quinquefasciatus*	larvicidal	
27	*Tagites terniflora*	*T. castaneum, S. oryzae*	contact	[22]
28	*Cymbopogon citratus*	*T. castaneum, S. oryzae*	contact	
29	*Elyonorus muticus*	*T.castaneum, S. oryzae*	contact	
30	*Citrus aurantium*	*M. domestica*	adulticidal	[23]
31	*Coriandrum sativum*	*M. domestica*	adulticidal	
32	*Eucalyptus cinerea*	*M. domestica*	adulticidal	
33	*Mentha piperita*	*M. domestica*	adulticidal	
34	*Syzygium aromaticum*	*M. domestica*	adulticidal	
35	*Cymbopogon citratus*	*T. ni*	topical	[24]
36	*Litsea pungens*	*T. ni*	topical	
37	*Cinnamomum glanduliferum*	*T. ni*	topical	
38	*Thymus vulgaris*	*T.ni*	topical	
39	*Ilex purpurea*	*T.ni*	topical	

The extracts derived from plant parts have been traditionally used by humans since ancient times to control pest problems [7]. Similarly, essential oils (Eos) along with many other metabolites of plant origin constitute components of the plant defence mechanisms and strong drivers of evolutionary events inducing selection pressures to herbivores. During the last decade, Eos have received much attention as pest control agents because of their insecticidal properties (Table 1), low mammalian toxicity and rapid degradation in the environment. They also tend to have broad-spectrum activity, relative specificity in their mode of action, easy to process and use. These Eos tend to be safe for animals and environment [6] making those potent compounds as suitable biological control agents. Mreover, Eos are found abundantly in some plant families such as Apiaceae, Pinaceae, Lamiaceae, Myrtaceae, Rutaceae, Umbelliferae, Asteraceae, Annonaceae, Zingiberaceae, and Lauraceae [8-11] etc. These oils are complex mixtures of chemical compounds thereby induce several mechanisms of toxicity to insects. Protein denaturation, enzymatic inhibition and membrane disintegration are some of the suggested modes [12,13] instigated by Eos.

Keeping in view the above objectives, 3 essential oils such as basil, clove and thyme were used to investigate the larvicidal activity against the larvae of *M. domestica* by using residual film method. Further, the effect of treatment on various biochemical aspects such assugar, glycogen and protein content in *M. domestica* larvae were also evaluated.

Materials and Methods

Chemicals and reagents

The Eos manufactured by Samed Agro Services Pvt. Ltd., Maharashtra, India, were procured from local markets. These oils were diluted in acetone to make the working solutions and stored in air tight containers at 4 °C until use. All other chemicals/reagents were of highest purity and molecular or analytical grade.

Rearing of housefly

The *M. domestica* nucleus culture was obtained from Entomology Section, national chemical laboratory (NCL), Pune (M.S.), India. The adult flies were reared at constant temperature of 28±2 °C and a relative humidity of 58-68% in plastic jars

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(35 x 15cm), covered with cheese cloth to prevent the escape. The flies were fed *ad libitum* by using a cotton swab soaked in milk which was changed after every 24hrs. The cotton swab also served as a substratum for oviposition by adult flies. After proper oviposition the eggs were transferred to another set of jarsfor hatching and larval development.For emergence from the pupation the pupae were separated to determine the growth stage of the flies.

Larvicidal assay

The larvicidal assay was carried out by using residual film method of Busvine [25] with few modifications. To determine the effect of different doses of Eos, 3rd instar larvae of housefly were used. Initially, a range of doses were taken to assess the effective concentrations of Eosrequired for the mortality of larvae. Similarly, many desirable doses were tested to determinethe LC_{50} and LC_{90} values. For residual film method, 1ml of Eos with desired dose, dissolved in acetone (v/v) were spread on filter paper discs kept inside theglass petri-dish of 90mm diameter. The Eos were applied in such a way to make a uniform film over the filter paper discs. For solvent evaporation, the treated petri-dishes were air dried for few minutes followed by release of larvae (n=10) and then incubating the plates at desired temperature and humidity for 24hrs. For control experiments, only acetone solution was sprayed on filter papers. The actual dose of Eopresent in 1ml mixture was calculated as dose per square centimetreby using the following formula.

Effect of Eos on nutritional reserves of the larva

Effect on total body sugar and glycogen content: Since the treatment of Eos affects the digestive physiology and metabolism of the insects, we tested the effect on sugars, glycogen and protein content of the larvae. In Larvicidal bioassays, 10 larvae were exposed to the LC_{50} concentrations of Eos for 24 hours. After the treatment, the larval bodies were collected and further processed for biochemical analysis by using method of Van Handel and Day [26] with slight modifications. Briefly, the housefly larvae were homogenized in 0.2ml anhydrous sodium sulphate solution following the addition of 0.8 ml solution containing equal volume of methanol and chloroform (1:1, v/v). The mixture was centrifuged at 3000rpm for 2min and the pellet obtained was used for glycogen estimation. The supernatant obtained was again suspended in 3ml of deionized water and then agitated at 3000rpm at 4 °C for 3min. The aqueous layer of the sample was utilized to determine the sugar and glycogen contentsby usingAnthrone method with glucose and glycogen as standards respectively.

Estimation of total protein contents

The effect of Eoson total protein content of the larvae was also studied by estimating the protein with Bradford method [27], using bovine serum albumin (BSA) as standard. Briefly, samples were prepared after crushing the larvae in 1.0ml of lysis buffer, followed by centrifugation at 5000rpm for 5 minutes at RT. The reaction mixture contained 0.2ml. Supernatant and 2ml Bradford reagent, incubated for 10min at RT.The standard curve was generated after measuring the absorbance in a spectrophotometer (Carry 60, Agilent technologies, USA) at 595nm. All bioassays were replicated three or more independent times.

Statistical analysis

Results obtained are reported as mean ± standard error means of three or more independent replicates which were subjected to one way ANOVA using Tukey's test ($p<0.001$). Analysis of data was carried outin SPSS software version 19 [28] and Microsoft Excel.

Results

Larvicidal potential of tested Eos

The present study demonstrates the efficacy of thyme, clove and basil Eos against the larvae of *M. domestica* . The highest effectiveness was observed with thyme Eo showing a lethal dose (LC50) of 2.82mg/ml (Table 2). Though, the efficacy of basil and clove oils were quite significant with respect to control groupsbut much lesserwhen compared to thyme essential oil. The lethal doses such as LC50 and LC90 for clove leaf EO were 3.79mg/ml and11.45mg/ml respectively while in case of thyme Eo LC50 and LC90 values were 2.82mg/mland 6.87mg/ml respectively. However, EO from basil showed a higher dose for LC50(5.99mg/ml)and LC90 (12.74mg/ml) values the chi-square values were found to be very significant at $p<0.001$ level indicating the heterogeneity of the house fly populations. With increase in the dosage of Eos, the mortality of larvae was also increased showing a linear effect on larvae depicting a paramount efficacy of tested Eos against the *M. domestica* populations (Figure 1a-1c).

Table 2: Each value represents mean of five or more replicates, LCL: lower confidential limit, UCL: upper confidential limit, df: degree of freedom, LC_{50} and LC_{90} were lethal concentration at which 50% and 90% population dies respectively. *Significant at $p< 0.001$.

Essential Oil	LC_{50} (mg/ml)	95% Confidential		Regression Equation	LC_{90} (mg/ml)	Chi square
		limit LCL	(LC_{50}) UCL			
Thyme	2.82	2.6	3.06	y=3.49+3.33x	6.87	7.49*(7)
Clove	3.79	3.44	4.19	y=3.45+2.67x	11.45	8.32*(7)
Basil	5.99	5.55	6.44	y=1.96+3.90x	12.74	5.48*(7)

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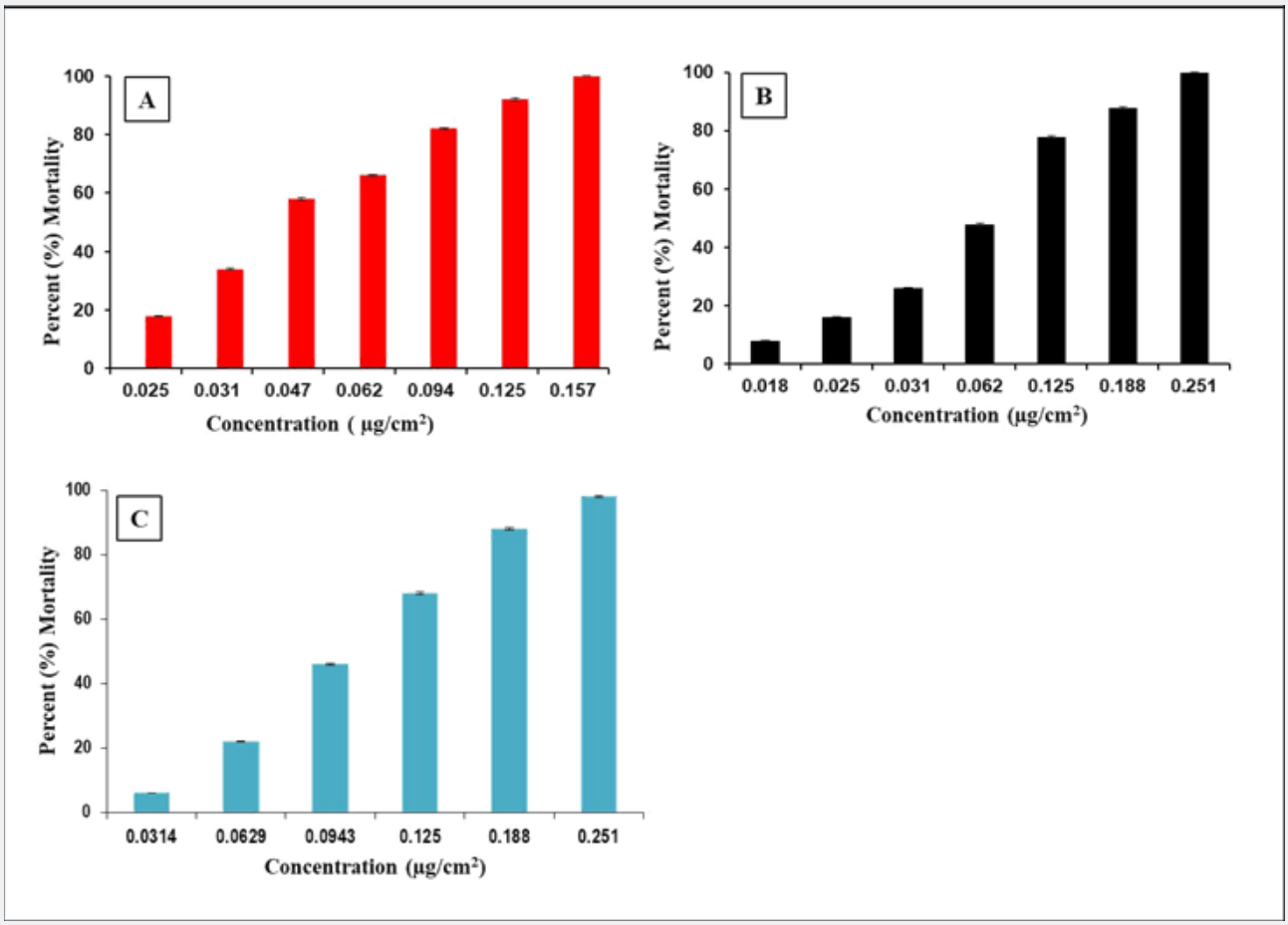

Figure 1: Larvicidal activity of essential oil of A) Basil; B) Clove and C) Thyme Eos on the larvae of *M. domestica.* Significant *p< 0.001 when compared to control.

Effect of Eos on the nutritional reserves of the larvae

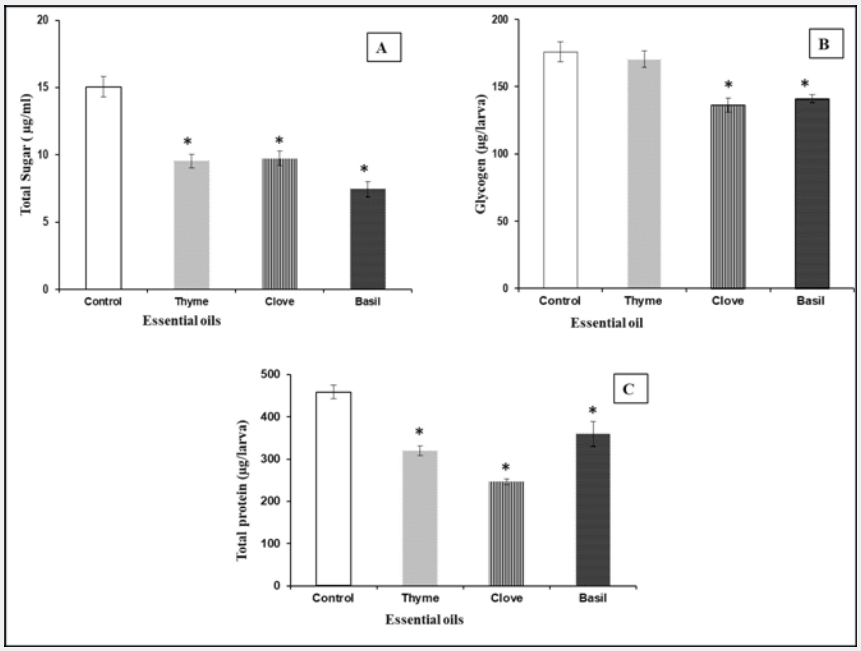

Figure 2: Effect of LC_{50} doses on nutritional reserves of 3rd instar larvae of M. domestica, A) total- Sugar; B) Glycogen and C) protein contents. Significant *p< 0.001 when compared to control.

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The chemical components present in the Eos always play a diverse effect on the metabolism of the larvae. To determine these changes we checked the effect of Eos on the glycogen,sugar and protein contents of larvae. To observe the changes in nutritional reserves, the larvae were sacrificed after exposure to different LC_{50} concentrations of Eos as shown in Figure 2. For each test, a control set was used where larvae were treated with acetone only. The control larvae showed a higher amount of sugar 15.05µg, glycogen 175.80µg, and protein content 458.17µg which were reduced to much lower levels treated larvae. All the 3 tested oils showed remarkable influence on metabolism of 3rd instar larvae of *M. domestica* . Among which , basil Eo was found to be much effective showing a 2 fold decrease in sugar content due to treatment. The effect on total body proteins by clove oil was noticeable which was reduced by 46.28% after the treatment. However, the effect on glycogen content of the larvae was not as significant as sugar and protein. From these changes it is quite evident that Eos cause subtle changes at cellular or molecular levels.

Discussion

The M.domestica is an eusyanthropic fly species linked to the human habitat, and a chief offender among the filth breeding and feeding flies worldwide [29]. The indiscriminate feeding habits along with structural morphology (presence of hair and sticky pads) make these flies ideally suited to carry and disseminate pathogens [29-31]. The species of *M. domestica* is involved in the dissemination of enteric pathogens such as *Escherichia coli, Shigella* spp., *Salmonella* spp., *Helicobacter spp., V. cholerae*. So there is a dire need to control these disease transmitting vectors using naturally occurring biocontrolagents of plant origin. Essential oils are considered the best alternative for the control of pests and vectors because of their inherent insecticidal properties and their action involves many elements of acetylcholinesterase inhibition and octopaminergic effects [32]. Additional effects can be seen in behaviour modification (attraction/repellency), contact toxicity for different life stages [33].

These Eos exhibit larvicidal, pupicidal or adulticidal properties while some of them may be antifeedants, ovipositional deterrents and insect growth regulators for housefly as well as for some other pests. In addition to contact toxicity [34], some plant derived essential oils are volatile and can act like fumigants offering the prospect for use in store-grain industry [35-38]. Some studies have focused on the use of essential oils from plants as potential bioactive agents against mosquitoes viz., *A. aegypti* [39]. Many plants have been reported to bear the potential insecticidal actions on different stages of *M. domestica* via crude extracts or extracted active compounds [40-42]. Some studies have also reported the effects on life stages like metamorphosis fecundity, life span of house flies [42]. Pavela and co-workers [19], screened 34 essential oils, extracted from plants, against *M. domestica* where the *Pogostemon cablin* essential oil was found to be the most efficient at a lethal dose of 3µg/fly. Larvicidal efficiency was determined for the most significant oil thymol namely the lowest doses LD50 32.9 and 14.2 mg/l for the 3rd and 4th instars of Culex quinquefasciatus [19]. Similarly, Palacios et al. [23] studied the effects of 21 medicinal and edible plants against housefly in which they detected limonene (92.5%) and 1, 8-cineole (56.9%) as principle components responsible for insecticidal activity. Medicinal plants with pulegone, menthone, limonene and 1, 8-cineole are also reported to be toxic to house flies. Due to the medicinal values of many plants there is an increased interest in developing plant derived novel insecticides as an alternative tool to the synthetics.

In larvicidal assay thyme essential oil showed higher activity than basil and clove oils. During the study it was observed that mortality of larvae was dose dependent, as the dose concentration was increased, the % mortality of the larvae was also increased. Further the effect of higher doses was also distinguishable due to tanning of body colour from whitish to brownish black during the treatment. However, at low doses larvae showedmortality but without any change in the body colour. Hitherto, mechanism of action of Eos in insects is not clear and is reported to be diverse. According to Cantrell and his colleagues [43] larvicidal compounds act either by absorption to the cuticle, via the respiratory tract, or enter through the process of ingestion into the gastrointestinal tract. Once inside the body of larvae, these substances can reach the site of action or cause systemic effects by diffusion into different tissues [44]. In addition, the lipophilicity of the different constituents of Eos enables disruption and penetration through the lipoprotein matrix of the insect cell membrane [45]. Some Eos are known to cause the deterrent or anti-feeding behaviour in insects suggesting a neurotoxic action [44] while some act as insect growth regulators through analoguesor antagonistic effects to endogenous hormones. In the present study it was found that even relatively short-term exposure of larvae to lethal doses can markedly increase their mortality over time, and thus reduce the total number of viable adults, leading to a possible significant reduction in the overall populations. Which is in congruence with the observations, reported by Shalaby [46] and Ansari [15], who described that short-term exposure of larvae and adults to Eos may cause a significant reduction in fecundity and fertility. In the present investigation, the larvicidal activity of Eos followed the order thyme > clove > basil.

Recently, Bhatnagar et al. [47] stated that clove oil and eugenol can be very effective for controlling mites, termites and mosquitoes even at lower concentrations. Eugenol induced 100 % mortality in mosquitos such as *Anopheles stephensi, Aedes aegypti,* and *Culex quinquefasciatus* at a dose of 7L/ha in 30-35 minutes. Eugenol is known to kill mosquito larvae (specifically *Ochlerotatus caspius*) with LC_{50} values of 7.53mg/L for 24 hours and 5.57mg/L for 48 hours [48].

Proteins are mainly involved in the architecture of the cell which is the chief source of nitrogenous metabolism. Insects have very little carbohydrates, and proteins that serve as a reservoir

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to meet the increased energy demands of the body. It is clear from the present study that highly significant reductions in the level of total protein, glycogen and sugar as compared to control treatment was induced by the Eos. The decreases in total protein level in different developing stages suggest the high protein hydrolytic capacity due to elevation of protease activity. Some studies have suggested that inhibition of DNA synthesis due to the action of Eos might affect protein synthesis machinery of the insect. Similarly, Senthilkumar & his team [49] who reported a drastic fall in the concentrations of carbohydrates, proteins and lipids. An immediate explanation to maximum reduction in sugar and glycogen content after treatment could be due to their involvement to combat the chemical stress. Hence, treatment with Eos might have interrupted this process and resulted in larval mortality. The data obtained from the present study clearly indicate that these 3 Eos were quite effective as larvicides for providing a better and potential alternative for the control of *M. domestica* infestations.

Conclusion

In present study 3 different essential oils were used to assess the larvicidal activity by using residual film method. The effects of tested Eos on nutritional reserves like, total body sugars, glycogen and protein content of *M. domestica* were also determined. In biochemical analysis, all 3 Eos showed remarkable influence suggesting the disruption of the defence mechanism of 3rd instar larvae of *M. domestica* . Consequent upon the findings of the present study, these essential oils can find a place in eco-friendly strategy for housefly management programme upon further field trials.

Acknowledgement

This work has been carried out under the scope of BCUD grant (15-SCI-001422), sanctioned by S. P. Pune University, Pune (M.S.), India. MD is indebted to the University Grants Commission, New Delhi, India, for Maulana Azad National fellowship. Corresponding author acknowledges the administration of S. P. Pune University, Pune for the grant sanctioned under DST PURSE and DRDP Schemes.

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How to cite this article: Jyoti M C C, Mudasir A D, Radhakrishna S P**.** Evaluation of Some Essential Oils against the Larvae of House Fly, Musca domestica by Using Residual Film Method**.** Adv Biotech & Micro. 2018; 9(1): 555752. DOI: 10.19080/AIBM.2018.09.555752

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