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Laboratory-based Evaluation of The Potential of *Beauveria bassiana* Crude Metabolites for Mosquito Larvae Annihilation

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Abstract: *Malaria is a disease of concern with respect to morbidity and mortality. Intent of the study is to use the metabolite of Beauveria bassiana to control the survival of larvae of malarial parasite. For Dichloromethane extract,20%, 20%, 60% and 100% larval death occurs after one and half hour for the extract 7.5 mg, 15 mg, 22.5 mg and 30 mg respectively whereas, for Chloroform extract 40%, 60% and 100% larval death occurs after one and half hour for 7.5 mg, 15 mg and 30 mg extract respectively. Dichloromethane and Chloroform extract of Beauveria bassiana showed potential anti-larval activity.*

Key Words:*Malarial parasite, Larvae, Beauveria bassiana, Dichloromethane, Chloroform*

I. Introduction:

Malaria imposes great socio-economic burden on humanity and with six other diseases (diarrhea, HIV/AIDS, tuberculosis, measles, hepatitis B, and pneumonia), accounts for 85% of global infectious disease burden.[1,2] Malaria afflicts ~90 countries and territories in the tropical and subtropical regions and almost one half of them are in Africa, South of Sahara. About 36% of the world population (i.e., 2020 million) is exposed to the risk of contracting malaria.[3] The World Health Organization estimates 300-500 million malaria cases annually, with 90% of this burden being in Africa. In addition, the estimated annual mortality attributed to malaria ranges from 700,000 to 2.7 million globally and > 75% of them areAfrican children and expectant mothers. In the Southeastern Asian Region of WHO, of ~1.4 billion people living in 11 countries (land area, 8,466,600 km^2; i.e., 6% of global area), 1.2 billion are exposed to the risk of malaria, most of whom live in India.[13] However, Southeast Asia contributed to only 2.5 million cases to the global burden of malaria,of this, India alone contributed 76% of the total cases.[4,5,6]

The use of biological pesticides such as entomopathogenic fungi is growing in popularity because it is able to alleviate many of the concerns associated with chemical poisons. The use of entemopathogenic fungi have been revised significantly.[7-13] First, entomopathogenic fungi are found ubiquitously in the soil throughout the world, therefore they would not be considered as "introduced" organisms into the environment. Second, although *Beauveria bassiana* is considered a broad-spectrum insect pathogen, strains can be developed that are more hosts specific. With research into pathogenicity and strain specificity, it is anticipated that fungal biological control agents can be selected to target specific insect pest.[14]

Beauveria is one of the frequently isolated entomopathogenous fungi with a cosmopolitan distribution. *Beauveria bassiana* is an ubiquitous saprobe and can be found in soil or decaying plant material, where it grows as multicellular mycelia by absorbing nutrients from the decaying matter. Mosquitoes have been listed as one of the natural host of *Beauveria bassiana*.[15]*Beuveria bassiana* is non toxic to mammals, birds, or plants; and its use is not expected to have deleterious effects on human health or the environment.[16]Conidia of *Beauveria bassiana* are effective in killing mosquito larvae when applied as conidia dust in breedingsites. Besides infecting larvae, thefungus has also proved to be virulent to adult mosquitoes.[17,18,19]

Recently, theoretical and experimental studies have shown the potential of entomopathogenic fungi as next generation agents for the control of malaria mosquitoes. Currently there are no vaccines against malaria; however, studies have shown the potential for fungal entomopathogens to reduce the spread of this disease.[19]However, most of this work has focused on targeting adult mosquitoes. Larval control has a convincing history of malaria eradication and recent studies have also shown this approach to be highly effective.[1, 20, 21, 22-24] It is, therefore, worthwhile to investigate the ability of entomopathogenic fungi *Beauveria bassiana* to control mosquito larvae. The objective of this study was to assess the efficacy of bioactive compound extracted from *Beauveria bassiana* by using chloroform and dichloromethane.

II. Materials And Methods:

2.1 Isolation of *Beauveria bassiana*:

The fungus *Beauveria bassiana*was isolated from soil taken from experimental farms of Panjabrao Deshmukh Krishi Vidyapeeth, Nagpur.The serial dilution method resulted observable growth intoplates of 10^{-3}, 10^{-4} and 10^{-5} dilution. Using standard taxonomic keys, monographs, atlases and various websites isolates were characterized, identified and assigned to respective genera and species. White mucoid colonies were suspected as *Beauveria bassiana* were subcultured on potato dextrose agar. Identification was carried out on the basis of morphological and cultural characteristics.[25,26]

2.2 Extraction of Bioactive Compound:

2.2.1 Dichloromethane Extraction (Set-1):

Beauveria bassiana was inoculated into Saborauds Dextrose Broth. A 5 ml inoculum (9.75 X 10^6 spores/ml) was transferred in 5 flasks containing 250 ml Saborauds dextrose broth each. The flasks were kept in orbital shaker at 120 rpm at 25^0C, for 10 days. After incubation fungal mycelial balls were formed in media.The media were pressed through muslin cloth and mycelia were collected. Collected mycelia were soaked in 200 ml methanol for 48 hours. It was filtered through muslin cloth and allowed for evaporation of methanol upto dryness on heating water bath. The 150 ml distilled water was added to the concentrated extract and bioactive compound was extracted with 150 ml dichloromethane. The organic dichloromethane was evaporated on heating in water bath. Dark brown compound so obtained was used for further study.

2.2.2 Chloroform Extraction (Set-2):

Beauveria bassiana was inoculated into Saborauds Dextrose Broth. A 5 ml inoculums (10.25 x 10^6 spores/ml) was transferred in 5 flasks containing 250 ml Saborauds dextrose broth each. The flasks were kept in orbital shaker at 120 rpm at 25^0C, for 10 days. After incubation fungal mycelial balls were formed in media.The media were pressed through muslin cloth and mycelia were collected. Collected mycelia were soaked in 200 ml methanol for 48 hours. It was filtered through muslin cloth and allowed for evaporation of methanol upto dryness on heating water bath. The 150 ml distilled water was added to the concentrated extract and bioactive compound was extracted with 150 ml chloroform. The organic layer of chloroform was evaporated on heating water bath. Brownish-yellow compound obtained was used for further study.[27]

2.3 Field collection of mosquito larvae:

Mosquito larvae were collected as larvae from breeding sites in Nagpur city(MS), India in the July and August (2012) months of monsoon. Breeding sites varied from tyre tracks, irrigation pools, and shallow water to open concrete water tanks. Larvae were collected using the dipping method with the aid of copper ladle, transferred into plastic jars with perforated lids for ventilation and transported to the laboratory for rearing.

2.4 Weather Conditions in the study area:

Nagpur has tropical wet and dry climate with dry conditions prevailing for most of the year. It receives an annual rainfall of 1,205 mm (47.44 inches) from monsoon rains during June to September. Summers are extremely hot, lasting from March to June. On record temperature ranges highest47.9°C, to lowest 3.9 °C.

2.5 Laboratory rearing of mosquitoes:

Larvae were cultured in plastic containers, 5 cm height by 27 cm width by 36 cm long with a large surface area and a water depth not more than 4 cm at 25 ± 2^oC and 70 - 75% relative humidity. A 12 hours light and dark cycle was maintained[28]. A 20-25 mg farex was provided as larval feed every 24 hours in each of the larval rearing basins.

2.5 Antimosquito larval activity test:

2.5.1 Set-1: Total 8 beakers containing 10 ml distilled water and 10 mosquito larvae were taken. Out of them 4 were used as control and 4 were used as experimental. The 100 mg extract containing bioactive compound was dissolved in 2 ml methanol i.e., 50 mg per ml. From this 1.5 ml extract was taken and dissolved in 8.5 ml distilled water i.e., 7.5 mg/ml. Then it was tested against mosquito larvae in various concentrations such as 1 ml – 7.5 mg, 2 ml – 15.0 mg, 3 ml – 22.5 mg and 4 ml – 30.0 mg. For control 15% methanol was prepared and applied as 1 ml, 2 ml, 3 ml and 4 ml.

2.5.2 Set-2: Total 8 beakers containing 10 ml distilled water and 10 mosquito larvae were taken. Out of them 4 were used as control and 4 were used as experimental. The 375.4 mg extract containing bioactive compound was dissolved in 5ml DMSO. From this1 ml extract was taken and dissolved in 9 ml of distilled water i.e.,

concentration of extract is 7.5 mg/ ml. Then it was tested against mosquito larvae in various concentrations such as 1 ml – 7.5 mg, 2 ml – 15.0 mg, 3 ml – 22.5 mg and 4 ml – 30.0 mg. For control 1ml DMSO was dissolved in 9ml distilled water and applied as 1 ml, 2 ml, 3 ml and 4 ml.[29]

III. Results:

In the present study it was planned to have an insight into the sourcing of entomopathogenic fungi from nature. Entomopathogenic fungi can be found infecting living as well as dead insects in a variety of habitats. So an exercise was carried out to isolate *Beauveria bassiana*, laboratory culturing and metabolite extraction, and to evaluate its activity against field collected mosquito larvae. The fungus *Beauveria bassiana* was isolated successfully from soil. In set-1, total extract obtained was 100mg. The extract was applied to test antimosquito larval activity were 7.5 mg, 15 mg, 22.5mg and 30 mg. Till the first half hour there was no death in the beaker. After first half hour the movements of larvae become slower in the beaker containing 15 mg and 22.5 mg extract and 1 larva died in the beaker. After 1hr the movement of larvae in all the beakers slower down and larval death occurs in all four beakers.There was 20%, 20%, 60% and 100% larval death occurs after one and half hour in the beakers containing 7.5 mg, 15 mg, 22.5 mg and 30 mg dichloromethane extract respectively, except, in set-4 with 4ml of dichloromethane extract at 11:35 am and 1 ml extract at 12:35pm showed slow activity whereas, at 1:05pm set-3 with 4ml extract showed 90% larval death. In set-1 beaker containing 30 mg dichloromethane extract showed 100% positive mosquito larvicidal effect within one and half hour (Table 1) (Fig.1).

In set-2, total extract obtained was 375 mg. The extract was applied to test antimosquito larval activity were 7.5 mg, 15 mg, 22.5mg and 30 mg. Till the first hour there was no larval death in the beaker. After first hour the movements of larvae become slower in all the beakers and 2, 3 and 3 larvae were died in the beaker containing 15 mg, 22.5 mg and 30 mg extract respectively. After 2hrs the movement of larvae in all the beakers becomes slower and there was again 2 and 3 larvae died in the beaker containing 15 mg and 30 mg extract respectively. After two and half hour there was 4 more larvae died in the beaker containing 30 mg extract. There was 40%, 60% and 100% larval death occurs after one and half hour in the beakers containing 7.5 mg, 15 mg and 30 mg chloroform extract respectively, except set-2 with 4ml extract at 2:00 pm has given 20% larval death and at 3:00 pm gives 40% larval death respectively (Table 2) (Fig. 2).

Interpreting results showed thatthe beakers containing 30 mg chloroform and dichloromethaneextract has given 100% positive mosquito larvicidal effect within two and half hours.Unusual results in few sets considered to be a false result shown by larvae might be due to physiological defects and low immunity of the larvae.From the results, it can be concluded that fungal extract of *Beauveria bassiana* containing bioactive components have the ability to kill mosquito larvae.

IV. Discussion:

The results of this study showed dichloromethane and chloroform extract of *Beauveria bassiana* have the ability to produce the bioactive compound for inhibiting mosquito larvae which confer to its lethality. In set-1 beaker containing 30 mg dichloromethane extract shows 100% positive mosquito larvicidal effect within one and half hour (Table 1). In set-2, the beakers containing 30 mg chloroform extract shows 100% positive mosquito larvicidal effect within two and half hours[28, 30] (Table 2). On evaluation of 17 species, LC_{50} values obtained to be in the range of 3-24 µl/ml against *Culexquinquefasciatus* 3 rd instar larvae on 48hrs.[31]A crude extract of tolypin caused 100% mortality in the larvae of *Culex. pipiens* and *Anopheles maculipennis* at a concentration of 11.1/m1.[33] Bioassay of dichloromethane extract from mycelium of *B. bassiana* at 100 ppm showed activity against *Aedes aegypti* larvae.[32] The extract had Beauvericin and two analogues (Beauvericin A and B). For the study of *Beauveria bassiana* metabolite extract, 86% mortality observed with beauvericin in *Aedes aegypti* larvae after 48 h exposure at 20 g/ ml,[34] but only 39% when half the dose was used (10 g /m1. With LD50 values of 10 to 100 ppm a mixture of 70% destruxin A and 30% B from *Metarhizium anisopliae* was shown to be toxic to mosquito larvae.[35]Thus, entomopathogenic fungi *Beauveria bassiana* extracted with suitable solvents are a promising tool for control of larval populations of malaria mosquitoes. These results provide evidence that the entomopathogenic fungus *Beauveria bassiana* has potential for useas an alternative vector control tool against insecticide-resistant mosquitoes. Many entomopathogenic fungi of the Hyphomycetes are known to produce toxic insect secondary metabolites in nutrient-rich media. Some of these metabolites from Hyphomycetes have been isolated and identified[36-39]and their chemical structures have been elucidated (e.g. oosporein, beauvericin and beauveriolides). Studies have shown that quantities of these metabolites produced in vivo are usually much less than those secreted in nutrient media.[40]

These mosquito-pathogenic fungi need to be studied further extensively with respectto large-scale production of conidia, the stability of these formulations underlaboratory and field conditions needs to be evaluated. Secondary metabolites have shown promising larvicidal activity, the active fraction needs to be studied in detailand has a potential of commercial exploitation.

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Table 1: Antimosquito Larval Activity of Dichloromethane Extract (DCM)

Time/Extract	Set	C-1	1ml (7.5mg)	C-2	2ml (15mg)	C-3	3ml (22.5mg)	C-4	4ml (30mg)
11:35 a.m	1	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-	Slow activity
	5	-	-	-	-	-	-	-	-
12:05 a.m	1	-	-	-	Slow activity	-	Slow activity	-	1 Death
	2	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-	-
12:35 a.m	1	-	-	-	Slow activity	-	Slow activity	-	Slow activity
	2	-	-	-	Slow activity		Slow activity		Slow activity
	3	-	-	-	Slow activity	-	-	-	-
	4	-	Slow activity	-	Slow activity	-	Slow activity	-	Slow activity
	5	-	-	-	Slow activity	-	Slow activity	-	Slow activity
01:05 a.m	1	-	2 Death	-	2 Death	-	6 Death	-	9 Death
	2	-	2 Death	-	2 Death	-	6 Death	-	9 Death
	3	-	2 Death	-	2 Death	-	6 Death	-	8 Death
	4	-	2 Death	-	2 Death	-	6 Death	-	9 Death
	5	-	2 Death	-	2 Death	-	6 Death	-	9 Death
Larval Death in %		No Death	20%	No Death	20%	No Death	60%	No Death	100% (80% only for Set 3 of 01:05 a.m)

Where, C-1, C-2, C-3, and C-4 are control 1,2,3 and 4 respectively.

Table 2: Antimosquito Larval Activity of Chloroform Extract (CHL)

Time/Extract	Set	C-1	1ml (7.5 mg)	C-2	2ml (15 mg)	C-3	3ml (22.5mg)	C-4	4ml (30mg)
1:00 p.m	1	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-	-
2:00 p.m	1	-	-	-	2 Death	-	3 Death	-	3 Death
	2	-	-	-	2 Death	-	3 Death	-	2 Death
	3	-	-	-	2 Death	-	3 Death	-	3 Death
	4	-	-	-	2 Death	-	3 Death	-	3 Death
	5	-	-	-	2 Death	-	3 Death	-	3 Death
3:00 p.m	1	-	-	-	2 Death	-	Slow activity	-	3 Death
	2	-	-	-	2 Death	-	Slow activity	-	4 Death
	3	-	-	-	2 Death	-	Slow activity	-	3 Death
	4	-	-	-	2 Death	-	Slow activity	-	3 Death
	5	-	-	-	2 Death	-	Slow activity	-	3 Death
3:30 p.m	1	-	-	-	-	-	-	-	4 Death
	2	-	-	-	-	-	-	-	4 Death
	3	-	-	-	-	-	-	-	4 Death
	4	-	-	-	-	-	-	-	4 Death
	5	-	-	-	-	-	-	-	4 Death
Death in %		No Death	-	No Death	40%	No Death	60%	No Death	100%

Where, C-1, C-2, C-3, and C-4 are control 1,2,3 and 4 respectively.

Fig. 1: Larval Death In Percentage With Respect To Dichloromethane Extract (DCM) Concentration in mg.

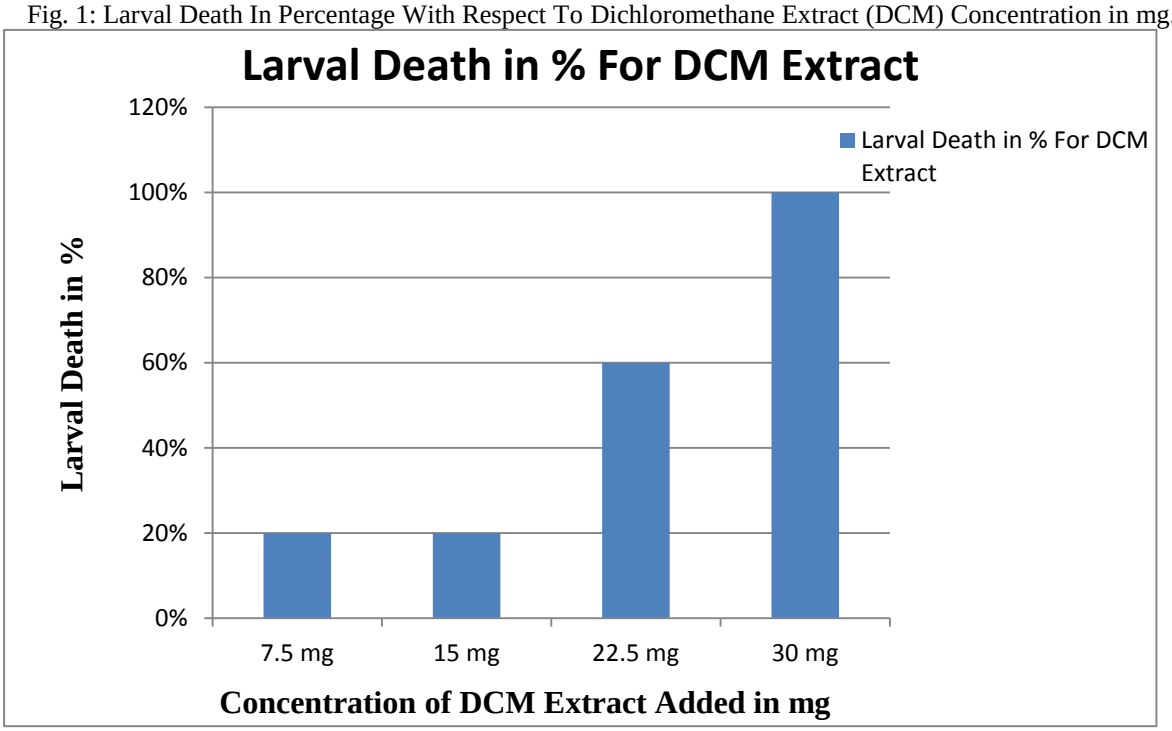

Fig. 2: Larval Death In Percentage With Respect To Chloroform Extract (CHL) Concentration in mg.