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CONTROL OF THE GRATER WAX MOTH GALLERIA MELLONELLA (L.), USING THE ENTOMOPATHOGENIC FUNGUS BEAUVERIA BASSIANA

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ABSTRACT

Beekeeping is an important activity to increase the family income in rural areas. However, honey bees pests and their control measurements is a great challenge. Despite the extensive use of insecticide to control the greater wax moth Galleria mellonella is the most distructive insect pests of honey bees in the tropic. Hence the present a study was aimed to test the entomopathogenic fungus Beauveria bassiana as microbiological control agent against G. mellonella. The third larvae instars were tested against different fungal suspension spores concentrations which were prepared from sub culture in the Microbial Pesticides formulation Unit (National Centre for Research) during the period from (2014-2015). The obtained result showed G. mellonella larvae were highly susceptible to the infection of B. bassiana. High mortality (20-100%) was achieved using different concentrations at exposure time of 11 days under laboratory condition (60-70 RH and 25C°±2). Mortality mean was significantly different among concentrations ($F= 35.75$, $P < 0.05$) and significantly higher in all the concentrations in comparison to control. The LC_{50} that can kill 50 % of G. mellonella larvae after treatment with 5µL of B. bassiana ITCC 6628 at different concentrations was estimated to be 01.40×10^7 conidia /mL .The LT_{50} was estimated at different concentration. The highest pathogenicity was obtained by concentration of 01×10^9 conidia /ml at estimate LT_{50} value of 7 day and 12 hours. The present study clearly showed that B.bassiana is a promising biological control agent against G. mellonella**,** but this finding needs more investigation in the field of non-selectivity and formulation improvement of B. bassian.

Keywords: Beauveria bassiana**,** Galleria mellonella, Entomopathogenic, Beekeeping

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1. INTRODUCTION

In addition to their valuable products, the honeybees, *Apis mellifera,* are key pollinators for agriculture and the maintenance of natural biodiversity (Aebi et al., 2012). However, their health status is currently of considerable concern, due to worldwide massive losses of managed colonies (Neumann and Carreck 2010). As continuity of scientific development, the man put great efforts in pest and disease control increases as result of bee keeping industry progressed.

Honey bee is attack by many pests among of which is insect pest including (Ants, termites, beetles, wasps and moths). The most serious and noises insect pest of beekeeping in the Sudan were moths, namely the greater wax moth (*Galleria mellonella*) (El-Niweiri et al 2005). According to our field observation in Khartoum and River Nile States apiaries, there was great damage caused by the greater wax moth and the beekeepers always complain from it. The greater wax moth chewed their way down to the midrib of the comb (tunnels), this tunneling destroys the wax cell of the comb and also they can cause dead to bee brood.

Wax moth control is a comprehensive problem particularly in warm climates, the best practices is to make the colony stronger and healthier because weak colonies increases the wax moth population rapidly. The most important Chemical fumigants used to control the *G.mellonella* is paradichlorbenzene, but it is unfortunately does not kill all stages of the pest and does not clean up a severe case of moth; it remains only a preventative (Sanford, 1985).Physical control (protecting store combs by climate manipulation, gamma-ray irradiation and sterilization, and temperature manipulation) are the common methods used. The biological control treatment with *Bacillus thuringiensis*, used as a suspension sprayed onto the combs and its effect on the larvae of the wax moth last for several weeks (Sanford, 1985). Fungal agents are among the most promising group of biological control against insect pest (Reithinger et al., 1997). Over 500 species of fungi are known to have insect pathogenic properties. Interestingly, *Beauveria* and *Metarhizium* (*Deuteromycotina, Hyphomycetes*) represent the most frequently used genera (Burges and Hussey, 1971) and are known to infect a broader range of insect pests of crops belonging to Lepidoptera, Homoptera, Hymenoptera, Coleoptera and Diptera. Most research on fungi has been directed to *Beauveria* and *Metarhizium* (Greathead and prior,1990; Whitten and oakeshott, 1991). Entomopathogenic fungi (EPF), compared to other entomopathogenic microbial organisms can infect their host via contact i.e. Invade via epicuticle of integument, and do not need to be ingested by the insect to cause infection (Goettel et al., 2005; Ali et al., 2010) .These fungi are cosmopolitan and not leave undesirable residues hence can be used, even chose to harvest. Besides that, these are compatible with other pest management tactics. Additionally, their production is easy and economical and do not require high input technology

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(prior, 1998). Virulence is the most important indicator to measure the potential of fungi against pests and the basis of choosing highly virulent fungi in laboratory bioassays (Li et al., 2012).

In this research we would concentrate in biological control to suppress this pest because it is devastating and fortunately no chemical pesticide is registered for its control in the Sudan, thus biological control is the best solution because it's safe, cheap and environmental sound. Recently the world is focus on organic agriculture instead of chemicals.

The present study was therefore aimed to investigate the entomopathogenic fungus *Beauveria bassiana* as microbiological control agent against the grater wax moth *Galleria mellonella*.

2. MATERIALS AND METHODS

2.1. Rearing of the greater wax moth

Rearing greater wax moth was done as described by Bronskill (2013م). Different larval instars of the greater wax moth (many larvae) were collected from infected bee colony from different apiaries (Shumbat, Alfaki hashm and Wad Alblah). Selected apiaries were proved to be free of any kind of insecticide treatment. The collected larvae were then kept in a plastic container (bucket) in a dark room for experimental use. Inspection to check, different development and growth stages of the greater moth larvae was daily, and about 1kilo gram of clean free infected wax combs was added every three days this was continue for a period of (30-35 days approximately),then the 3rd larval instars were isolated and collected for the experimental studies .

2.2. Isolation of the pathogen

Four soil types were obtained from Toti island (Toti 1,Toti 2), Alzhari and Jebelawlia were supposed to be contaminated with the fungus *B. bassiana*, after removing roots and gravel soil, samples were weighted as (50,100,150,300,and 350 gm), respectively according to soil types, varies from loamy to clay soils.

All samples were placed into an empty plastic cups containers, About *7 Galleria mellonella* 3rd instars' larvae were prepared for boiling water test to prevent them to reach pupation stage development, the technique used is insect bait method (Zimmermann 1986) by boiling 500 ml of water up to 56c° then dipping them for 10 seconds, and after that dipping them in a normal cold water for 30c°, then placed in a dry tissue paper in a Petri dishes (9cm in diameter), under dark room condition for 3-5 hours to retain back its viability.

For pathogen treatment about 16 cups were filled with sieved soil then about 50ml of normal (absolute) water poured in cups for soil wetting for obtaining of good relative humidity favorable

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for infection pathogen growth, after that 7 larvae treated (in boiled water) were placed in each cup, and 5 gm of free infected wax were added to each cup to serve as a food for the tested larvae. All cups were taken to a humid place which is more conducive condition favorable for growth and development of the fungus.The larvae was inspected daily for fungal growth and kept down the soil to insure maximum fungal growth.

2.3. Media preparation

Beauveria isolates were cultured in Potato Dextrose Agar media (P. D. A.) prepared as fallow:-

Unpeeled potato is washed and cut to many cubes and then one litter of distilled water is added, and then boiled for one hour.

The potato water was extracted by a clean piece of fabric into 4 flasks; about 400 ml of the extracted water is added to 600(ml) of distilled water to each flask to complete the volume of the extracted potato water to 1000 ml (1litre). 8gm of dextrose and 8gm of agar is added to each flask containing 1000ml of extracted potato water.

Finally for each preparation of media one capsule of anti bacterial agents (chloramphenicol) is added to prevent bacterial growth. Then prepared media will be interned inside the autoclave for media disinfectant to prevent microorganism contamination that is by using autoclave and disinfectant is done to a period of 15 minutes at 121 c and pressure per square inch (psi), up to 15 lbs and incubated for reusing.

The prepared media was dissolved in a microwave for a period of 15 seconds and cool dawn, and then poured into sterile Petri dishes beside the flame.

After one day the already prepared media of three strains (A) ITCCNo.6628, (B) ITCCNo.6645, and Sudanese (S) show (Fig: 1) , then culture fungal of *B. bassiana* is scraping by a needle to have some spores of fungus to grow in this media in which placed in an incubator for fungal developmental growth for a period of 16 days.

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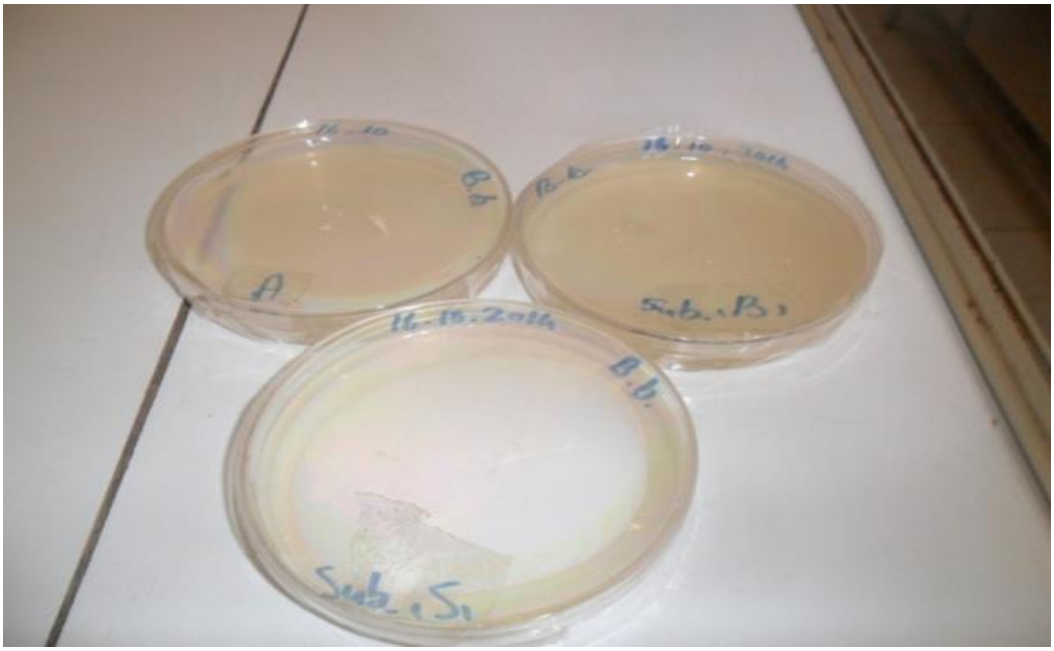

Fig: (1) Preparation of media (PDA) for three fungal strains of *B.bassiana* (A, B, S)

2.4. Screening Test:

Preliminary screening test was conducted to evaluate the pathogenicity of different *Beauveria* isolates including indigenous and imported ones to the wax moth larvae (Table 1). Sudanese strain *B. bassiana* and the Indian strain *B. bassiana* ITCC 6628 was tested against 3rd larval instars stage. Then these larvae were first equally contaminated with fungal powder spores into sub culture of fungus as single concentration for maximum challenge bioassay. Untreated wax combs (5gm) were added as diet in a Petri dishes (9cm in diameter) and immediately were placed into the incubator with 60-70% RH and 25±2C° for maintaining a conducive condition for a good fungal growth, then the same test were done but without contaminated larvae (free infected larvae) while the wax combs were contaminated with fungal powder spores as larval diet and placed into the incubator for the same testing purpose. The isolate that give mortality over 50% in 7 days was selected for further investigation.

2.5. Preparation of Indian sub culture

A bout 4 Petri dishes (9cm in diameter) containing potato dextrose agar (PDA) were prepared as sub culture by scratching the pure sub culture of fungus strain ITTCCNO.6628 to each Petri dishes, then they inoculated using needle, then the inoculated culture placed inside the incubator at temperature 25 C° to a period 7-10 days to achieve maximum growth and sporulation (fresh) (Fig: 2).

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Table (1): Source information of different strain of entomopathogenic fungus *(B.bassiana*).

Isolate	Host	Origin
B. bassiana No.6628	*Phyllocoptruta oleivora*	Indian
B. bassiana No.6645	*White grub*	Indian
B. bassiana	Beetle (Unidentified species)	Sudan

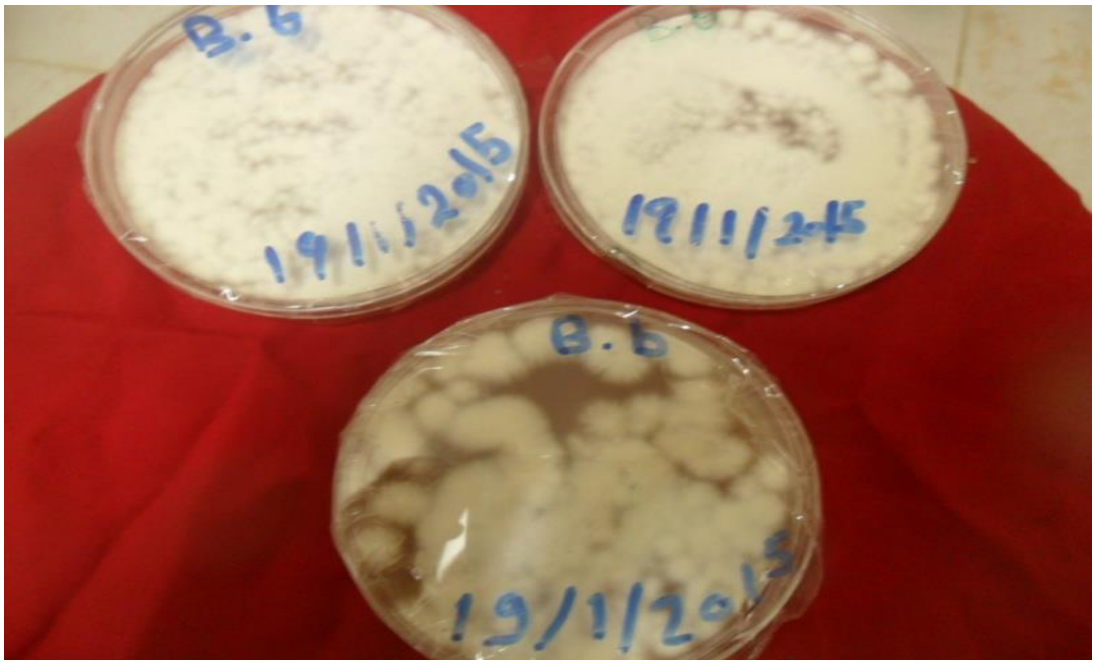

Fig. (2) Indian sub culture of *B.bassiana* fungus strain, ITCCNo.6628 (Fresh)

2.6. Preparation of concentration of *B.bassiana* (Indian strain ITCCNO.6628)

2.6.1. The laboratory tools used for different concentration:-

Four conical flask with 250ml volume

One stock flask with 500ml volume

Five test tubes

Many tips blue and micropipette

One measuring cylinder

One box of filter papers

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One tea plastic (125mm diameter)

(0.05%) Tween 80

The whole laboratory tools from (1 to 8) were used for several concentration preparations, the conidia were harvested by flooding 10 ml of sterile distilled water containing 0.5% Tween 80 on agar plate, for better distribution of conidial spores and for conidial improvement homogeneity, and then the suspensions was serial diluted for 5 times using sterile distils water, the mixture stirred by hand to make it more homogenous, after that the conidial concentration of final suspension was determined by direct count Neubauer haemocytometer, then the spores concentration were adjusted for 01 $\times 10^5$, 01 $\times 10^6$, 01 $\times 10^7$, 01 $\times 10^8$, 01 $\times 10^9$ and 0 1 $\times 10^{10}$ conidia\ml and each concentration was considered one treatment. About 1 ml of each concentration suspension was prepared then about 30 larvae were exposed to the suspension fungus by dipping technique for 2 second using a tea plastic with mesh screen. The control of the larvae were treated with water contained 0.01% triton. After that drying for one minute using sterile filter paper to avoid cross contamination. Three replicates were used for each concentration (01 $\times 10^5$, 0 1 $\times 10^6$, 0 1 $\times 10^7$,0 1 $\times 10^8$, 01$\times 10^9$ and 01$\times 10^{10}$ conidial \ml).

2.6.2. Susceptibility of GWM larvae to the fungal

In each treatment 10 larvae were kept in clean sterilize Petri dishes(9cm in diameter) and in each Petri dishes about 5grm of free infected wax as diet, and then all treated larvae in Petri dishes and the control experiment were all placed inside the incubator with adjusted temperature (25±2) and relative humidity (60%-70%) for about 11 days. A larvae was considered dead if it did not move after being probed with a pin turn to black and became softness. Dead wax moth larvae from the each treated unit (Petri dishes) were surface sterilized in 90% ethanol, cultured on SPDA with streptomycin, and incubated for 36 h at 26 °C. The presence of mycelia growing from a larva cadaver was considered to indicate fungal-caused mortality. Numbers of dead larvae were counted after specified time intervals (3, 5, 7, 9, 11 Days).

2.6.3. Evaluation of LD50 andLT50

In addition to determine the magnitude of population response as a relationship to concentrations, which is called the median lethal concentrations LC50, we also evaluate the response in relationship to time. In this case, we used a fixed concentration and determine the time it takes to kill or adversely affect 50% of the population (LT50).

2.7. Statistical analysis

At the end of this experiment the cumulative mortality of wax moth larvae caused by the fungi tested in each concentration was recorded, and subjected to analysis of variance (ANOVA) one

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way using a completely randomized design. Significant differences among means were separated with Fisher's protected LSD tests. To determine the LC50, LC95 and LT50, LT95 of the *Beauveria* bassiana ITCC 6628, cumulative wax moth larvae mortalities were subjected to probit analysis using the SPSSver.22

3. RESULTS

3.1. Isolation of the pathogen

When using GWM bait method technique for pathogen isolation from different soil types (Loamy to Clay), also anther isolation technique was used to detected the *B.bassiana* fungus by using suspected dead larvae symptoms technique. Unfortunately in both trials no *B.bassiana* fungus was detected. They were only found to be contained a saprophytic fungal growth. Thus an alternative Sudanese strain of *B.bassiana*, isolated from unidentified beetle in addition to Apure culture ready for use of *B.bassiana*, strain (A) ITCC No.6628, (B) ITCC No.6645, were used for the experiment.

3.2. Screening results

In the screening test, the preliminary result after 11 days showed that there was no significant differences between Sudanese and Indian strains and the result were nearly the same when screening tests against 3rd instars larvae of GWM , (Fig:3). However the Indian strain ITCCNo.6628 was found to be have accumulative mortality higher than 50% after 7days, therefore it has been chosen for further investigation in this study.

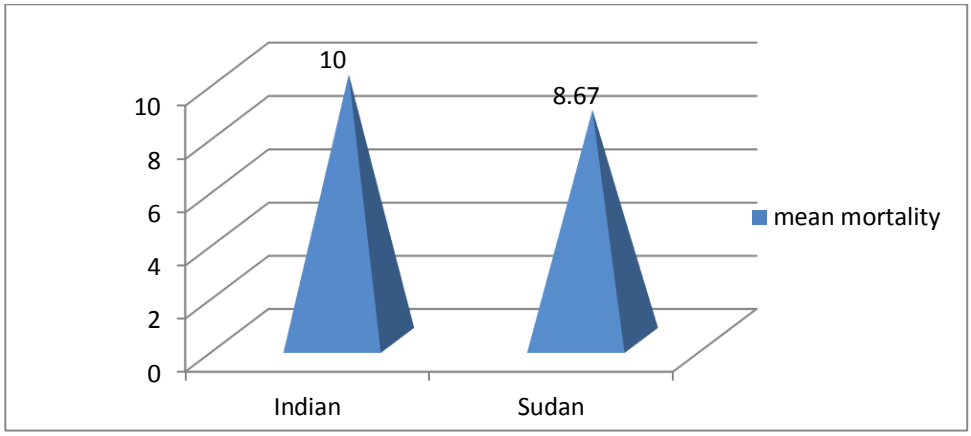

Fig (3) Mortality mean of the 3rd instars larvae of *Galleria mellonella* after feeding in wax treated with *Beauveria bassiana* Sudan and Indian strains

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3.3. Pathogenicity of Beauveria bassiana against Galleria mellonella

Pathogenicity of the fungus B.bassiana strain ITCCNo.6628 against 3rd larvae of GWM has been assessed under laboratory condition at 25°C and 60-70% RH. The GWM larvae were found to be susceptible to the fungal when it was dipped in the spores suspension mixed with 0.05% Tween 80 and using six different concentrations (01×10^5, 01×10^6, 01×10^7, 01×10^8, 01×10^9, 01×0^{10} conidia\ml). The mean mortality was significantly different among concentrations, $F= 35.74$, $P < 0.0001$(Table2) and was significantly higher in all concentrations comparing to the control $P < 0.05$ (Table 5)

Table (2): Analysis of variance (ANOVA) for different concentration of B.bassiana strain ITCCNo.6628 mixed with 0.05% Tween 80 against 3rd larval instars of GWM.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	255.33	6	42.55	35.74	.000
Within Groups	16.67	14	1.190		
Total	272.00	20			

The first characteristic symptoms of cadavers by using these fungus spores suspension was soft and black after 3 days of treatment Fig (4), and then observed the fungal growth observed in different part of soft skin area of the insect body and then covers with white mycelium (external sporulation). These symptoms occurs after 5 days of treatment Fig (5)

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Fig: (4) Symptoms of dead larvae of GWM showed softness and blackness due to *B.bassiana* fungal attack.

The highest mortality percentage of 100% reported at the concentration of (10^{10} conidia /ml) after 11 days post exposure, followed by 93.33 % , 83..33%, 66.67 %, 56.67% and 20 % at concentrations (10^{9}, 10^{8}, 10^{7}, 10^{6}, and 10^{5} conidia\ ml) respectively however , the mortality percentage generally increase as concentrations increased Fig (6).

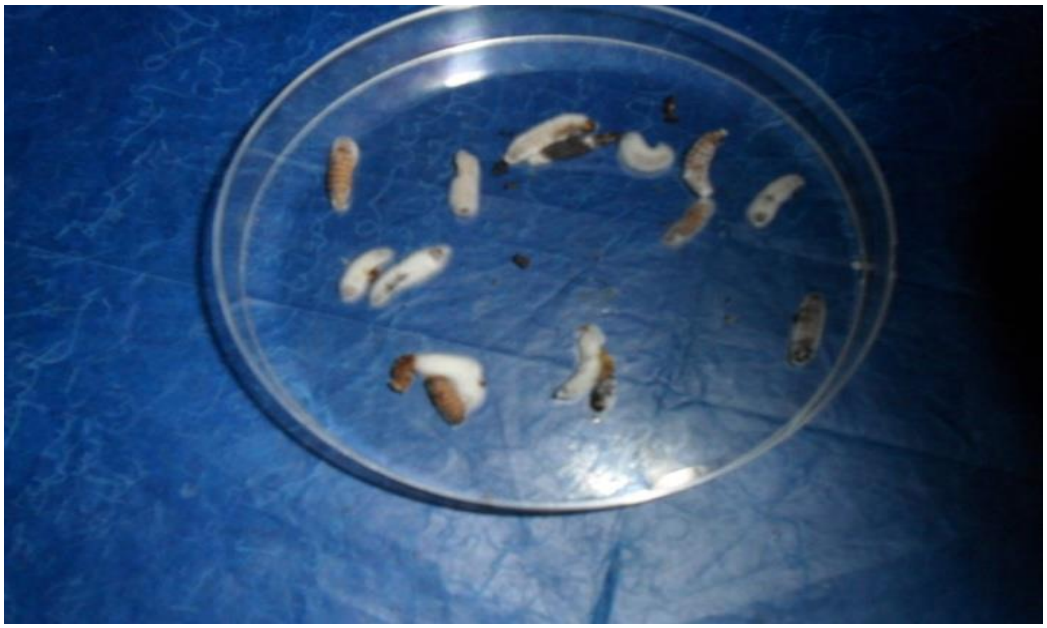

Fig : (5) White mycelium growth on dead larvae of GWM due to *B. bassiana* attack.

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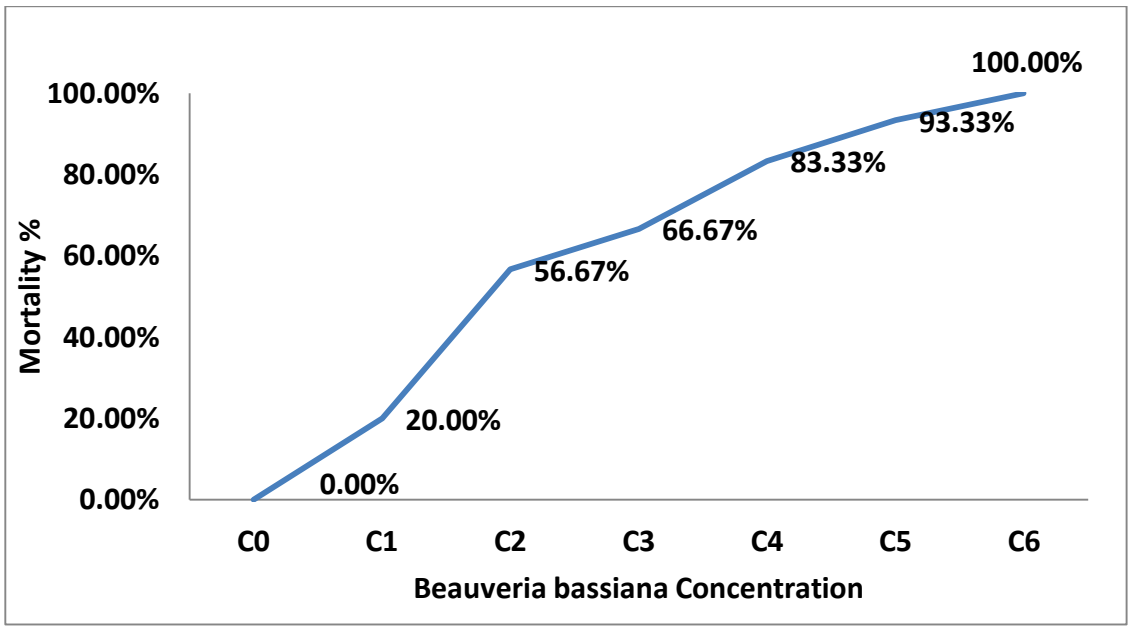

Fig (6) Mortality percentage of 3rd instars larvae of *Galleria mellonella* treated with different concentrations of *Beauveria bassiana*. C0, C1, C2, C3, C4, C5 and C6 refer to concentrations 01×10^5, 01×10^6, 01×10^7, 01×10^8, 01×10^9, 101×0^{10} conidia\ml

3.4. Evaluation of LC_{50} and LT_{50}

3.4.1. LC_{50}

The median lethal concentration (LC_{50}) that can kill 50 % of the greater wax moth *Galleria melleonella* larvae after treatment with 5µLof *Beauveria bassiana* ITCC 6628 at different concentrations was estimated to be 01.40×10^7 conidia/mL (Table 3..). However, at concentrations above the established LC50, *Beauveria bassiana* can cause 95% mortality at LC95 value of 01×10^{10} conidia /ml(Table .3.)

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Table (3). LC50 and LC95 estimated for Greater wax moth *Galleria melleonella* after 11 days of exposure to 5µLof *Beauveria bassiana* ITCC 6628 at six concentrations of 01 ×10^5, 01 × 10^6, 01 × 10^7, 01 × 10^8 ,01 × 10^9 and 01 ×10^{10} conidia/mL under laboratory conditions.

LC (conidia/ml)	95% Confidence Limits for Concentration		
	Estimate	Lower	Upper
LC_{50}	01.40 × 10^7	04.50× 10^6	3.60× 10^7
LC_{95}	01 × 10^{10}	02.50× 10^9	01× 10^{11}

3.4.2. LT50

The estimated LT_{50} values of cumulative mortality with time of 3 ,5,7 and 9, day concentrations at 01× 10^6, 01× 10^7 01× $10^8$and 01× 10^9 conidia /ml using probit analyses showed that, . *B. bassiana* killed 50% of the of wax moth larvae within, 15.80, 11.63 , 08.63.and 07.50day at the concentrations 01× 10^6, 01× 10^7 ,01× $10^8$and 01× 10^9 conidia/m, respectively. The highest pathogenicity is obtained by concentration of 01× 10^9 conidia /ml at estimate LT_{50} value of 7 day and 12 hours. (Table 4…)

Table (4). LT50 estimated for Greater wax moth *Galleria melleonella* after treatment with 5µLof *Beauveria bassiana* ITCC 6628 of different concentrations at different time exposures.

LT_{50} (day)	95% Confidence Limits for Time		
	Estimate	Lower	Upper
LT_{50} [a]	07.50	05.30	13.04
LT_{50}[b]	08.63	06.32	20.04
LT_{50}[c]	11.63	8.40	47.72
LT_{50}[d]	15.80	10.73	96.367

[a]1 × 10^9 conidia/mL

[b]1 × 10^8 conidia/mL

[c]1 × 10^7 conidia/mL

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[d]1×10^6 conidia/mL

Table (5): Comparisons between mortality mean of *Galleria mellonela* larvae treated with different concentrations of *Beauveria bassiana* and the control (zero concentrations) using LSD.

Control	(J)*Beauveria* concentrations	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
.00	0×10^5	-2.00-*	0.89	.041	-3.91-	-.09-
	01×10^6	-5.67-*	0.89	.000	-7.58-	-3.75-
	01×10^7	-6.67-*	0.89	.000	-8.58-	-4.75-
	01×10^8	-8.33-*	0.89	.000	-10.24-	-6.42-
	01×10^9	-9.33-*	0.89	.000	-11.24-	-7.42-
	01×10^{10}	-10.00-*	0.89	.000	-11.91-	-8.09-

*. The mean difference is significant at the 0.05 level.

4. DISCUSION

4.1. Isolation of the pathogen

This study evaluated for the first time the pathogenicity of one local fungal isolate, and two imported ones against wax moth larvae. Although the present result did not reveal any *Beauveria bassiana* strain from dead larvae or by trap technique from soil, the ready isolated local *Beauvaria (*isolated from beetle) showed proposing result. Good isolation can be obtained from different areas of beekeeping in different states of Sudan. Recently it was proved that the *B.bassiana* fungus obtaining by trap using *G. mellonella* has high pathogencity than that obtained from soil (Zayed, 2003). Therefore further investigation on isolating local *Beauveria bassiana* from wax moth in Sudan is suggested.

4.2. Screening Result

Although the preliminary result did not show significant differences between the Sudanese strain and the Indian strain in the mean mortality; the study was conducted using only Indian strain. This is because Indian strain ITCC.6628 was already identified, tested and established as entomopathogenic (ELbashir ea al 2014) and our local Sudanese strain has recently discovered

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and still under investigation (Satti and Gorashi 2013). Moreover the Indian strain ITCC.6628 matched our criteria of selecting and screening the suitable strain, which determined by mortality higher than 50% after 7days.

4.3. Pathogenicity of *Beauveria bassiana* against *Galleria mellonella*

All the six concentrations were considered pathogenic to wax moth larvae. Concentrations 1×10^7 1×10^9 to 1×10^{10} were highly pathogenic to wax moth larvae, with more than 80% mortality, whereas 10^5 to 10^6 concentrations were the least pathogenic, causing larvae mortality <60%. This result obviously demonstrated the Pathogenicity of wax moth larvae to the fungal *B. bassiana* at different concentration, ranging from 10^5 to 10^{10} conidia/ml used. The result was agreed with data obtained by (Zayed 2003), who reported that larvae of the GWM was susceptible to these fungus, and similar work by Charnley et al (1989) reported that adult of GWM was susceptible to these fungal suspension concentration (10^4 to 10^7).The mortality is appeared to be concentration dependent and this result is almost similar with the data obtained by Steinhaus (1963), and also agreed with Liu *etal* (2002), and Wright *etal* (2005) who reported that the mortality in infected aphids with *B.bassiana* increased with increase in concentration of conidia suspension.

The symptoms of dead larvae that observed in this study were also found to be in line with other finding. Alian (1992) reported that soft and black were first observed after treatment by *B. bassiana* fungus. Paschke(1965) and Kaaya(1998) stated that dead larvae (soft, black) were the general symptoms after treatment with *B. bassiana*. Mortality was considered to be due to mycosis only when external growth of mycelia (external sporulation) following incubation of dead insect was apparent (Emiru Seyoum *et al*., 1994; Emiru Seyoum, 2001). And the same mycelium symptoms were reported by Barson (1977) who noticed that white mycelium was appeared firstly on the dead larvae of *Scolytus scolytus* (*L.)* when infected with *B.bassiana* and then covered the body surface.

This study confirm that the Indian strain ITCC.6628 has lethal effect on the greater wax moth larvae with an estimated LC50 value of 01.40×10^7 conidia/mL and would kill 95% of the greater wax moth larvae population at LC95 value of 01×10^{10} conidia /ml. Similar work by Alice Sinia (2012) Indicated that *B. bassiana* GHA had an estimated LC50 to varroa mites of $9.6 \text{ x}10^6$ conidia/mL.

The LT_{50} value, using spore concentrations high enough for Indian strain ITCC.6628 to kill most larvae (1×10^9 conidia/ml) was found to be 7.5 days. This result was in the range of LT_{50} of other finding. However, concentration of *B. bassiana fill* in the same range might be variable according to the *B. bassiana strain*. A concentration of 2.5×10^6conidia/ml was able to kill most

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larvae in 6.4 days (Zayed 2003). LT_{50} value of 6 days was also achieved at concentration (1×10^8 conidia/ml by a wild-type *B. bassiana* in Canada (Fan, et al 2012). Normally fast mortality period with lower concentration is expected to be obtained by *B. bassiana strain* isolated from or by the greater wax moth larvae itself as indicated by Zayed (2003).

Results of this research study are very encouraging, the fungus proved to be effective as a microbial control for larvae of *G.mellonella* infesting comb under laboratory conditions. Satisfactory protection can be gained by successive treatments. Although the entomopathogenic fungi *B.bassaina* showed promising results as potential biocontrol agents, their successful use as biocontrol agent in the field depends on environmental conditions. Their pathogenicity is influenced by both biotic and a biotic factors (Davidson et al. 2003). Biotic factors include the fungal strain, its physiology, the defense mechanisms of the host, and the developmental stage and cuticular characteristic of the host. Abiotic factors, such as temperature and humidity, influence spore germination and host colonization (Goettel and Inglis 1997). In this context, indigenous isolation *B. bassiana* from target pest wax moth will be of great importance. Therefore the present finding needs more investigation to improve isolation in the field of non-selectivity and formulation improvement. Indeed *B. bassiana* did not record as a pathogen of honeybees (Steinhaus, 1963, Muller-Kogler, 1965). However, much work should be done in the field of non-selectivity.

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