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RESEARCH ARTICLE

EFFECT OF PURIFIED METABOLITES OF PENICILLIUMMARNEFFEI AND GEOTRICHUMCANDIDUM AGAINST LARVAE OF CULEXQUINQUEFASCIATUS (DIPTERA:CULICIDAE).

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Penicillium marneffei and *Geotrichum candidum* are known to be dimorphic and keratinophilic fungi and effective mosquito control agents. The current research evaluates the efficacy of the fungi P. marneffei and G. candidum as biological agents in the control of larval instars of the mosquito Cx. quinquefasciatus. Different concentrations of P. marneffei and G. candidum fungi metabolites purified by column chromatography had various effects on the mortality of the larvae of this mosquito. The larval mortality rates (first, second, third and fourth) instars were respectively LC50=0.71, LC50=0.587, LC50=0.684, LC50=0.746 at the concentration of 1.8 ml/cm ^{2}, and after 72 hours of treatment by P. marneffei; while for G. candidum, the larval mortality rates (first, second, third and fourth) instars were respectively LC50=0.579, LC50=0.649, LC50=0.755, LC50=0.855 at the concentration of 1.8 ml/cm ^{2}, and after 72 hours of treatment.

Introduction:-

Certain species of the genus Culex mosquitoes are well known as vectors of some potent pathogens.Culexquinquefasciatus(Say) is considered a major vector of many viruses such as the St. Louis Virus (CDC June, 2007) as well as filarial worms Wuchereriabancrofti which are responsible for the deaths of hundreds of millions of people in 73 countries around the world (WHO,2013). These diseases can be controlled by targeting the causative parasites and pathogens. It is easier to control vectors than parasites. Chemical control has been one of the most widely used conventional methods for mosquito control since chemical pesticides are relatively inexpensive and usually lead to immediate control. However, the use of chemical insecticides has been greatly impeded due to the development of physiological resistance in the vectors, and environmental pollution resulting in bio amplification of food chain contamination. Therefore, there is a need for new and effective alternative approaches for sustainable mosquito control (Rajesh et al .,2014) such as microbial control using fungi which are pathogenic to insects. This is considered important for several reasons, including ease of launch in nature and the application of genetic engineering techniques by(Yuen et al .,2013).P. marneffei and G.candidum are fungi associated with insects, but the extent of their impact on them is unknown (Al-Jubouri, 2008; Reetha et al .,2005). P. marneffei has many important enzymes that can be considered virulent factors which are (Histidine Kinase, Catalase Peroxidase, Superoxide dismutase (SOD), Glyceral dehyde-3-phosphate dehydrogenase (GAPDH), IsocitrateLyase, melanin pigment melanin) but it is not known if that fungus secretes toxins or not (**James ,2006).** G.candidum has several toxic compounds that have phenolic characteristics, especially Indolactic acid and Phenolactic acid (AL-Khalidi, 2014). Furthermore, this fungus secretes two protease enzymes, Metallo peptidase and Serine peptidase (Kaliski et al.,2006). It also secretes the enzyme Lipase in the form of Lipase1 and Lipase11.It should be noted that these enzymes are important virulence factors working to penetrate the cell wall and aiding in the analysis of the target insect and these enzymes are resistant to antibiotics (Chandan et al., 2003). The isolating of fungi from mosquito

larvae cadavers in Iraq and the use of these fungi for the control of Cx. quinquefasciatus mosquitoes have not been previously documented. The present study describes the larvicidal effect of extracellular metabolites of G.candidumafter purification against all instars of Cx. quinquefasciatus. The use of metabolites, purified by column chromatography, is an effective method against the phenomenon of resistance, and can be used in small quantities as a potent fungal larvicide.

Materials and methods:-

Fungi strains:-

The fungal strain of P. marneffei and G. candidum were isolated from culex larvae cadavers. These strain were maintained in the laboratory in SDA medium at 25°C.

Preparation of broth and culture of P. marneffei and G. candidum:-

The broth was prepared for the culture of P. marneffei and G. candidum. The Subauraud dextrose broth (SDB) was prepared according to the method of Soni and Prakash (2011). Five 250-ml conical flasks, each containing 100-ml Suboraud dextrose broth (Dextrose 40 g, peptone 10 g, deionized water 1,000 ml), were autoclaved at 20 psi for 20 min. The broth was supplemented by 50 µg/ml chloramphenicol as a bacteriostatic agent. P. marneffei and G. candidum colonies grown on potato dextrose agar plates were transferred to each flask using an inoculation needle. The conical flasks inoculated with P. marneffei and G. candidum were incubated at 25°C for 15 days.

Maintenance of mosquito larvae in laboratory:-

The colonies of Cx. quinquefasciatus were maintained in the laboratory at a temperature of 25°C, with a relative humidity of 75±5% for a 14-h photoperiod. The larvae of Cx. quinquefasciatus were maintained in separate enamel containers.

Isolation and purification of extracellular metabolites:-

Cell-free culture filtrates were obtained by filtering the broth through successive Whatman No.-1 filter papers after incubation. Thereafter, the metabolites were purified by column chromatography as shown in Fig.1A. In a typical experiment, a 4-ml sample was prepared in 1-ml solvent (ethanol/deionized water) and was chromatographed on a silica gel (100–200 mesh size). Elution was done with various ratios of ethanol and metabolites (ethanol/metabolites-2:8) and purified thrice. Then, 5-ml fractions were collected from all ratios. These were tested on a 2x10-cm plate of silica gel, and the plate was left to dry. Then, the spot sites were identified and exposed to iodine vapour and UV radiation as shown in Figs.1B and1C. The values of the relative movement and Relative Flow (RF), were then set according to Harborn (1984).

Larvicidal investigation of purified metabolites against Cx. quinquefasciatus larvae:-

To investigate the larvicidal activity of filtered metabolites, different ratios of ethanol and metabolite were first assessed against first, second, third, and fourth instars of Cx. quinquefasciatus. A ratio of 2:8 was found to be a significant potential against larvae of Cx. quinquefasciatus. (Soni and Prakash, 2011).

Bioassay:-

The larvicidal activity of Cx. quinquefasciatus was assessed by using the standard method (WHO 2005). All mosquito larvae of Cx. quinquefasciatus were separated and placed in a container in microbe-free deionized water. After that, different test concentrations of metabolites in 100-ml deionized water were prepared in 250-ml beakers.. Bioassays were conducted separately for each instar five different concentration (0.5,0.8,1,1.5 and 1.8 ml/cm^2)of purified metabolites. To test the larvicidal activity of extracellular purified metabolites, 20 larvae of each stage were separately exposed to 100-ml of test concentration. Similarly, the control was run to test the natural mortality, except that concentrations of culture medium were used instead of the fungal filtrates (Koch and Pasture). Thereafter, we could further examine the mortality which was determined after 24,48, and 72 h of the treatment. No food was offered to the larvae during the experiments. The experiments were replicated thrice to validate the results.

Data management and statistical analysis:-

The data on the efficacy were subjected to probit analysis (Finney 1971). The control mortality was corrected by Abbott's formula (Abbott 1925). The relationship between probitandlog concentrations were established as probit equations and probit regression lines were drown for each of larval stage.

Results:-

The findings were significant and showed that increasing filtration metabolites could effectively control larval populations of mosquitoes. The efficacy study shows highest mortality at 2:8 (ethanol/metabolites) ratio after 24, 48, and 72 h of exposure.The first and second instars were highly susceptible to 2:8 ratio with both fungi P. marneffei and G. candidum . The larval mortality rates of first instar were LC50 =1.21 ,LC50 =0.762 , LC50 =0.71 , after 24, 48, and 72 h and of second instars were LC50 = 1.621, LC50 =0.961 , LC50 =0.587 , for the same time intervals as above, while for the fungus G. candidum the larval mortality rates of first instar were LC50 =1.744 , LC50 =0.588 , LC50 =0.579 , for the same time intervals as above, and of second instar were LC50 = 1.8, LC50 =1.064 , LC50 =0.649 , for the same time intervals as above, while the fungus P. marneffeidemonstrated a clearly superior performance with mortality rates of third instarLC50 =(-) , LC50 = 1.162, LC50 =0.684 , for the same time intervals as above ,and fourth instar LC50 =(-) , LC50 =1.1728 , LC50 =0.746 , for the same time intervals as above. G. candidumhadmortality rates of third instarLC50 =1.736 , LC50 =1.606 , LC50 =0.755 , for the same time intervals as above , and fourth instar LC50 =(-) , LC50 = (-), LC50 =0.855 , for the same time intervals as above (Table1). During the experiment, P. marneffeiandG.candidum metabolites were used as mosquito larvicides and found highly effective. The colony of the selected fungi were maintained in their specific media for a certain period of time. The culture filtrates were obtained by a filtering process after incubation periods through Whatman filter paper and purified by column chromatography; then this purified metabolite was tested against Cx. quinquefasciatus larvae. The test fraction (2:8) was prepared by mixing ethanol with metabolites in different ratios.

Table: Probit equations and susceptibility of Culexquinquefasciatus larvae against extracellular metabolites of P. marneffei and G. candidum after 24, 48, and 72 h after column chromatography.

Fungi	LC	first			second			third			Fourth		
		24	48	72	24	48	72	24	48	72	24	48	72
P. marneffei	50	1.21 (41.015 - 44.11)	0.762 (46.9 – 48.99)	0.71 (52.77-58.78)	1.621 (46.92-52.77)	0.961 (46.92-52.77)	0.587 (52.77-54.99)	-	1.162 (48.84-48.84)	0.684 (48.84-50.84)	-	1.1728 (45 –52.77)	0.746 (46.92 - 48.84)
	90	-	-	-	-	-	-	-	-	-	-	-	-
	Regression equation	Y = 1.468 + 2.695 X	Y = 1.711 + 2.952X	Y=1.566 +4.447X	Y = 1.377 +2.514X	Y = 1.564 + 2.913X	Y =1.48 + 4.614 X	———	Y = 1.505 + 2.709X	Y = 1.166 + 4.251 X		Y = 1.353 + 2.461 X	Y = 1.334 + 3.642 X
G. candidum	50	1.744 (46.92 - 52.77)	0.588 (45 – 46.92)	0.579 (50.77 - 57)	1.8	1.064 (45-50.77)	0.649 (48.84 - 52.77)	1.736 (48.84 - 50.77)	1.606 (46.92 - 53.07)	0.755 (45 - 46.92)	-	-	0.855 (45 - 50.00)
	90	-	-		-	-	-	-	-		-	-	
	Regression equation	Y = 1.143 + 2.543 X	Y = 1.622 + 2.887 X	Y = 1.502 + 4.248 X	Y = 1.014 + 2.503 X	Y = 1.509 + 2.704 X	Y = 1.28 + 4.254 X	Y = 0.839 + 2.655 X	Y = 1.27 + 2.55 X	Y = 1.392 + 3.526 X			Y = 1.321 + 3.353 X

(-) mortality rates did not occur

Discussion:-

Unlike other mosquito control agents, the entomopathogenic fungi are unique because they have the ability to directly infect the host insect by penetrating into the cuticle and do not need to be ingested by the insect to cause disease. There are major advantages when fungi are used as biocontrol agents for mosquitoes. The fungi which can cause disease for insects have a very narrow range of hosts, and considerable progress has been made in recent years in development of environmentally benign spores and mycelium-based biocontrol agents for mosquito populations. Fungal biocontrol agents have reduced the levels of harmful synthetic chemical pesticide in agriculture, horticultural, and natural ecosystems (Khan et al .,2012). Overall, the results indicate a direct correlation between the concentrations used and the length of exposure on the one hand, and the destruction rate for the four larval stages on the other, wherein the destruction rates increased with increasing concentrations and exposure periods. However, there was an inverse relationship between the destruction rate and the larval age, with lower destruction rates observed for older larvae as found in Figs. (1,2,3,4).

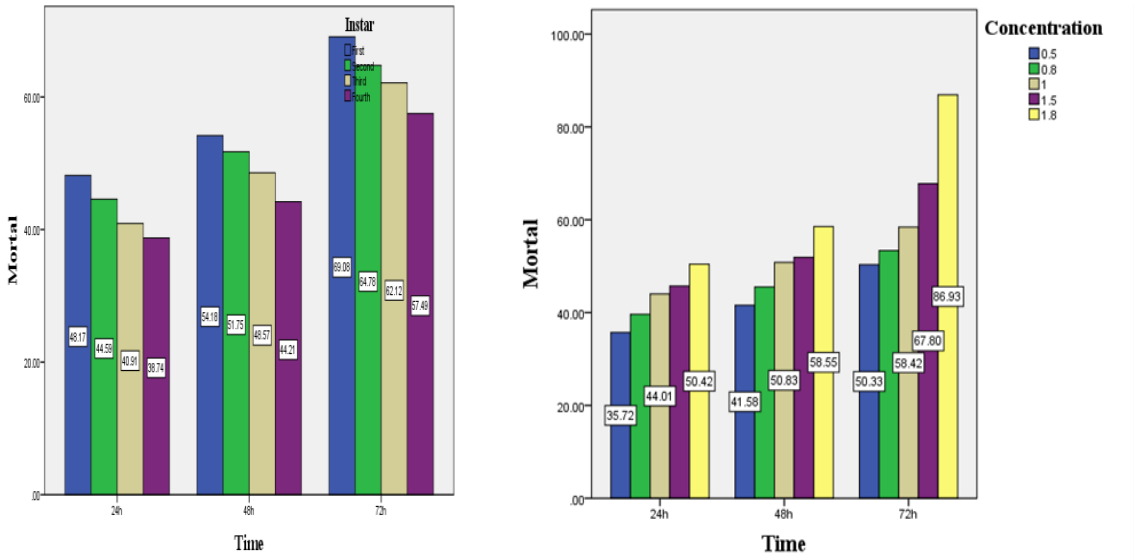

Fig.1: Relationship between percentage mortality and concentrations of P. marneffei filtrate metabolites against larvae ofCulexquinquefasciatus after 24, 48, and 72 h of exposure.in the laboratory after column chromatography.

Fig. 2: Relationship between percentage mortality and larvae age of P. marneffei filtrate metabolites against larvae ofCulexquinquefasciatus after 24, 48, and 72 h of exposure.in the laboratory after column chromatography.

Soni and Prakash (2010) demonstrated that the use of purified secondary metabolites is more potent and effective as a fungal larvicide than fungal suspensions or unpurified secondary metabolites, and that this method is suitable for field trials.

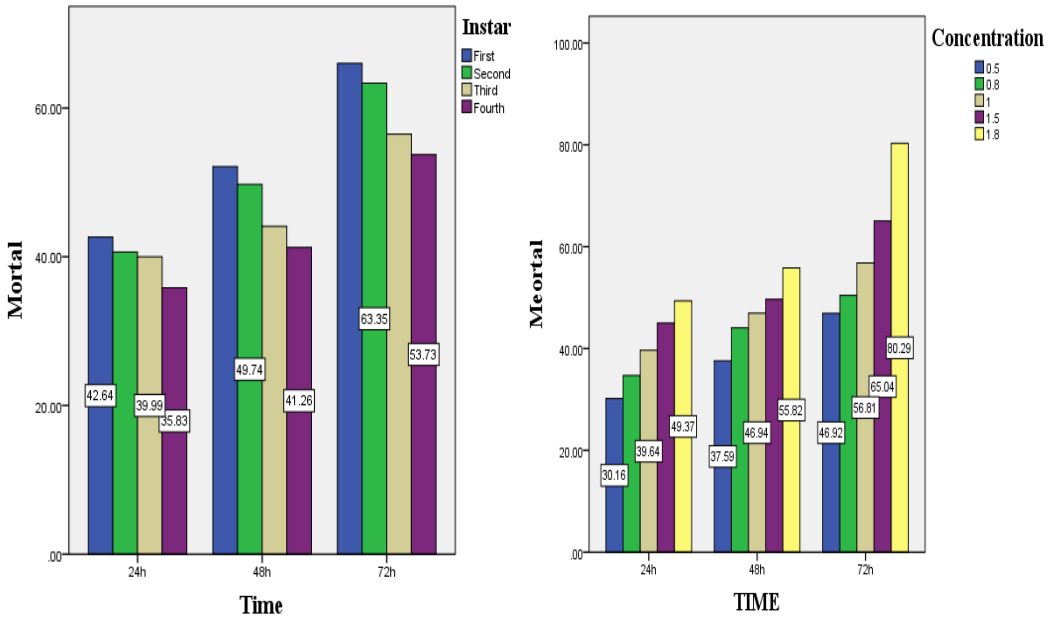

Fig. 3: Relationship between percentage mortality and concentrations of G. candidumfiltrate metabolites against larvae ofCulexquinquefasciatus after 24, 48, and 72 h of exposurein the laboratory after column chromatography.

Fig. 4: Relationship between percentage mortality and larvae age of G. candidumfiltrate metabolites against larvae ofCulexquinquefasciatus after 24, 48, and 72 h of exposurein the laboratory after column chromatography.

These results are in agreement with what Soni and Prakash (2010) found, whereby C.keratinophilum secondary metabolites purified by column chromatography led to the greatest mortality rates of Cx. quinquefasciatus at a concentration of 8:2 (metabolites/ethanol) which were LC50=26.66ppm and LC90=121.96 and LC99=231.86 after 72 hours of exposure. These results were contrary to what Soni and Prakash (2010) found when they used the fungus A.niger MTCC2587 which had caused a 100% mortality rate at all three larval stages of the Cx. Quinquefasciatus mosquito. The results also agreed with the findings of Soni and Prakash (2012) on the use of metabolites purified of the fungus V. lecanii against larval stages of mosquitoes Cx. quinquefasciatus and A. aegypti, where the greatest mortality of larval first, second and third instars was 90% at the same concentration and for the same duration of exposure. Exposing the larvae of Cx. quinquefasciatus and Ae. aegypti mosquitoes to purified secondary metabolites of the fungi T. ajelloi and L.gignatum has demonstrated that the latter had a significant impact on the larvae of the Cx. quinquefasciatus mosquito, while T. ajelloi was more effective with the larvae of Ae. aegypti (Singh and Prakash, 2012).

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