Aspergillus sydowi Metabolites Efficacies against the Mosquito Larval (*Culexpipiens* and *Aedescaspius*) Population and Cytotoxicity after Purification with Column Chromatography

Abutaha Nael[1], Al-mekhlafi Fahd A.[1,3], Semlali Abdelhabib[2], Baabbad Almohannad[1] and Wadaan Mohammed A.[1]
1. Department of Zoology, College of Science, King Saud University, SAUDI ARABIA
2. Department of Biochemistry, College of Science, King Saud University, Riyadh, Kingdom, SAUDI ARABIA
3. Department of Agricultural Production, College of Agriculture and Veterinary Medicine, ThamarUniversity, YEMEN
*nabutaha@ksu.edu.sa

Abstract
The present study was carried out to evaluate the larvicidal activity and cytotoxicity of Aspergillus sydowi extract. The LC_{50} calculated of methanol crude extract and fraction 6 was 250.86±4.15 and 56.61±0.89 µg/ml for Aedes caspius and 271.02±7.01 and 103.74±0.99 for Culex pipina respectively after 24 hours of exposure. Larvae of Ae. caspius treated with fraction 6 of A. sydowi methanol extract underwent a lethal disruption of the peritrophic membrane and an extensive cellular microvillar disruption. Expression of Bcl-2 representative of antiapoptotic protein was greatly reduced in control when compared to treated group conversely; Bax the pro-apoptotic protein was higher in treated group.

The cytotoxicity of fraction 6 and 7 showed 80.25% and 86.3 % survival at the highest concentration (37µg/ml) tested against Hepg2. On the other hand, Jurkat cells lines showed higher sensitivity to the fraction tested. Fraction 6 showed 45.17% survival where as fraction 7 showed 86.34 % survival at the highest concentration tested. The HPLC chromatogram revealed that fraction 6 obtained is not pure and requires further purification and characterization to examine larvicidal and cytotoxic effect.

Keywords: *Aspergillussydowi*, Mosquito, Histopathology, Cytotoxicity, HPLC.

Introduction

Mosquitoes are vector agents that carry disease from person to person without catching the disease themselves. Mosquito alone spreads some of the vector borne diseases like Dengue, Japanese encephalitis, filariasis, yellow fever, malaria and chikungunya affecting the socio-economical status of many nations.[21]

Repeated use of synthetic insecticides for mosquito control has disrupted natural biological control systems and led to resurgences in mosquito populations. It has also resulted in the development of resistance[7], undesirable effects on non-target organisms; environmental and human health concern.[4,10] The biological control of immature stages now appears to be the most powerful means of reducing target populations of Culicidae and other dipteran pests. The use of biological control agent can reduce the drawback associated with insecticide[16]. Recent estimates suggest that to date, we only know approximately 5% of the total species of fungi and maybe as little as 0.1 % of bacteria. And among the already described, only a small fraction has been examined for their biological activity profile[8].

Microbial insecticides are being considered as alternatives to chemical insecticides because of their selective toxicity and ready decomposability in the ecosystem. Also, unlike the inherent dangers associated with the process of production of synthetic insecticides, the process for the manufacture of microbial products is safe, well-contained with less pollution[17]. So far the extracellular secondary metabolites from three hundred and fifty fungi and ninety four actinomycetes have been screened for larvicidal activity against *Culex quinquefasciatus*[27].The metabolites of *Chrysosporium tropicum* have been found highly pathogenic as adulticides against *Anopheles stephensi*, *Culex quinquefasciatus* and therefore, the fungi are weapons with great potential in mosquito vector control.[20]

Apoptotic cell death is a physiological process critical for organ development, tissue homeostasis and elimination of defective or potentially dangerous cells in complex organisms.[13,18,22] In apoptosis process, the Bcl-2 protein has been characterized as a prosurvival, apoptosis-suppressing factor whereas the Bax protein was identified as an apoptosis-promoting factor.[11,29] The antagonistic action of these two proteins has been described as a cellular rheostat of apoptosis sensitivity such that the intracellular levels of the Bcl-2 and Bax proteins can direct the death and survival responses of a cell to an apoptotic signal.[15]

The present study was conducted to evaluate thecytotoxicity, the immunohistochemical localization of Bcl-2 and Bax in tissue sections and mosquito larvicidal activity of *Aspergillus sydowi* methanol extracts and its fractions against *Culex pipiens*and *Aedes caspius* larvae. Larval susceptibility to *Aspergillus sydowi* fraction 6 of crude extract was also compared at the histopathological level to elucidate the effects of this potential bioinsecticide.

Material and Methods

Sampling and isolation method: Potato dextrose agar (PDA) Petri plates supplemented with 50 µg/ml chloramphenicol were put at the height of 1.5 m above the ground level during sampling for 15 minutes. The Petri Plate Gravitational Settling Method was employed for the isolation of fungi[2,19]. Fungi were incubated at 30 °C for one week in the dark. As soon as the fungal growth appeared, colonies were transferred to PDA without chloramphenicol to obtain pure cultures and maintain them for identification. Stereomicroscope and light microscope were used to determine the colonial features and the morphological structures of the fungi.

Cultivation and Extraction of Fungal Culture: Five plates were inoculated with approximately 6 mm disks of 4 days old culture and kept at 28º C for 10 days. The five plates were homogenized and transferred to 1 liter flask with 500 ml methanol and left to stir overnight at 140 rpm at 30°C. The mixture was filtered through Whatmann No.1 filter paper. The mixture was then transferred to a round bottom flask and dried using a rotary evaporator at 40º C. The methanol extract was separated by column chromatography on Silica gel 60 (E-Merck, Darmstadt, Germany) using chloroform and methanol to yield 6 fractions of chloroform (F_1-F_6, 15ml each) and one fraction of methanol (F_7 200ml). Each fraction was evaporated in a rotary evaporator (40°C) and the residue was dissolved in methanol to the required concentrations to assay for cytotoxicity and larvicidal activity.

Mosquito culture: *Cx. pipiens* and *Ae. Caspius larvae* were obtained from a colony maintained at Department of Zoology, College of Science, King Saud University. They were reared indoor at 27 ± 2°C, 50 % ± 5% relative humidity, a 14:10 light: dark photo-period and they were fed daily with fish feed until become pupae. Pupae were transferred from the trays to a cup containing tap water and were maintained in our insectary. They were moved into mosquito cage where the emergent adults were fed with a 10 % glucose solution in a jar with cotton wick. The adult were given a blood meal from a mouse placed in resting cages overnight for blood feeding by females *Cx. pipiens*. Glass Petri dish lined with filter paper with 100 ml tap water were kept inside the cage for oviposition.

Bioassay: From the crude extract and the fractions obtained, different concentrations ranged between 500 to 62.5µg/mL and 200 to 12.5µg/mL were prepared respectively. Each test solution was placed in Multi-Well Plates (12 Well) and left until dried. Later, it was dissolved in one ml of tap water and tested against the 4th instar larvae (*Ae. Caspius* and *Cx. pipiens*). Each experiment was conducted in triplicate and tap water was used as a negative control. The number of dead larvae was counted after 24 h of exposure and the percentage of mortality was reported for the average of three replicates. The LC_{50} was calculated only for the test extracts that showed 100% mortality. The percentage mortality was determined and means ± SE of data are reported.

Immunohistochemical staining: Paraffin-embedded, formalin-fixed mosquito mid gut of treated and untreated fourth instar larvae of *Ae. Caspius* were sectioned (4µm) and incubated with anti-Bcl-2 and Bax (1:100) (Santa Cruz Biotechnology) overnight at 4˚C after blocking endogenous peroxidase activity with 0.3% H_2O_2. Sections were then incubated with a secondary antibody for 30 min. After washing off the free antibodies with PBS, autoradiography was performed with DAB (3, 3'-diaminobenzidine) followed by counterstaining with hematoxylin. Negative blank controls were prepared during the staining in which the first antibody was omitted and replaced with PBS. For each antibody, all slides were stained simultaneously to avoid inter-assay variation.

Cytotoxicity assay: Human T cell leukemia (Jurkat) and humanhepatocarcinoma cell line (HepG2) cells were cultured in RPMI-1640 supplemented with 10% FBS and 1% L-glutamine. The cultures were maintained in a humidified incubator with 5% CO_2 at 37°C. The cytotoxicity of all the fractions obtained was determined in each experiment using MTT colorimetric assay. Briefly, the cells were grown in 96-well plates to a density of 5 x 10^5 cells/well. The cells were treated with different concentration ranged between 37.0 - 0.15 mg/ml after 4 days of incubation, cells were washed with phosphate buffer saline and 20µl of MTT (5 mg/ml in PBS) solution was added to each well and further incubated for 2 h. The medium was then removed from each well and isopropanol containing 0.04 M HCl was added to dissolve the formazan produced in the cells. The optical density of the formazan product in solution was measured with a microplate reader at 570 nm. The experiment was conducted in triplicate. Data were calculated as percent of cell viability by the following formula:

$$\%\ \text{cell viability} = \frac{\text{Mean absorbance in test wells}}{\text{Mean absorbance in control wells}} x100$$

HPLC Analysis: The HPLC analysis was performed on reversed-phase high-performance liquid chromatographic system with gradual elution mode using a mobile phase of acetonitrile: water (70:30, v/v) on Hypersil gold phenyl column (150×4.6 mm, 5µm particle size) with 1 ml/min flow rate at 254 nm using UV detector.

Results and Discussion

Mosquito is considered the vector of many threatening diseases; therefore, the need for different control agent is very essential. The development of resistance in many of the vector mosquito species makes it indispensable to continue the search for new agents.

In our present investigation, the toxicity of the fourth instar larvae of *Cx. Pipiens and Aedes caspius* to the crude extract

and the fractions tested of *Aspergillus sydowii* were noted and statistical data are presented in table 1 and 2. The efficacy study of the methanol crude extract of *Aspergillus sydowii* showed 100% mortality at the concentration of 500µg/ml after 24 hours of exposure. The LD_{50} calculated for *Aedes caspius* and *Culex pipiens* was 250 and 271 µg/ml respectively. On the other hand, the LD_{50} calculated of fraction 6 for *Aedes caspius* and *Culex pipiens* was 56.61±0.89 and 103.74±0.99 µg/ml respectively. However, all other fractions tested were inactive.

In our study, it was found that *Ae. caspius* is more susceptible than *Cx. pipiens* to the extracts tested. This is in accordance with previous studies which attributed these differences to the physiological characteristics of the different species tested.[1,5,22,25] Fraction number 6 showed very high potency after 24 hours of exposure with 100% mortality rate against *Ae. caspius*. The mortalities increased with increasing concentrations of the extracts tested in dose dependent manner. This confirms the report of Shadia et al[23] that there is a positive correlation between concentration and the percentage of the larval mortality.

The histopathological effect of the extract in gastric caeca region was studied. The choice of this region is justified because it is directly in contact with toxic element of extract. The study examined a series of cross section through the abdominal regions of normal untreated and treated fourth instar larvae of *Ae. Caspius*. The normal midgut of *Ae. Caspius* consisted of columnar epithelium cells; each one is cylindrical containing a large, coarsely granular nucleus that occupies a middle position in the cell. The columnar epithelium has a striated border (microvilli) covered by the peritrophic membrane (Fig. 1).

Larvae of *Ae. caspius* treated with fraction 6 of *A. sydowi* methanol extract underwent a lethal disruption of the peritrophic membrane, cytoplasmic vacuolization and an extensive cellular microvillar disruption (Figure 1). Cytoplasmic vacuolization in these cell types may result from anosmotic imbalance. Alves et al[6] analyzed the activity of several insecticides that target the ion channels and reported that cell vacuolization was a common response of *Culex quinquefasciatus* to intoxication with insecticides. Abed et al[3] reported that the midgut columnar cells of *A. aegypti* responded with intense cytoplasmic vacuolization following treatment with *Citrus reticulata* resin oil.

Treatment with the fugal extract resulted in reduced survival of mosquitoes. These data suggest that apoptosis can serve as defense in *Ae. aegypti* against the toxicity of *A. sydowi* extract and may potentially be exploited to control mosquito and hence disease transmission.

Comparison of Bcl-2 and Bax presence in histological section in control and treated group showed a clear difference (fig. 1). In control group, the expression of Bcl-2 protein was detected in cytoplasm and nucleus of the cell with strongly intense staining, however Bcl-2 expression was greatly reduce when compared to treated group. Bcl-2 expression prevents cells from apoptosis (fig. 1 B and E).

The expression of Bax protein was higher in treated group than in controls which are expected in treated tissue (fig. 1 C and F). Bax- associated protein appear to be dominant inhibitors of Bcl-2 action, they promote apoptosis via mitochondrial membrane damage facilitating the release of other apoptotic mediators, especially cytochrome c, resulting in caspase cascade activation followed by cell death.[9] Our findings indicated that the intensity of Bcl-2 and Bax differs in control and treated group and their expression is differentially regulated.

The cytotoxicity of the fractions tested against Jurkat and HepG2 cell lines is shown in figure 2. Fraction 6 and 7 showed 80.25% and 86.3 % survival at the highest concentration tested against Hepg2 (37µg/ml). On the other hand, the Jurkat cells showed higher sensitivity to the fractions tested (45.17% and 86.34 % survival).

The other fractions showed no cytotoxicity when compared to control. The inhibitory concentration that kills fifty percent of cells (IC_{50}) has been taken into consideration in order to categories potency of the crude extract. According to the American National Cancer Institute (NCI), the criteria of cytotoxic activity for the crude extracts is an IC_{50}< 30 µg/ml.[12]

The fraction 6 of methanol extract exhibited the highest antiproliferative potential among the fraction tested against jurkat cell lines and the value of IC_{50} (21.04 ± 1.9µg/ml) fall within the NCI criteria, thus is considered a promising anticancer potential (Figure 2). In fact, fractions 6 exhibited selective toxicity on jurkat cell lines. For example, the IC_{50}value of fraction 6 was 21.04 ± 1.9 µg/ml. This selectivity could be due to the sensitivity of the cell line to the active compounds in the extract or to tissue specific response.[14] Further studies need to clarify cytotoxic activity and selectivity of the extract against different cancer cell lines.

The HPLC chromatogram revealed that the fraction 6 is not pure compound and require further purification (Figure 3). Therefore, further standardization is required to isolate the compound/s responsible for the tested activity.

The results of this study will contribute to the increase the opportunity for natural control of various medicinally important pests by natural sources. Natural sources are less expensive and biodegradable. Further studies can be carried out on bioassay-guided fractionation of the active extract for the isolation of active constituent, structural elucidation of the active constituent, optimization of production parameters and also on scaling up of the process.

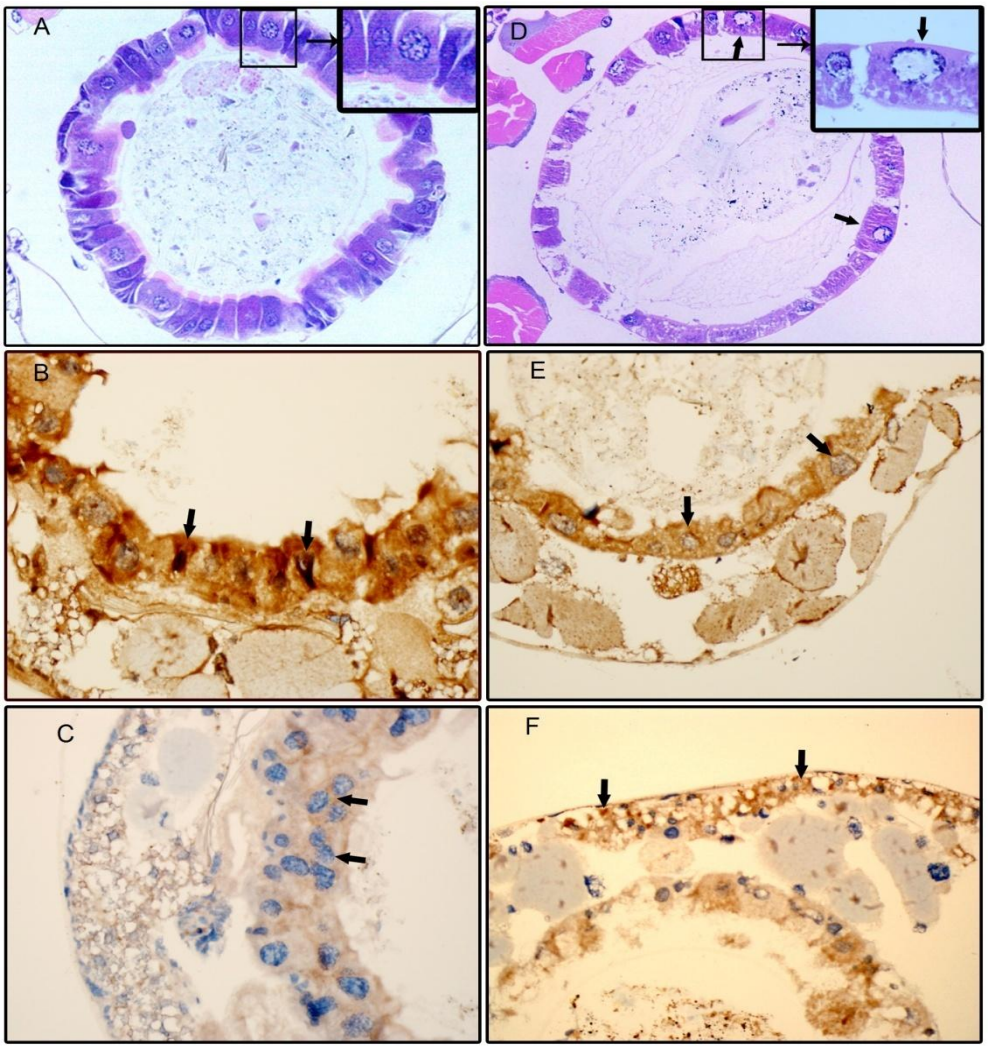

Fig. 1: Haematoxylin and eosin-stained mosquito midgut cross-sections of 4th instars midgut of *Ae. Caspius* (A and D). (D, E and F) Larvae after 24 h exposure to *Aspergillus sydowii* extract at concentration of 56.61 µg/ml. Immunohistochemical staining with Bcl-2 (B and E) and Bax (C and F). (A), (B) and (F) represent the Negative control for methanol, Bcl-2and Bax. Note epithelium cells (EC), lumen (L), Peritrophic membrane (PM), microvilli (MV), Nucleus (N).

Table 1
Larvicidal activity (% mortality) of different concentration of crude methanol extract of *Aspergillussydowi* against 4th instar larvae of *Ae. Caspius*and *Cx. Pipiens*.

Mosquito species	Mortality* (mean ± SE)%				LD_{50}
	Concentration (µg/ml)				
	500	250	125	62.5	
Ae. caspius	100±0	53.33±6.67	33.33±3.33	0	250.86±4.15
C x. pipiens	100±0	53.33±3.33	13.33±3.33	0	271.02±7.01

Table 2
Larvicidal activity (% mortality) of different concentration of fraction 6 of *Aspergillus sydowi* against 4th instar larvae of *Ae. Caspius*and *Cx. Pipiens*.

Mosquito species	Fraction		Mortality* (mean ± SE)%				LD_{50}
			Concentration (µg/ml)				
		200	100	50	25	12.5	
Ae. caspius	6	100±0	100±0	53.33±3.33	6.67±3.33	0±0	56.61±0.89
cx. pipiens	6	100±0	40±0	33.33±3.33	13.33±3.33	0±0	103.74±0.99

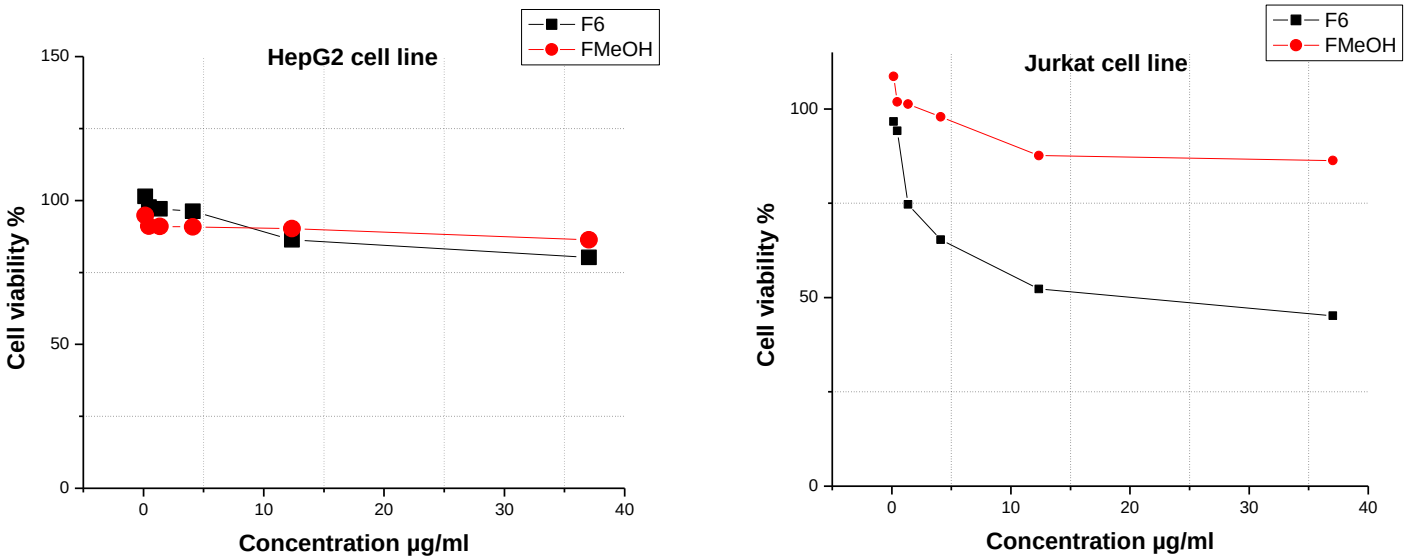

Figure 2: Growth inhibitory effects of fraction 6 and fraction 7 of methanolic extract on the two cancer cell lines, hepatoblasotoma (HepG2) and Human T cell leukemia (Jurkat).

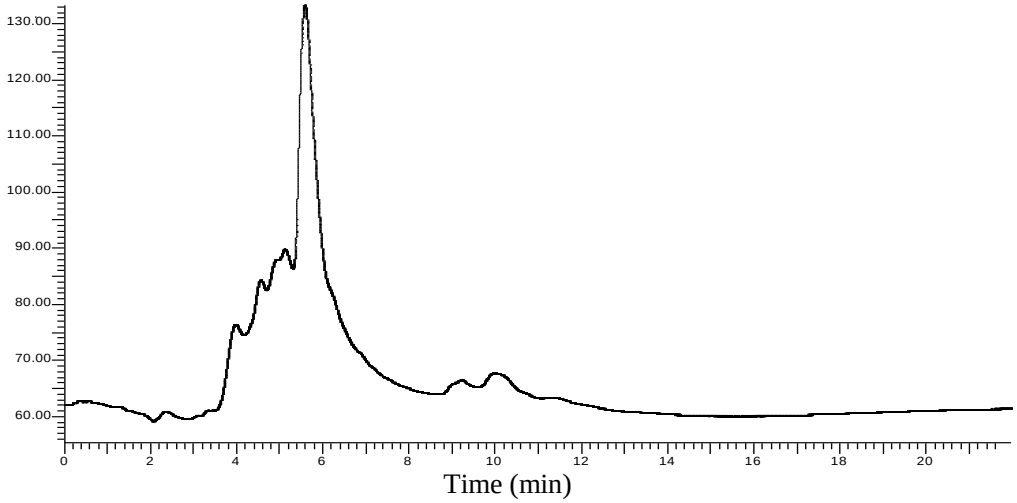

Fig. 3: Peaks of secondary metabolites obtained by HPLC analysis of fraction 6 of methanol extract, UV monitoring at 254 nm.

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