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In Vitro Anti-Cancer Activity of Larval Hemolymph and Fat Body of Flesh Fly *Sarcophaga argyrostoma* (Diptera: Sarcophagidae)

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Abstract

Insects are well recognized as a source of potentially useful compounds for modern medicine. Development of anticancer drugs from natural resources has been performed throughout the world. In the present study, anticancer activity of the hemolymph and fat body of *Sarcophaga argyrostoma* third larval instars is assayed against human breast cancer cell line (MDA-MB-231 cells). The cytotoxicity of the hemolymph and fat body samples were determined. The results showed that growth of MDA-MB-231 cells was inhibited at different concentrations upon 24 h of exposure. There is no inhibitory activity against Vero cells under these experimental conditions. Protein profile of the hemolymph and fat body were extracted and separated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein analysis showed differences in number of electrophoretic protein bands with different molecular weights between treated and control larvae. The present work assumes that hemolymph and fat body tissue have cytotoxicity against MDA-MB-231 cells and these results exhibit that larvae from *S. argyrostoma* could be used as a good source for developing anti-cancer agents and knowledge of these anti-cancer compounds will lead to better control of human disease.

Keywords

Sarcophaga argyrostoma, Hemolymph, Fat Body, Cytotoxicity, SDS-PAGE Analysis

1. Introduction

Cancer is one of the leading causes of death worldwide. Changes in cancer treatments, such as chemotherapy, surgeries and radiations, have numerous pitfalls [1] which are very unaffordable and expensive, toxic to healthy cells leading to many side-effects, ineffective in induction of cell death and drug resistance [2]. Therefore, it is of great importance to develop new and specific drug treatments for cancer, so researchers around the world are working to find better ways to prevent, detect and treat cancer [3] [4]. Insects are among the oldest and the most successful groups of animals occupying this planet. They make up about 55% of total biodiversity and are important natural source of antimicrobial (AMPs) and anticancer peptides (ACPs) [5]. An essential feature of the success of insects has been their ability to have evolved and, in many situations, thrived in environments replete with potentially parasitic and pathogenic competitors [6]. So, insects offer a source of novel natural compounds that may have therapeutic utility in cancer and other diseases [7]. Dipteran insects are known to respond to any threat by producing various humoral defence proteins [8] [9]. The flies belonging to the Diptera grow and survive in dirty places. It is reasonable to think that the insect could have strong antimicrobial peptides [6], and may have direct impact on cancer cells [10] [11]. The physical barriers such as outer exoskeleton, chitinous linings of the midgut and trachea resemble the first line of defence against any pathogen. At the molecular level, insects have developed an efficient host defence against any foreign substances that involves both humoral and cellular mechanisms [12]. Cellular defense in insects is provided by blood cells, hemocytes. So one of the most important insect defence reactions was blood coagulation where it prevents the animal from excessive bleeding when injured and aids in limiting the spread of microbes in the haemocoel [13]. The humoral mechanisms include production of defensin peptides by the fat body, the functional homologue of the mammalian liver, as well as by haemocytes and surface epithelia [14] [15]. A growing number of studies on different insects had shown that Lipopolysaccharide (LPS) could activate the induction of anticancer peptides [16]. They reported that injection of *Escherichia coli* Lipopolysaccharide (LPS) into silkworm larvae was resulted in changes in polypeptides in the hemolymph and fat body of the challenged larvae. The anticancer activity of excretion/secretion of *Lucilia sericata* and *Chrysomya albiceps* maggots against seven human cancer cell lines have been detected [11]. The current study focused on the anticancer activity of hemolymph and fat body of *S. argyrostoma* maggots that have been injected with LPS and control ones via investigation of inhibition of cell proliferation using human breast cancer cell line (MDA-MB-231 cells). We suppose that the active molecules in flesh fly larvae may be bioactive peptides with anti-cancer activities that were induced as a result of immunization of the larvae with Lipopolysaccharide injection. The sodium dodecyl sulphate-polyacrylamide gel electrophoresis SDS-PAGE was performed to detect the variations induced in protein banding pattern for hemolymph and fat body

samples. This work demonstrated the first report to evaluate cytotoxicity of hemolymph and fat body from larvae of *S. argyrostoma* against MDA-MB-231cells; this may lead to find a new natural source for cancer treatment.

2. Materials and Methods

2.1. Insect Colony

Sarcophaga argyrostoma larvae were collected and maintained for several generations at laboratory of Zoology Department, Faculty of Science, Al-Azhar University, under controlled conditions (25˚C ± 5˚C, 60% ± 10% RH and 12-12 light-dark photoperiod). Adults were fed on 10% sucrose solution while maggots were reared on bovine meat [17].

2.2. Larval Challenge

Newly moulted third instar larvae were injected with 20 ug/larva of LPS, from, *E. coli* 0111:B4 (Sigma) [16] between the first and second abdominal segments using a sterile thin-needled microsyringe. The hemolymph and fat body samples were collected from normal or control larvae and immunized ones (20 larvae/sample) at 24, 48, 72 h post injection.

2.3. Hemolymph Collection

After anesthesia with CO_2, hemolymph was collected from each group by cutting off the anterior tip of the larvae with sterile fine scissors and placed in an ice-cold eppendorf containing a few crystals of phenylthiourea (PTU) to prevent melanisation [18], hemocytes were separated from the hemolymph by centrifugation for 10 min at 1000 g at 4˚C [19], then the supernatant was stored at −20˚C until needed.

2.4. Fat Body Collection

The fat bodies were isolated from immunized and control larvae after anesthesia with CO_2. The anterior and posterior tips of the third-instar larvae were cut off with fine scissors. The fat body was excised from the larvae under a binocular microscope and placed into a physiological saline-containing Petri dish on ice. The Malpighian tubules and tracheas were carefully removed using tweezers [19]. The fat body samples were rinsed well in phosphate buffer saline. The suspension was sonicated several times for 20 s each time and centrifuged at 15,000 g for 15 min at 4˚C [16]. Finally the supernatant was preserved at −20˚C until needed.

2.5. Anti-Cancer Assay

2.5.1. Cancer Cell Line

Human breast cancer cells, MDA-MB-231 and Vero cells were obtained from VACSERA Tissue Culture Unit, Egypt, maintained and undergo to the cytotoxicity assay at Regional center for mycology and biotechnology, Al-Azhar University.

2.5.2. Cytotoxicity Evaluation

For cytotoxicity assay, the cells were seeded in a 96-well plate at a density of 1×10^4 cells/well at 37˚C for 48 h in a 5% CO_2 incubator and humidified atmosphere in a sterile environment until half-confluent monolayer formed. Hemolymph and fat body samples were used at different concentrations (**Figures 1-3**) as treatment medium against human cancer cell. They also tested against Vero cells which are normal kidney cells for evaluation of its cytotoxicity against the normal cells. One hundred µL MTT dye was added to each well then incubated for 2 hrs. Cells were washed by 100 µL PBS and 150 µL MTT de-staining solution was added on microtiter shaker for at least 10 min or until all MTT dye that has been extracted, formed the homogeneous solution.. The optical density was measured with the microplate reader (SunRise, TECAN, Inc, USA) to determine the number of viable cells and the percentage of viability was calculated as [(ODt/ODc)] × 100% where (optical density) ODt is the mean optical density of wells treated with the tested sample and ODc is the mean optical density of untreated cells. The relation between surviving cells and sample concentration is plotted to get the survival curve of each tumor cell line after treatment with the specified compound**.** The 50% inhibitory concentration (IC50), the concentration required to cause toxic effects in 50% of intact cells, was estimated from graphic plots of the dose response curve for each conc. using Graphpad Prism software (San Diego, CA. USA) [20]. Data were expressed as mean ± standard error (SE) of 3 replicates for each sample and analyzed by using SPSS, compared by the Two-way analysis of variance (ANOVA).

2.6. Electrophoresis

Proteins were extracted from the test samples of third larval instar using Tris buffer system as described by [21], Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method described by Laemmli [22]. The gel was electrophoresed at 25 - 30 m. constant current, at 200 V, and then stained with Coomassie brilliant blue. A solution of 10% acetic acid and 45% methanol was used for de-staining. Protein standard (marker) medium range from 270 KDa to 6.5 KDa (Fermentas) was used to estimate the molecular weights of the separated bands. Gels were photographed scanned and analyzed using Gel Doc VILBER LOURMAT system.

3. Results

3.1. Anti-Cancer Activity

The results of the antitumor activity of crude hemolymph and fat body samples from immunized and control maggots against human breast cancer cell line (MDA-MB-231 cells) were presented in (**Figure 1**) and (**Figure 2**), which showed the cell viability average percentages of the tumor cells. Results revealed that with increasing concentration of hemolymph and fat body samples, the percentage of cell viability of the tumor cells decreased. The samples that were

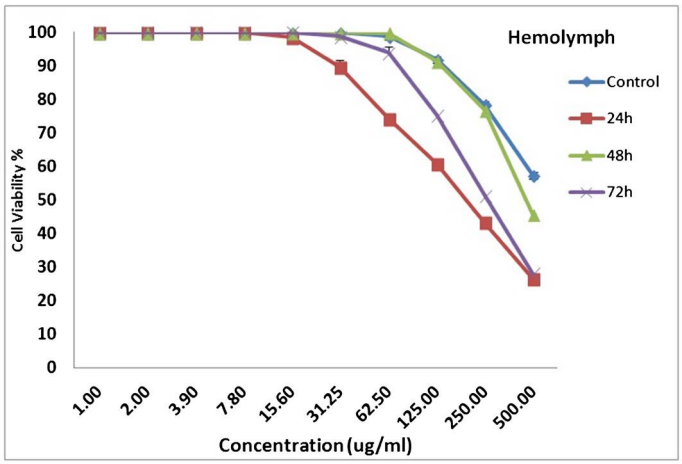

Figure 1. Cytotoxicity curve of *S. argyrostoma* hemolymph against MDA-MB-231 (human breast cancer cells).

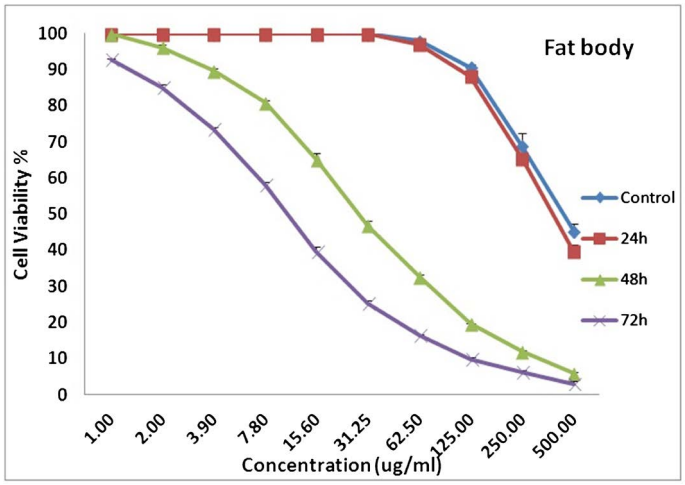

Figure 2. Cytotoxicity curve of *S. argyrostoma* fat body against MDA-MB-231 (human breast cancer cells).

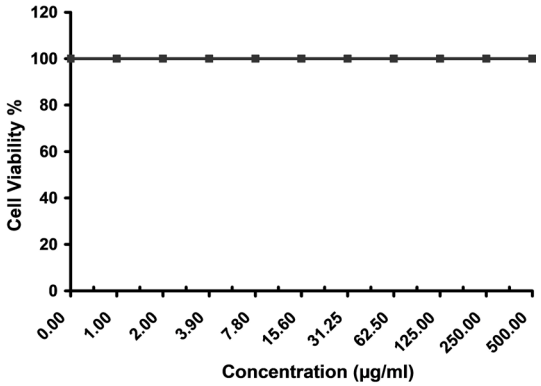

Figure 3. Cytotoxicity curves of *Sarcophaga argyrostoma* hemolymph and fat body samples against Vero cell.

collected from the normal maggots during the first, second and third days of third larval instars had the same effects on the cell viability. In the treated he-

molymph samples, the maximal activity was detected at 24 h p.i., then the activity decreased at 48 h p.i. and finally elevated again at the 3rd day p.i. Generally, the value of IC_{50} of control hemolymph (>500 μg/ml) was the highest if compared to treated ones (202 ± 5.9 μg/ml, 465 ± 14.5 μg/ml and 264 ± 9.1 μg/ml) at 24, 48 and 72 h p.i., respectively (Figure 1). The fat body samples exhibited remarkable increase of anticancer activity against tumor cell line if compared with hemolymph samples, with the maximal activity at third day post injection. Also all treated samples showed significant increase in the antitumor activity if compared to normal fat body samples. Figure 2 showed that the lowest IC_{50} values were recorded as a result of cell line treatment with immunized maggot fat body after 48 and 72 h p.i. (28.6 ± 0.9 μg/ml and 11.2 ± 0.6 μg/ml respectively). However samples of immunized larvae with different concentration exhibited no inhibitory activity against Vero cells under these experimental conditions (Figure 3).

3.2. Electrophoresis

The hemolymph and fat body of *S. argyrostoma* maggots were subjected to SDS-PAGE analysis and results showed variations in SDS-PAGE protein profiles between the normal and treated larvae.The electrophoretic protein patterns of the maggot hemolymph (Table 1 and Figure 4(a)) showed appearance of different

Table 1. The molecular weight analysis of hemolymph proteins of *S. argyrostoma* maggots.

MW	Samples/days					
	Control	24 h p.i	Control	48 h p.i.	Control	72 h p.i.
80.758	+	+	+	−	+	+
68.083	+	+	+	+	+	+
51.062	+	−	+	+	+	+
49.226	+	+	+	+	+	+
48.010	+	+	+	+	+	+
47.273	+	+	+	+	+	+
45.067	+	−	+	+	+	+
44.382	−	+	−	−	−	−
36.325	−	−	−	−	−	+
34.161	−	−	−	−	−	−
32.934	−	−	−	−	−	+
30.965	−	+	−	+	−	−
28.315	+	+	+	+	+	+
23.007	+	+	+	+	+	+
16.196	−	−	−	−	−	+
14.159	−	+	−	+	−	+
9.407	+	−	+	−	+	+
6.500	+	+	+	+	+	+
4.502	−	−	−	−	−	+

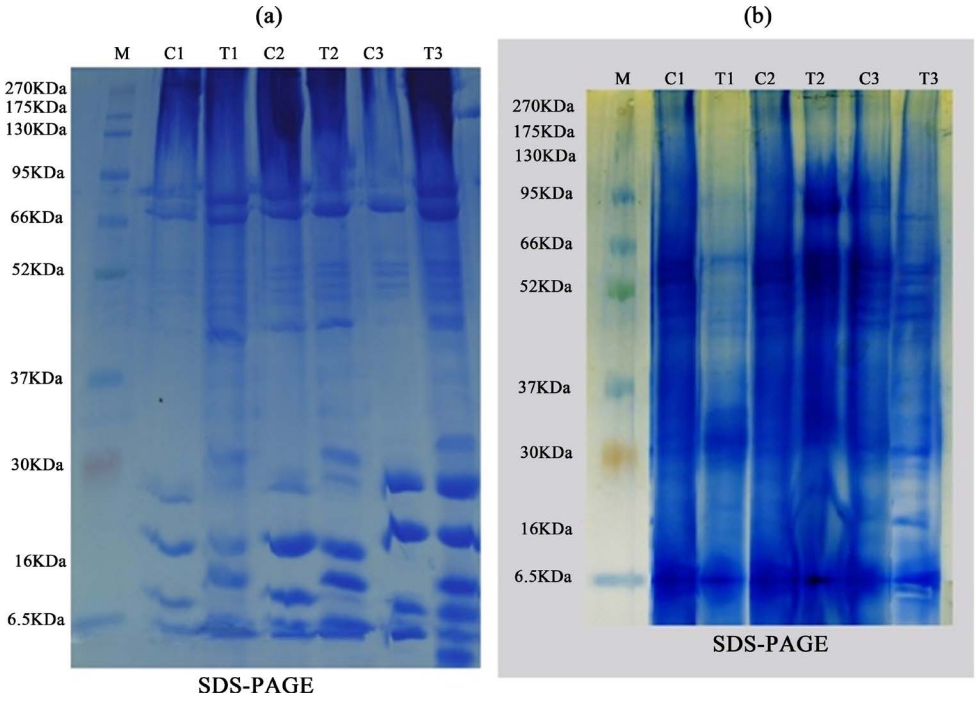

Figure 4. Protein profiles of hemolymph (a) and fat body (b) peptides of *S. argyrostoma* maggots using SDS-PAGE. LaneM: Marker, C1, C2, C3: hemolymph of normal larvae during the three days, T1, T2 and T3: samples of treated larvae at 24, 48 and 72 h after injection with Lipopolysaccharide.

19 bands with molecular weight ranged between 80.758 to 4.502 kDa. The hemolymph of normal larvae had the same protein pattern through the three days with 11 bands. On the other hand, in the treated hemolymph the number of detected bands was 12, 11, 16 bands at 24 h, 48 h and 72 h p.i., respectively. Three new bands with molecular weights of 44.382, 30.965 and 14.159 kDa were detected at 24 h p.i. and three bands with a molecular weight of 51.062, 45.067 and 9.407 kDa were disappeared compared to the control. Also, two bands with molecular weight 30.965 and 14.159 kDa were observed at 48 h p.i but other two bands with molecular weight 80.407 and 9.407 kDa disappeared as compared to the control. On the third day p.i. there were 16 bands with four unique bands of molecular weight 36.325, 32.965, 16.196 and 4.502 kDa were not found in the control and other treated samples. The protein profiles of the fat body tissues exhibited the number of total protein bands in the profiles was 16 (**Table 2** and **Figure 4(b)**), and as occurred in the hemolymph, the normal larvae didn't show any variation in the protein pattern in the three fat body samples (Seven bands with molecular weight ranges from 59.742 to 6.552 kDa). At 24 h p.i. eight bands were distinguished, three of them which had molecular weight 92.423, 48.365 and 32.165 kDa weren't detected in the control. On the other hand nine bands were observed during the second day after injection with the bands of molecular weight 88.102, 61.312, 48.365 and 28.847 kDa weren't found in normal ones. The highest number of bands was recorded at 72 h post injection, (Twelve bands ranged from79.221 to 4.502 kDa in their molecular weight) with four unique bands with molecular weight 79.221, 45.217, 18.276 and 4.502 kDa.

Table 2. The molecular weight analysis of fat body proteins of *S. argyrostoma* maggots.

MW	Samples/days					
	Control	24 h p.i	Control	48 h p.i.	Control	72 h p.i.
92.423	−	+	−	−	−	−
88.102	−	−	−	+	−	−
79.221	−	−	−	−	−	+
61.312	−	−	−	+	−	−
59.742	+	+	+	+	+	+
53.299	+	+	+	+	+	+
51.028	+	+	+	+	+	+
48.365	−	+	−	+	−	+
47.024	+	+	+	−	+	−
45.217	−	−	−	−	−	+
32.165	−	+	−	−	−	−
30.976	+	−	+	+	+	+
28.847	−	−	−	+	−	+
21.869	+	−	+	−	+	+
18.276	−	−	−	−	−	+
6.552	+	+	+	+	+	+
4.502	−	−	−	−	−	+

4. Discussions

Anticancer drugs development from natural resources is ventured throughout the world [4]. The anticancer effects of insects and their biochemical derivatives proved to be potential therapeutic tools, but little literature is available. Insects are a large, unexplored and unexploited source of potentially useful compounds for modern medicine [23]. Insects produce wide range of protein and peptides as a first fast defense line against pathogen infection. These agents act in different ways including insect immune system activation or by direct impact on the target tumor cells [24].

The present study tested the anticancer activity of fleshfly larvae, to evaluate cytotoxic and anticancer potential of crude hemolymph and fat body of third larval instar of *S. argyrostoma* that were immunized via injection with Lipopolysaccharide. Lipopolysaccharide was expected to activate adaptive immune system in *S. argyrostoma*, which plays a crucial role in fighting any foreign bodies, to produce defense peptides which they are synthesized mainly in insect fat body (functional equivalent of mammalian liver) beside certain blood cells, and then rapidly released into hemolymph as reported by Yakovlev *et al.* [25]. Damage to the integument or any infectious challenge was found to result in a several-fold increase in the antimicrobial activity of the fat body and extensive release of antimicrobial peptides [26].

The insect hemolymph also gains antimicrobial peptides (AMPs) after the insect has been wounded or after microbial infection [27] or to respond to the components of the microbial cell wall [9]. In addition to their antimicrobial effect, it is also proved that AMPs have anticancer activities [28] [29] [30] [31] [32].

The present results suggested that crude hemolymph and fat body of S. *argyrostoma* larvae have anticancer activity against human breast cancer cell line (MDA-MB-231 cells) in concentration dependent manner. These findings agreed with previous studies which proved that crude extracts from flies' maggot exhibited antitumor activity [33]. The hemolymph of *Sarcophaga* larvae could be induced cytotoxic effects on different tumor cell lines in the presence of murine macrophage [7] [23] [34] [35] [36] [37] [38]. The activity of the hemolymph samples in the present study has the maximal activity at 24 h p.i.; the activity has decreased at 48 h p.i. and then elevated again during the 3rd day, while the fat body samples exhibit gradual increase in their activity against tumor cell line. In addition, the results showed that median inhibitory concentration IC_{50} values of fat body samples from maggots during 3rd day were 11.2 ± 0.6 µg/ml, and showed higher anticancer activity against MDA-MB-231 cells than the antitumor activity of hemolymph samples which have the lowest IC_{50} value 202 ± 5.9 µg/ml. These differences between IC_{50} values of fat body and hemolymph may be due to the fact that the defense proteins are synthesized by the fat body, and then released in to the hemolymph in order to overcome the challenge. Tzou *et al.* [39] and Parvy *et al.* [40] estimated that the fat body is the main sources of defensin protein in tumours bearing larvae of *Drosophila*; defense peptides can specifically target tumor cells by rescuing tumor volume and tumor cell death. The obtained results agreed with Shehata *et al.* [11] and Hasaballah *et al.* [7] who found that excretion/secretion of *L. sericata, Chrysomya albiceps* and *M. domestica* maggots could have antitumor activity against different human tumor cell lines with low values of IC_{50}. Several bioactive peptides which derived from insects can exert important pharmacological potentialities in human physiology which in turn can be an effective tool for correcting the genomic alterations. It was necessary to detect the changes in the protein pattern as a result of immunization of the larvae using electrophoretic analysis. Scanning of the protein gel indicated that, immune challenge of the insects induced an increase of the peptides in the fat body and hemolymph causing appearance of additional proteins. Also the common bands differed in intensity between the control larvae and immunized ones. A peptide of molecular weight 4.502 kDa appeared in the hemolymph and fat body at third day post injection. This peptide may belong to sapecin family which was found in some insects, including different species of the Diptera [41]. This sapecin belongs to the insects defensin family which is small cationic peptides and full of cysteines [19].

The findings of the present work showed that the crude hemolymph and fat body of *S. argyrostoma* have antimicrobial peptides which possess cytotoxic ac-

tivities to inhibit the cancer cells.

It is sufficed to say that the tested hemolymph and fat body samples showed relatively high cytotoxicity against MDA-MB-231cells and these results could be the first demonstration that larvae from *S. argyrostoma* may have anticancer activities and it would be a great source for anticancer compounds which can be useful for human welfare.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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