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THE CYTOTOXIC EFFECT OF EXCRETORY/SECRETORY PRODUCTS AND WHOLE LARVAL HOMOGENATES OF THIRD INSTAR – LARVAE OF *CHRYSOMYA MEGACEPHALA* AGAINST DIFFERENT MAMMALIAN CELL LINES

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Abstract: The present study evaluate the cytotoxic effect of crude extracts from both excretory/secretory products and whole larval homogenates of third instar larvae of *Chrysomya megacephala* against breast (MCF-7), hepatocellular (HEPG2), colon (HCT), larynx (HEP-2), cervical (HELA) carcinoma cells. Both extracts were compared with normal vero cell line to measure their cytotoxic activity using cell viability. The results revealed that the crude extract from whole larval homogenates is less toxic on Vero cell lines than the crude from excretory/secretory products. On the other hand, the crude extract from excretory/secretory products is more effective against different mammalian cell lines.

Keywords: *Chrysomya megacephala,* Maggots, Cancer, Alternative natural drugs.

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INTRODUCTION

Insects and other arthropods provide ingredients that have been a staple for traditional medicine for Centuries in parts of East Asia, Africa and South America. While many of these ingredients have not been evaluated experimentally, an increasing number have been shown in preliminary trials to have beneficial properties. The most well- studied medical application of arthropods is the use of maggots-the larvae of flies that feed on necrotic tissue [1].

The benefits associated with the use of insect larvae or maggots in the healing of wounds were first observed and recognized centuries ago but it is only recently, especially with the increase in antibiotic resistant microorganisms, that such use, termed "biosurgery", has been readdressed and the renaissance of maggot therapy begun [2]. The constituents of the larval excretory/secretory (ES) products are thought to be central to the way in which maggots promote wound healing with several mechanisms proposed to explain their actions. Debridement is thought to be partially achieved via the proteolytic action of collagenase and serine proteases present in the ES which degrade the necrotic tissue into a form which is ingestible to the larvae and which act to remove the slough from the wound surface [3]. The stimulation of extra-cellular matrix (ECM) remodeling and closure has also been proposed to be attributable to the action of one or more 'chymotrypsin-like,' serine protease/s present in the ES which acts to degrade fibronectin into bioactive peptides. It is thought that these bioactive peptides are then able to stimulate adhesion and migration of fibroblasts [4] and are possibly responsible for the acceleration in healing [5]. The ES may also have an effect on fibroblast proliferation [5].

Despite recent advances in treatment modalities, cancer remains a major source of morbidity and mortality throughout the world. Moreover, the incidence of many cancers, including cancers of the skin, prostate, breast, and kidney, continues to increase [6]. However, the use of conventional chemotherapeutic agents that typically target rapidly dividing cancer cells is often associated with deleterious side-effects caused by inadvertent drug-induced damage to healthy cells and tissues [7, 8].

The extensive success from maggot therapy in healing wounds due to their ability to produce different proteolytic enzymes that liquefy the necrotic tissue [9], push us to think what further applications maggot therapy can be used for and how more patients can benefit, specifically patients with cancer. As expected, maggots have been found successful in cancer treatment, but solely for cancerous wounds.

Chrysomya megacephala [10], the Oriental latrine fly, is a common blow fly species of medical importance in many parts of the world and it is one of the most common blowflies in Egypt [11]. Larvae of this species are known to cause myiasis in several mammal species, including humans [12]. Another facet of medical importance of this blow fly is its association with human corpses

and its relevance to forensic entomology, *C. megacephala* were found connected with cases of human death [13]. Larvae of this species are known to cause facultative myiasis in several mammal species, including humans [12]. This species, along with other surgical maggots, has a long history of medical use in wound and ulcer healing.

In this paper, we study the effect of crude extracts from whole larval body and its excretory/secretory products of *C. megacephala* against different mammalian cell-lines.

Studying the effect of crude extracts from third instar larvae of *C. megacephala* against different mammalian cell lines is reported here for the first time. The aim of this study is to focus on new studies on the use of natural products especially from insects against cancer which is more safe and cheap because the chemotherapeutic agents that target rapidly dividing cancer cells cause damage to healthy cells.

MATERIALS AND METHODS

Rearing of insect: The laboratory colony of *C. megacephala* used in this study was established in the Department of Entomology, Faculty of Science, and Helwan University. *C. megacephala* was reared following the reported protocol [14]. They were identified according to mentioned method [15]. Adults from the stock colony of *C. megacephala* were kept in cages (38×38×56 cm) at 25±3°C, 14h photoperiod and 60–70% R.H. The cages were made with a wooden floor, a glass roof, and wire gauze on three of the sides. The fourth side was wooden with a circular hole fitted with a cloth sleeve to facilitate daily feeding, cleaning of the cage, and removal of eggs. Adults were supplied daily with granular sucrose, water, and pieces of liver.

Water was supplied by dipping a piece of cotton as a wick in a bottle filled with water, and the liver was provided in a Petri- dish. Egg batches were removed daily and transferred to a fresh piece of chicken placed in a rearing enamel bowl (35 cm in diameter) covered with muslin secured with a rubber band. At the prepupal stage, dry autoclaved sawdust was added to the bowl as a medium for pupation. Pupae were sieved from the sawdust and transferred to adult cages described above for adult emergence.

Preparation of crude extracts from insect larvae: Takes place by two methods:

1-Collection of larval secretions: The ES products were collected according to modifications of reported method [16]. Native excretions/secretions (nES) were collected by incubating third instar larvae of *C. megacephala* in a small quantity (50,000 larvae/ 500 ml) of sterile distilled water for 1h at 30°C in darkness. The sterile liquid was siphoned from the containers and centrifuged at 10,000×g for 5 min to remove particulate material, after which the supernatant was collected and lypophilized for cytotoxicity tests.

2- Preparation of larval homogenate: Whole third instar of insect larvae were (20,000 larvae in 500 ml) homogenized in distilled water and centrifuged, the supernatant was collected and lypophilized for cytotoxicity tests.

Cytotoxicity assays: The cytotoxicity of both extracts were tested against breast carcinoma MCF-7 cell line, colon carcinoma HCT cell line, hepatocellular carcinoma HEPG2 cell line, larynx carcinoma HEP-2 cell line and cervical carcinoma HELA cell. It was also tested against Vero cells which are normal kidney cells for the evaluation of its cytotoxicity against the normal cells. The method of cytotoxic activity assay was carried out according to reported method [17] in the Regional Center for Mycology and Biotechnology, Al-Azhar University. The Cells were seeded in 96-well plate at a cell concentration of $1x10^4$ cells per well in 100 µl of growth medium. Fresh medium containing different concentrations of the test sample was added after 24h of seeding. Serial two-fold dilutions of the tested chemical compound were added to confluent cell monolayers dispensed into 96-well, flat-bottomed microtiter plates (Falcon, NJ, USA) using a multichannel pipette. The microtiter plates were incubated at 37°C in a humidified incubator with 5% Co_2 for a period of 48h. Three wells were used for each concentration of the test sample. Control cells were incubated without test sample and with or without dimethyl sulfoxide (DMSO). The little percentage of DMSO present in the wells (maximal 0.1%) was found not to affect the experiment. After incubation of the cells for 24hrs at 37°C, various concentrations of sample (50, 25, 12.5, 6.25, 3,125 & 1.56 µg) were added, and the incubation was continued for 48h and viable cells yield was determined by a colorimetric method. After the end of incubation period, media were aspirated and the crystal violet solution (1%) was added to each well for at least 30 minutes. The stain was removed and the plates were rinsed using tap water until all excess stain is removed. Glacial acetic acid (30%) was then added to all wells and mixed thoroughly, and then the absorbance of the plates were measured after gently shaken on Microplate reader (TECAN, Inc.), using a test wavelength of 490nm. All results were corrected for background absorbance detected in wells without added stain. Treated samples were compared with the cell control in the absence of the tested compounds. All experiments were carried out in triplicate. The cell cytotoxic effect of each tested compound was calculated [18, 19].

RESULTS

The present study evaluate the effect of in-vitro anti-cancer activity of crude extracts from both ES products and whole larval homogenates of third instar larvae of *C. megacephala* against breast (MCF-7), hepatocellular (HEPG2), colon (HCT), larynx (HEP-2), cervical(HELA) carcinoma cells. Both extracts were compared with normal Vero cell line to measure their cytotoxic activity using cell viability (Table 1).

On measuring the cytotoxic effect of ES from third instar larvae of *C. megacephala* on Vero cell line, the results showed that on increasing the concentration of ES, the cell viability decreases and that 5.5 µg/ml concentration of crude extract is the maximum concentration to be used to induce 50% of cell viability (Table 1).

The anti-cancer activity of ES from third instar larvae of *C. megacephala* on breast (MCF-7), hepatocellular (HEPG2), colon (HCT), larynx (HEP-2) and cervical (HELA) carcinoma cells showed potent cytotoxic effect. The maximum concentration from ES that induce 50% cell viability is 3.1 µg/ml, 3.7 µg/ml, 4.6 µg/ml, 2.2 µg/ml and 2.3 µg/ml for breast (MCF-7), hepatocellular (HEPG2), colon (HCT), larynx (HEP-2) and cervical(HELA) carcinoma cells respectively (Table 1).

On the other hand, measuring the cytotoxic effect of crude extract from whole body homogenates of third instar larvae of *C. megacephala* on Vero cell line, the results revealed that 11 µg/ml is the maximum concentration can be used to produce 50% of cell viability (Table 1). The maximum concentration from whole body homogenates of third instar larvae of *C. megacephala* that induce 50% cell viability is 10.0 µg/ml,7.4 µg/ml, 8.6 µg/ml, 10.2 µg/ml and 7.1 µg/ml for breast (MCF-7), hepatocellular (HEPG2), colon (HCT), larynx (HEP-2) and cervical(HELA) carcinoma cells respectively (Table 1).

Table (1): Cytotoxic effect of the excretory/ secretory (ES) and the whole extracts (WH) of third instar larvae of *C. megacephala* against breast carcinoma MCF-7 cell line, hepatocellular carcinoma HEPG2 cell line, colon carcinoma HCT cell line, larynx carcinoma HEP-2 cell line, cervical carcinoma HELA cell line and normal Vero cell lines. IC_{50} (µg) (50% inhibitory concentration).

Sample conc. (µg)	MCF-7 cell line		HEPG2 cell line		HCT cell line		HEP-2 cell line		HELA cell line		VERO cell line	
	Viability %		Viability %		Viability %		Viability %		Viability %		Viability %	
	ES	WH	ES	WH	ES	WH	ES	WH	ES	WH	ES	WH
50	4.98	9.74	6.17	10.98	5.62	12.49	3.98	14.82	4.21	11.96	8.86	20.74
25	8.74	23.19	10.83	22.62	9.86	21.84	7.44	23.17	8.13	27.84	13.79	32.18
12.5	19.12	42.48	18.85	35.19	14.28	34.36	11.39	39.86	13.79	39.12	28.52	45.35
6.25	33.86	61.62	34.74	53.49	38.51	59.73	23.76	67.12	28.54	51.63	43.78	64.21
3.125	49.23	74.53	53.19	65.21	60.94	79.35	41.52	82.97	39.66	68.28	69.34	82.97
1.56	61.62	88.15	69.62	74.35	78.57	89.12	55.29	91.08	58.42	82.95	88.16	93.54
IC_{50} (µg)	3.1	10	3.7	7.4	4.6	8.6	2.2	10.2	2.3	7.1	5.5	11

The present results also revealed that the crude extracts from whole larval homogenates are less toxic on Vero cell lines than the crude extracts from ES products while the ES products are more effective on different cell lines. The ES products produces 50% cell viability at minimum concentrations against different cell lines starting with HEP-2, HELA followed by MCF-7, HEPG2 and ending with HCT.

DISCUSSION

The present study showed that the extracts of whole larval homogenates are less toxic to Vero cell lines, but less effective on different cell lines while extracts from ES products are more toxic to Vero cell lines and it is more effective on different cell lines especially HEP-2, HELA, MCF-7, HEPG2 and HCT respectively. This may be due to that ES products are more purified than whole larval homogenates which contains cuticle, fat bodies and other body organs. From this it is said that there may be anti-cancer components produced by third instar larvae of *C. megacephala* which may helps for the treatment of HEP-2, HELA, MCF-7, HEPG2 and HCT carcinoma cells.

The larvae of maggots are known to produce a mixture of proteolytic enzymes that disrupt necrotic tissue [9]. In a previous study on larvae of *C. megacephala* it was stated that the analysis of nES products from early third instar larvae of *C. megacephala* produces a single band at 16 KDa and a broad band between 23 KDa and 45 KDa [20]. Most insect trypsins are 20–30 KDa as determined by SDS-PAGE [21]. Four proteolytic enzymes, comprising two serine proteases, a metalloproteinase and an aspartyl proteinase, were detected, with molecular weights ranging from 20 to 40 KDa, with activity across a wide pH range [9]. Also it was reported that the amplified product resulting from PCR using cDNAs isolated from both first and third larval instars of *C. megacephala* produced bands between 500 bp and 715 bp. For trypsin of both instars the amplified PCR product was at 573 bp while for chymotrypsin of both instars the amplified PCR product was at 715 bp [20].

Proteases play a prominent role in a wide array of physiological processes such as food digestion, blood clotting, embryogenesis, tissue reorganization (*e.g.* wound healing, regeneration, molting, metamorphosis *etc.*), defense mechanisms and immune responses. Most animal species synthesize a variety of protease inhibitors with different specificities, whose function is to prevent unwanted proteolysis. Proteases are involved in various disease states. For instance, the destruction of the extracellular matrix of articular cartilage and bone in arthritic joints is thought to be mediated by excessive proteolytic activity [22]. In emphysema, gingivitis, tumor invasion and inflammatory infections, it is suggested that tissue destruction is caused by proteases [22]. Among the enzymes involved in extracellular matrix degradation, a few serine proteases (elastase, collagenase, cathepsin G) are able to solubilize fibrous proteins such as elastin and collagen [23].

In the last decade, it became obvious that in invertebrates, serine proteases and their inhibitors are also involved in parallel physiological processes (*e.g.* blood clotting cascade in *Limulus* [24] and the innate immune response [25]. The serine proteases (SP) are the dominant class of proteolytic enzymes in many insect species [26]. SP carry out a diverse array of physiological functions, the best known being digestion, blood clotting, fibrinolysis, fertilization, and complement activation during immune responses [27]. They have also been shown to be associated with many diseases including cancer, arthritis, and emphysema. It was reported that chymotrypsin formed the first line of defense against cancer by stripping away the proteins around the cancer cells [28]. Also, it was stated that the proteases could kill cancer cells via one of the aforementioned mechanisms to disrupt the cell membrane [29]. Another possibility is the induction of apoptosis in cancer cells via mitochondrial membrane disruption following peptide uptake into the cytoplasm. In the bacterial cell membrane, fundamental differences exist between the cell membranes of malignant cells and normal cells that likely account for the ability of the peptides including cecropins to kill cancer cells, while sparing healthy cells. Other factors that contribute to the preferential killing of cancer cells by peptides include membrane fluidity and cell surface area [30]. The membrane fluidity of cancer cells is greater than that of untransformed cells, which may enhance the lytic activity by facilitating membrane destabilization [31]. Finally, cancer cells have a greater cell surface area than normal cells due to the presence of higher numbers of microvilli that may allow cancer cells to bind increased number of peptide molecules [32].

Moreover, some of the protease inhibitors isolated from invertebrate sources are quite specific towards individual mammalian serine proteases. This also offers huge opportunities for medicine [33].

Likewise, the biological role of proteases from invertebrates especially insects and their interactions may be a subject for investigation for the development of future cancer therapies. Developing drugs from proteases produced by invertebrates are better than those from mammals to avoid the developing of new trends of diseases that are resistant to drugs. In the future, it is likely that numerous specific proteases and their inhibitors will be tested clinically for the treatment of human disease such like emphysema, inflammation, dermitis and cancer. Future studies are needed to study and purify these proteases or their peptides from third instars' larvae of *C. megacephala* to investigate their effect on different cancer cell lines.

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