Research Article

Anticancer activity of defense peptides from *Galleria mellonella* on cervical hela cancer cell line

Jissin Mathew, C. Vani*, Amala Lizy

ABSTRACT

Aim: The statistical report shows that cervical cancer is the fourth most common neoplasm in women. Thus, the productions of natural drugs with good anticancer properties are highly necessitated. Several studies demonstrated the activities of *Galleria mellonella* in biological stream. The hemolymph peptides can exert immune properties *in vitro* and *in vivo*. M**aterials and Methods:** Keeping all research reports as background, we have formulated the study of hemolymph and supernatant extracts efficacy on HeLa cell line with various concentrations of 10 µg/ml, 20 µg/ml, 30 µg/ml, 40 µg/ml, and 50 µg/ml, respectively. In *in vivo, G. mellonella* is used as model in the field of entomological life. These characteristics along with its availability as anticancer, antimicrobial, antifungal, and antileishmanial activities made the *Galleria* hemolymph promising seal in the research world. **Results:** The results revealed the effectiveness of *G. mellonella* hemolymph and supernatant extracts in repressing the growth of HeLa cells. Noticeable inhibition was recorded in all concentrations of hemolymph and supernatant extracts. The HeLa cell line treated with supernatant of *G. mellonella* concentrations of 10 µg/ml, 20 µg/ml, 30 µg/ml, 40 µg/ml, and 50 µg/ml showed percentage inhibition apoptosis of 47.37 ± 1.92%, 49.7 ± 1.3%, 56.00 ±3.28%, 71.40 ± 3.9%, and 74.8 ± 2.6%, respectively. Moreover, the samples treated with hemolymph extracts concentrations of 10 µg/ml, 20 µg/ml, 30 µg/ml, 40 µg/ml, and 50 µg/ml recorded highest percentage of the inhibition of 63.68 ± 2.3%, 76.20 ± 2.9%, 84.73 ± 3.5%, 97.09 ± 0.12%, and 97.30 ± 0.04%, respectively. The IC_{50} value of the *Galleria* hemolymph was found 2.3 µg/ml and for *Galleria* supernatant 3.34 µg/ml, respectively. The molecular weight of the proteins present in hemolymph and the supernatant ranged between 28 KDa and 30 KDa compound and peak value of 5.282 was observed in hemolymph extracts in high-performance liquid chromatography analysis. **Conclusion:** To the best of our knowledge, it is the first report communicating investigation on maximum inhibition rate in HeLa cell line using the *Galleria* hemolymph extracts.

KEY WORDS: Anticancer, *Galleria mellonella,* High-performance liquid chromatography, 3-(4, 5-dimethythiazol2-yl)-2,5-diphenyltetrazolium bromide assay, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, Therapeutic effects

INTRODUCTION

Cervical cancer is the second leading cause of permanent ending of life in women. India accounts 1/4th global burden of cervical cancer; statistically, 17% of cervical cancer deaths occurs among women aged 30–69 years. Conventional cytology screening programs showed declination in the incidence of cervical cancer.[1] Worldwide estimation of 528,000 new cases and about 14 million new cancer variants are detected and 8 million deaths estimated due to lack of proper treatment.[1] The active form of apoptosis involves cell suicide controlled by network of genes, plays significant role in the pathogenicity.[2]

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Exploring the major pathways such as RAF/MEK/ERK, phosphatidylinositol-3 kinase, Wnt/b-catenin, apoptosis, and coupled membrane receptor signaling have eased the peculiarity for the development of therapeutic agents.[3,4] The primary cause of abnormal changes in the cervical cells or tissues associated with the infection of human papillomavirus, environmentally hazardous, and smoking. There are many Food and Drug Administration approved drugs available for the malignant treatment; further, the identification of natural compounds with non-toxigenic effect is challengeable.[4,5] Research report findings state that the chemotherapeutic drugs such as Avastin, Bleomycin, Hycamtin, keytruda, and Gemcitabine - cisplatin cause leukemia, respiratory disorder, and fetal abnormalities in pregnant women.[5] Many plant extracts have been studied for its anticancer studies. Our research hypothesis aimed the development of

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compounds from *Galleria mellonella* widely used as *in vivo* model for pathogenicity testing in entomology.

One such identification of defense peptides and proteins from *Galleria* hemolymph showed higher anticancer activity in human brain glioblastoma multiforme cell line T98G.[6] *G. mellonella* is an insect from the order Lepidoptera and the family Pyralidae (snout moths). It is, in fact, the caterpillar larvae, or wax worm, and not the adult moth that is used as an animal model. When compared with traditional mammalian model hosts, *G. mellonella* larvae are cheaper to establish and easier to maintain, as they do not require special laboratory equipment. In addition, the use of *G. mellonella* does not require ethical approval and their short life span makes them ideal for high-throughput studies.

This simple insect model can bridge the gap between *in vitro* studies and mammalian experimentation by screening out compounds with a low likelihood of success while providing greater justification for further studies in mammalian systems. Thus, broader implementation of the wax moth larva model into antiinfective drug discovery and development programs could reduce the use of mammals during preclinical assessments and the overall cost of drug development.[7]

G. mellonella serves as a good model for biochemical research. Given the size of the insect, it is possible to obtain easily hemolymph and other tissues as a source of many immune-relevant polypeptides.[8] Many of these novel proteins are believed to be virulence factors and deciphering their functions will increase our understanding in disease mechanisms and ultimately provide a base for the development of novel therapeutic agents.[6] The greater wax moth *G. mellonella* is one of the most widely used insects. *G. mellonella* larvae are commercially available and obtained in large numbers, and these can be reared on artificial diet also and are simple to use as they do not require any special laboratory equipment. There are no ethical constraints and their short life cycle makes them ideal for large-scale studies.[7,8] However, an extensive study about the organism has not been done regarding the usage of *G. mellonella* larvae itself as a source of anticancer agent even though certain antimicrobial peptides have been discovered.

Greater wax moth (*G. mellonella* L.) larvae are used as suitable host for reproduction of biocontrol agents such as entomopathogenic nematode, *Steinernema carpocapsae,* and natural enemies, namely *Microplitis croceipes* and *Archytas marmoratus*. An artificial diet was developed during 2007–08 for mass rearing of *G. mellonella*.[9] The present study demonstrates the anticancer activity of *G. mellonella* larval hemolymph and supernatant against HeLa cell line.

MATERIALS AND METHODS

Rearing of *G. mellonella* and Immunization of Insects[10]

Greater wax moth *G. mellonella* larvae were obtained from Sugarcane Breeding Institute, Tamil Nadu. The larvae were then reared on artificial diet containing following composition corn flour - 200 g, milk powder - 130 g, yeast - 70 g, honey - 100 ml, and glycerin - 150 ml for the mass production. The immune challenge was performed by piercing the larval abdomen with a needle dipped in *Escherichia coli* cells.

Isolation and Extraction of Hemocyte-free Hemolymph from *G. mellonella*[6,11]

The *Galleria* larvae were chilled for 15 min at 4°C and immune challenge was performed by piercing the larval abdomen with a needle dipped in a pellet of *E. coli* cells. The larvae were kept in dark at 24 h and the hemolymph was obtained by puncturing the larval abdomen with a sterile needle, a few crystals of phenylthiourea to prevent melanization. Then, the hemolymph was diluted with the extraction solution methanol: glacial acetic acid: water (90:9:1). The proteins precipitated were centrifuged at 10,000 rpm for 20 min at 4°C. The obtained supernatant and the pellets were dissolved in 0.1% trifluoroacetic acid and freeze dried.

Isolation of Crude Supernatant from Crushed *G. mellonella*[6]

About 20–30 larvae were obtained and surface sterilized using 0.1% formalin (9.9 ml sterile distilled water + 0.1 ml formalin). The larvae were crushed using motor and pestle by adding 2 ml of sterile distilled water. The sample was then centrifuged at 10,000 rpm for 20 min at 4°C.

In Vitro Studies for the Inhibition of Cervical Cancer[6,12]

Cervical cancer HeLa cell line was cultured in Roswell Park Memorial Institute with 10% fetal bovine serum and penicillin and streptomycin antibiotics and was maintained at 5% CO2 (Eppendorf New Brunswick, Model: Galaxy 170S, Hamburg, Germany) at 37°C. To evaluate the cytotoxic effects of the methanolic extracts of hemolymph and crude supernatant of *G. mellonella* 3-(4, 5-dimethythiazol2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed by subculturing and seeding 1×10^6 cells in each well in 96-well plate and incubated overnight at 5% CO2. From the sample stock solution (1 mg/ml) 50 µg, 100 µg and 150 µg/ml were added in triplicates and incubated overnight. After incubation, 20 µl of MTT was added and incubated for 4 h. After incubation, 100 µl of DMSO was added and mixed well. Absorbance reading at 570 nm was recorded and calculated using the formula.

$$\text{Cell viability\%} = \frac{\text{Test Sample OD}}{\text{Control OD}} \times 100$$

Other Techniques[12]

The total protein concentration of hemolymph methanolic extracts and crude extracts of supernatant was performed with reference to Lowry's method using bovine serum albumin as standard. Then, the extracts of supernatant and methanolic hemolymph were resolved in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and high-performance liquid chromatography (HPTC) was performed and the peak was recorded.

RESULTS

G. mellonella was allowed to rear and multiply on artificial diet for 1 week in dark conditions. The eggs layed from the wax moth (*G. mellonella*) were separated and placed onto another diet container. Approximately >500 eggs were layed and kept for hatching. The morphological characteristics of eggs were small, shiny, and yellowish golden color. The immunized seventh segment larvae were observed for 24 h incubation with *E. coli* cells [Figure 1].

After overnight incubation, from the immune-challenged *Galleria* larvae, hemolymph was obtained by puncturing the abdomen area in the third segment. About 0.5 ml of tiny white fluid hemolymph was obtained and stored. The hemolymph was extracted using methanol: glacial acetic acid: water in 90:9:1 ratio. The proteins precipitated were subjected for centrifugation at 10,000 rpm for 20 min at 4°C. The obtained supernatants were used for the estimation of peptides and anticancer activity.

Figure 1: Rearing of *Galleria mellonella* and immune-challenged *Galleria* larvae with *Escherichia coli*

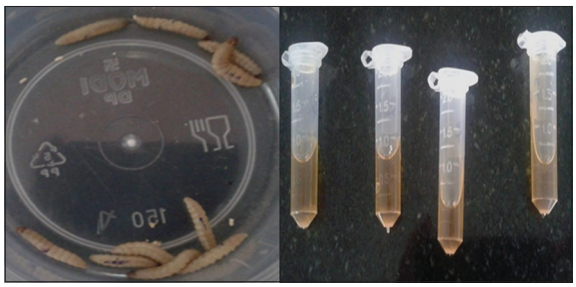

Figure 2: Screening of crude *Galleria* supernatant

The surface sterilized 20–30 larvae using 0.1% formalin were crushed and centrifuged at 10,000 rpm for 20 min at 4°C [Figure 2]. The supernatant was collected and proteins were estimated using Lowry's method.

The live cells [Figure 3] having mitochondrial succinate dehydrogenase reduce yellow MTT to insoluble formazan (dark purple) crystals. The samples treated with concentrations of 10 µg/ml, 20 µg/ml, 30 µg/ml, 40 µg/ml, and 50 µg/ml. The presence of viable cells was visualized by the development of purple color formazan crystals using ×40 inverted microscope. Then, the suspension was read using ELISA reader at 570 nm. The HeLa cell line treated with supernatant of *G. mellonella* concentrations of 10 µg/ml, 20 µg/ml, 30 µg/ml, 40 µg/ml, and 50 µg/ml showed percentage inhibition apoptosis of 47.37 ± 1.92%, 49.7 ± 1.3%, 56.00 ± 3.28%, 71.40 ± 3.9%, and 74.8 ± 2.6%, respectively. Moreover, the samples treated with hemolymph methanolic extracts concentrations of 10 µg/ml, 20 µg/ml, 30 µg/ml, 40 µg/ml, and 50 µg/ml recorded highest percentage of the inhibition of 63.68 ± 2.3%, 76.20 ± 2.9%, 84.73 ± 3.5%, 97.09 ± 0.12%, and 97.30 ± 0.04%, respectively [Figure 4]. The IC_{50} value of the *Galleria* hemolymph was found 2.3 ug/ml and for *Galleria* supernatant 3.34 ug/ml, respectively. The significance value $P \leq 0.01$ was observed in one-way analysis of variance using GraphPad Prism 6 software [Table 1].

The total protein concentration of the hemolymph methanolic extracts at 680 nm contains 146 µg/0.2 ml of proteins. Therefore, 100 ml of the test contains 73,000 µg/100 ml or 73 mg/ml. The total protein concentration of the supernatant contains 218 µg/0.2 ml of proteins. Therefore, 100 ml of test contains 109,000 µg/100 ml or 109 mg/ml. Thus,

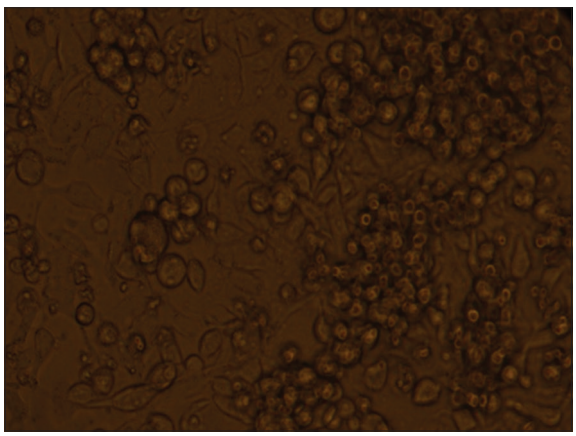

Figure 3: HeLa cells in Roswell Park Memorial Institute medium with 10% fetal bovine serum

Table 1: The relative quantitation of hemolymph and supernatant of *Galleria mellonella*

Sample	Concentrations (µg/ml)				
	10	20	30	40	50
Control	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0
Hemolymph	63.68±2.3^{a***}	76.20±2.9^{a***}	84.73±3.5 a***	97.09±0.12^{a***}	97.30±0.04^{a***}
Supernatant	47.37±1.9^{a***b***}	49.7±1.3^{a***b***}	56.00±3.2^{a***b***}	71.40±3.9^{a***b***}	74.8±2.6^{a***b***}

The values are expressed in mean±SD; significant* $P \leq 0.05$; highly significant ****$P \leq 0.01$; not significant^{ns} $P \geq 0.05$; ^{a}control versus hemolymph and supernatant; ^{b}hemolymph versus supernatant

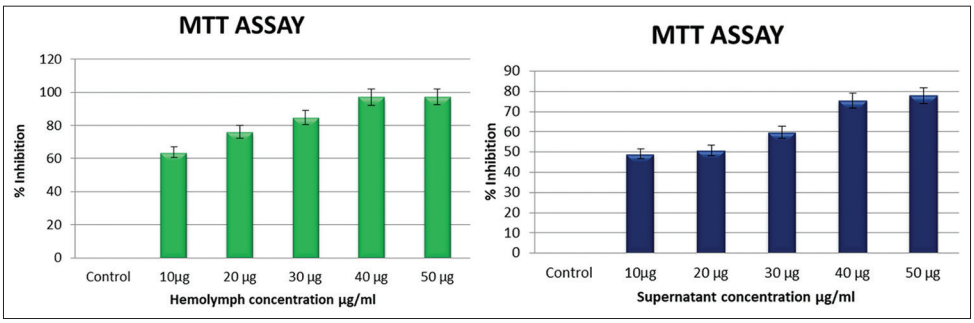

Figure 4: Cytotoxicity of various concentrations of the hemolymph and supernatant *Galleria* extracts against HeLa cells

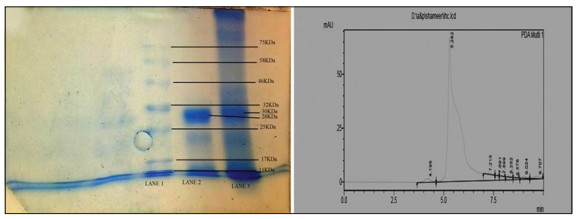

Figure 5: Detection of sodium dodecyl sulfate-polyacrylamide gel electrophoresis and high-performance liquid chromatography analysis

our research finding indicates that the presence of the large amounts of proteins and polypeptides is the factors responsible for its anticancer activity. The SDS-PAGE observation showed the presence of 28 KDa proteins in Lane 2 of supernatant extract and 30 KDa protein in Lane 3 of hemolymph methanolic extracts, respectively. The HPTC retention factor 5.282 of hemolymph methanolic extracts is presented in Figure 5.

DISCUSSION

G. mellonella can be reared and multiplied at low cost for its product yield which possesses many active compounds. The immune-challenged *G. mellonella* infected with nematode symbiont *Photorhabdus luminescens* enabled the production of novel antimicrobial epoxide, structure similar to a known antibiotic exerts antimicrobial activity against *Bacillus subtilis*, *E. coli*, *Streptococcus pyogenes*, and multiple drug-resistant *Staphylococcus aureus*.[7] Further studies on epoxide 1 from *G. mellonella* showed cytotoxic response against human cancer cell lines, MCF-7 WT, H460, and Jurkat. The hemolymph extracts at 50 µg/ml showed dramatic 97.30% of inhibition; our result was found to very similar to angiogenesis effect. One such study identified prophenoloxidase from *G. mellonella*. Research reports of *G. mellonella* as *in vivo* model estimated toxicity of chemicals, biocides, pesticides, and cosmetics reduce the risk of exposure to humans. The presence of hemolytic polypeptides can detect toxicity responses using as an *in vivo* and production of hemolymph peptides.[8] Thus, in this *in vitro* study, we conclude that the presence of hemolytic polypeptides might be the responsible factor for anticancer activity on HeLa cell line.

Another study demonstrates that the presence of antibacterial proteins designated as G4 and G5 in the hemolymph of *G. mellonella* showed larvicidal activity against six lepidopteran pests. The advantageous medical immune properties and biocontroling activity is making *G. mellonella* larvae more beneficial in the field of life sciences.[13] The research conducted in Spain concluded that the presence of polyethylene digesting enzymes on the larval gut breaks down polyethylene plastic bags by digestion and not chewing it. In this study, the team observed that the 100 *Galleria* larvae kept in polyethylene film *in vitro* degrade 92 mg of the plastics within 12 h. The achievement of plastic degradation was found higher rather than with the microbes.[14] These studies as reference led our research significantly showing its pharmaceutical properties for the prevention of many diseases *in vivo* and *in vitro*.

The ultrastructural characterization of hemocytes from *Galleria* larvae was carried out. The cytologic and morphological screening revealed four hemocytes that are plasmatocytes, granular cells, spherule cells, and oenocytoids. From this, plasmatocytes and granular cells were the most circulating cells in the *Galleria* hemolymph. Due to the presence of

phenoloxidase activity of plasmatocytes, granular cells and oenocytoids exhibited strong phagocytic defense against *E. coli*.[15,16] Our studies also suggest that the presence of hemolymph peptides may be the ultimate reason for its anticancer activity.

Furthermore, a study about the identification of peptides from immune-challenged *G. mellonella* proves its defense mechanism as antimicrobial agents. Eight peptides such as proline-rich peptide 1, defensing rich peptide, anionic peptide 1 and 2, cecropinD-like peptide, and apolipophorin were identified and characterized.[11,12] All these findings along with our results prove its beneficial implement in the environment.

Aside it's antimicrobial and antifungal activity of hemolymph peptides, the research explored hemolymph peptides for antiparasitic activity. They have identified moricin-B, moricin C4, cecropin-D, and anionic peptide-2 and tested against *Leishmania promastigotes* for antileishmanial activity.[17,18] Having all applications in various fields promise *G. mellonella* to be used as beneficial agent.

CONCLUSION

Insects have an open circulatory system. Moreover, hemolymph in insects is a fluid analogous to blood in humans. This hemolymph in addition to the plasma also contains other substances such as inorganic salts (Na, K, Mg, and Ca), organic salts, hemocyanin (for primary oxygen transfer), and proteins. These are the components of the hemolymph. However, on the whole, it may contain fatty acids, conjugates of peptides, saccharides, and other aromatic compounds digestive serine proteases and other active compounds.

The treatment of the HeLa cells with *G. mellonella* hemolymph and supernatant resulted in induction of cell death, as some cells exhibited the symptoms of apoptosis or necrosis. We conclude our results that the presence of peptides and proteins in the methanolic extract of hemolymph showed the highest percentage of inhibition 97.30% than crude supernatant.

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COMPLIANCE WITH ETHICAL STANDARDS

This article does not contain any studies with human participants or animals performed by any of the authors.

AUTHORS' CONTRIBUTIONS STATEMENT

Dr. Vani C and Jissin Mathew conceived and planned the experiments. Jissin Mathew carried out the experiments. Dr. Vani C and Jissin Mathew contributed to sample preparation and interpretation of the results. Jissin Mathew took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

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