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CYTOTOXICITY OF VENOM FROM ENDOPARASITOID *PIMPLA TURIONELLAE* L. (HYMENOPTERA: ICHNEUMONIDAE) ON GLIOBLASTOMA CELLS

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ABSTRACT

In the treatment of brain cancer, the inhibition of cancer cell proliferation using anti-cancer agents is a priority approach. Venom of endoparasitoid wasps also may be a candidate for the development of new therapeutic agents. In this context, endoparasitoid *Pimpla turionellae* L. (Hymenoptera: Ichneumonidae) venom may have an anti-carcinogenic effect on glioblastoma that resists traditional therapies by various mechanisms. Therefore, we aimed to investigate endoparasitoid venom cytotoxicity on glioblastoma cells. For this purpose, the cytotoxic potential of venom from *P. turionellae* was evaluated on rat C6 glioblastoma cell lines with methyl thiazolyl tetrazolium assay (MTT assay). *P. turionellae* venom was isolated from 15±2 day-old females. Different concentrations of *P. turionellae* venom (176.6-1.83 µg/ml) were applied to C6 rat glioblastoma cells in vitro. Results of MTT assay showed that the viability of C6 cells in vitro significantly decreased depending on the parasitoid venom concentrations. Therefore, *P. turionellae* venom showed cytotoxic activity in a time- and dose-dependent manner. In conclusion, the results from this research could be used as primary data of venom cytotoxicity for the investigation of new chemotherapeutic agents against malignant tumors.

Keywords: *Pimpla turionellae*, Endoparasitoid venom, Glioblastoma, Cytotoxicity, MTT

ÖZET

Beyin kanseri tedavisinde, antikanser ajanlar kullanılarak kanser hücrelerinin proliferasyonunun inhibe edilmesi öncelikli bir yaklaşımdır. Endoparazitoid arıların venomu da, yeni terapötik ajanların geliştirilmesine aday olabilir. Böylece bir endoparasitoid tür olan *Pimpla turionellae* L. (Hymenoptera: Ichneumonidae)'nın venomu, çeşitli mekanizmalarla geleneksel tedavilere direnç gösteren glioblastoma üzerinde antikanserojen bir etkiye sahip olabilir. Bu nedenle, çalışmamızda endoparazitoid venomunun glioblastoma hücreleri üzerindeki sitotoksisitesinin araştırılması amaçlanmıştır. Bu amaçla, *P. turionellae* venomunun sitotoksik potansiyeli, metil tiyazolil tetrazolyum testi (MTT assay) ile sıçan C6 glioblastoma hücre hattı üzerinde değerlendirildi. *P. turionellae* venomu 15 ± 2 günlük dişi bireylerden izole edildi. farklı konsantrasyonlardaki *P. turionellae* venomu (176.6-1.83 µg/ml) in vitro olarak C6 sıçan glioblastoma hücrelerine uygulandı. MTT testinden elde edilen bulgulara göre, uygulanan parazitoid venom konsantrasyonuna bağlı olarak C6 hücrelerinin in vitro canlılığının önemli ölçüde azalmıştır. Bu nedenle, *P. turionellae* zehiri zamana ve doza bağlı bir şekilde hücreler üzerinde sitotoksik aktivite göstermiştir. Sonuç olarak, endoparazitoid arı venomunun sitotoksisitesine ait elde edilen bu bulgular, malignant tümörlere karşı yeni kemoterapötik ajanların araştırılmasında primer veri olarak kullanılabir.

Anahtar Kelimeler: *Pimpla turionellae*, Endoparazitoid venom, Glioblastoma, Sitotoksisite, MTT

1. INTRODUCTION

Recently, investigation of animal venoms as new therapeutic agents has increased in cancer therapy [1-8]. Several biological functions such as pheresis, anti-carcinogenic activity, cell death by oncotic lytic mechanisms, structural and pharmacological properties, and cytotoxic effects on cancer cells, of the protein components of venom cocktails of various species have been shown by *different* researchers [1, 5, 9-12]. It is also known that venoms from Hymenopteran species such as bees and wasps contain low molecular weight compounds and neurotoxin, substances with antimicrobial action, a complex mixture of enzymes and cytolytic peptides [13-16]. Studies demonstrated that active pharmacological compounds obtained from wasp venoms exhibit anticonvulsant [17], cytotoxic, genotoxic, mutagenic

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[18], and cytostatic [19] influences on the mammalian cells. Moreover, Sisakht et al. [5] reported that bee venom has apoptotic and cytotoxic effects on human glioblastoma cells. However, similar to bee venoms, studies about the antitumoral potential of venoms secreted by hymenopteran endoparasitoid species on glioblastoma multiforme are unavailable in the open literature. Glioblastoma multiforme (GBM) constitutes more than half of cancer types in the central nervous system. The treatment approach begins with surgery, followed by radiation therapy and / or radiation combined with chemotherapy. However, regardless of the treatment approach, treatment resistance and recurrence develop because of apoptosis which causes changes in various genes present in GBM [20, 21]. Therefore, the inadequacy of classical drugs and methods, destructive properties on healthy cells, and associated side effects have led researchers to seek alternatives to fight glioblastoma in particular [22]. Thus, our study subject focused on the endoparasitoid venom which may be potential as an alternative biological agent, to prevent the proliferation of rat glioma cells.

One of the endoparasitoid species *P. turionellae* L. (Hymenoptera: Ichneumonidae) develops as pre-pupal and pupal solitary, idiobiont, and endoparasitoid of numerous pest Lepidopteran species [14]. Venom from endoparasitoid *P. turionellae* L. (Hymenoptera: Ichneumonidae) female, known not to contain viral products, displays paralytic, cytotoxic and/or cytolytic effects on the Lepidopteran hosts or insect cell line [14, 23]. Studies on the identification of venom components revealed that *P. turionellae* venom contains various biologically active components including biogenic amines, proteins, enzymes, and peptides [13, 24]. Ergin et al. [24] also suggested that *P. turionellae* venom contains various toxins or peptides which inhibit functions at neuromuscular junctions to paralyze host insects. For this reason, endoparasitic wasp venom may be used in the development of new biopharmaceuticals in GBM therapy. This study was therefore focused on determining the cytotoxic potential of endoparasitoid venom from *P. turionellae* on the rat C6 glioblastoma cell line.

2. MATERIALS AND METHODS

2.1. Insect Rearing

The model host *Galleria mellonella* (Lepidoptera: Pyralidae) colony was reared on an artificial diet described by Er et al. [23]. *P. turionellae* was reared on the model host *G. mellonella* pupae. Adult female parasitoids were fed with host pupae (10:4 once in three days) and 55% honey solution (v:v). Host stock culture was maintained at 27 ± 1°C, 60 ± 5 % RH in constant darkness and parasitoid culture was maintained at 24 ± 0.5°C, 50 ± 5% relative humidity, and 12: 12 photoperiods in Animal Physiology Laboratory at Eskisehir Technical University, Eskişehir, Turkey.

2.2. Isolation of Crude Venom from *P. turionellae*

Venom was isolated from venom glands of 15 day-old females according to Uçkan et al. [13]. Venom sacs from 40 female endoparasitoids were used for each replicate of cell viability (MTT). Females were frozen at –20°C for 10 minutes before dissection of venom sacs. Then, all venom sacs were removed by pulling the ovipositor apparatus with a fine forceps and crushed for 1-2 min by a sterile disposable pestle inside the 0.5 ml Eppendorf tube containing 40 µl of cold ISB (insect saline buffer, 2 mM $MgCl_2$, 4 mM $CaCl_2$, 10 mM HEPES, 10 mM KCl, 150 mM NaCl, pH: 7.0) on ice. Subsequently, all homogenized venom glands centrifuged at 12,000g for 10 minutes at +4°C to remove cell debris. The resultant supernatant was used as the crude extract of the venom and was stored at –80°C until use in experiments.

2.3. Total Protein Analysis in Crude Venom

To determine the total protein concentration of venom samples, the Bradford method (1976) was performed using bovine serum albumin as the standard curve at 595 nm by a microplate absorbance

reader (iMark™ Bio-Rad). To prevent venom inactivation, dissolved venom samples were used within twenty-four hours for MTT assay.

2.4 Cell Culture

C6 glioblastoma cells were used to determine the cytotoxicity of crude venom on mammalian cells. C6 cells were cultivated in 25 cm^2- culture flasks containing 5 ml of DMEM F12 (Gibco), supplemented with Sodium Pyruvate, 10% of fetal bovine serum (FBS) and 4.5 g/L D-Glucose 0.11g/L, 2 mM L-glutamine inactivated antibiotic antimycotic solution 1% of penicillin-streptomycin (Sigma, P4333) enclosed in a CO_2 incubator (5%), till they reached confluence. For the storage process, the cell culture medium was drawn from the cell flasks using a pipette and a PBS-EDTA solution was then used to wash the remaining cells in the cell flask. To facilitate the dislodging of cells from the flask base, 0.25% Trypsin-EDTA was added to the cells in the flask and incubated in 5% CO_2 at 37ºC for 2 min. Fresh medium was added to cells detached from the flask bottom and the mixture was centrifuged at 1200 rpm for 5 min. After centrifugation, the supernatant was withdrawn from the flask. To the remaining pellet in the flask fresh medium containing 10 % DMSO was added and the mixture kept at -80ºC for further use.

2.5. MTT Assay

The MTT assay (Thiazolyl Blue Tetrazolium Bromide – 158990010, Acros Organics) with C6 cells was performed with some modifications. In each well of a 96 well plate, $1x10^5$ cells were seeded and the plate was incubated for 24 h to attaching to the flask surface. The medium was discarded from the wells after incubation and 100 µl of fresh culture medium, ISB containing culture medium (without venom) and culture medium containing different venom concentrations (176.6, 83.3, 22.07, 5.5, and 1.83 µg/ml) were each individually added to the well plates. Cells were then individually incubated for 4 and 24 hours after which, control and experimental medium solutions were removed from the wells and 100 µl of 5 mg/ml MTT-DMEM-Fl2 solution was added. The cells were incubated again at 37 °C in a CO_2 incubator (EC160, Nüve) for 3 hours. After incubation, MTT solution was removed from the wells and DMSO solution (Sigma, 100µl) added. The well plate was placed on a shaker for 5 minutes after which the optical density of the cells in the wells was measured at 570 nm (750 nm reference) in the microplate reader (iMark™ Microplate Absorbance Reader, Bio-Rad). All assays were carried out using four wells in four replicates for each treatment.

2.6. Statistical Analysis

All results in this study were ascribed as mean ± standard error (Mean ± SE). Data analysis was performed using an SPSS software program (version 18.0 for Windows). Before statistical analyses, percentage data on cell viability were normalized by arcsine transformation. One-way analysis of variance (ANOVA) test was performed to compare the cytotoxic effects of the venom. To define significant differences among means, the least significant difference (LSD) test was used in the SPSS program. A t-test was also carried out to determine the significance of the effects of the venom concentrations on cell viability in response to two-time point (4 and 24 h). Significance was ascribed at $P < 0.05$.

3. RESULTS

MTT assay is one of the methods used for the determination of the cytotoxic or anti-proliferative effects of venom on viable cells. *P. turionellae* venom concentrations at 176. 6 µg/ml, 83.3 µg/ml, 22.07 µg/ml 5.5 µg/m, and 1.38 µg/ml were tested on the viability of C6 glioblastoma cells (%) at different exposure times (4 and 24 hours). According to MTT assay, cell viability did not show any significant change between control and ISB control at the two-time points (Figure 1). As shown in fig.1, *P. turionellae*

venom decreased the viability of glioblastoma cells in a concentration-dependent manner and the half-maximal inhibitory concentration (IC_{50}) value was determined as 114.36 ± 2.2 µg/ml for 24 h incubation. The viability of cells exposed to venom concentrations for 4 hours significantly decreased when compared with controls (F_{4h} = 62,397, df_{4h} = 6, 105, P_{4h} = 0,000) except for 1.38 µg/ml venom concentration. The percentage of cell viability decreased by about 67% at 4 hours post-treatment at the highest venom concentrations compared to the controls, while at the end of the 24 hours, the percentage decreased to about 51% at the same concentration (Figure 1). However, there was also no statistical difference detected between the viability of glioblastoma cells at the two highest venom concentrations at each time point. From the results obtained, it was determined that cell viability reduced in a concentration-dependent manner when compared with controls at 24 hours post-treatment (F_{24h} = 64,381, df_{24h} = 6, 105, P_{24h} = 0,000, Figure 1). In addition, cell viability (%) changed between the two exposure times at all venom concentrations except for the control groups and decreased significantly as exposure time increased (P <0.05, t-test, Figure 1).

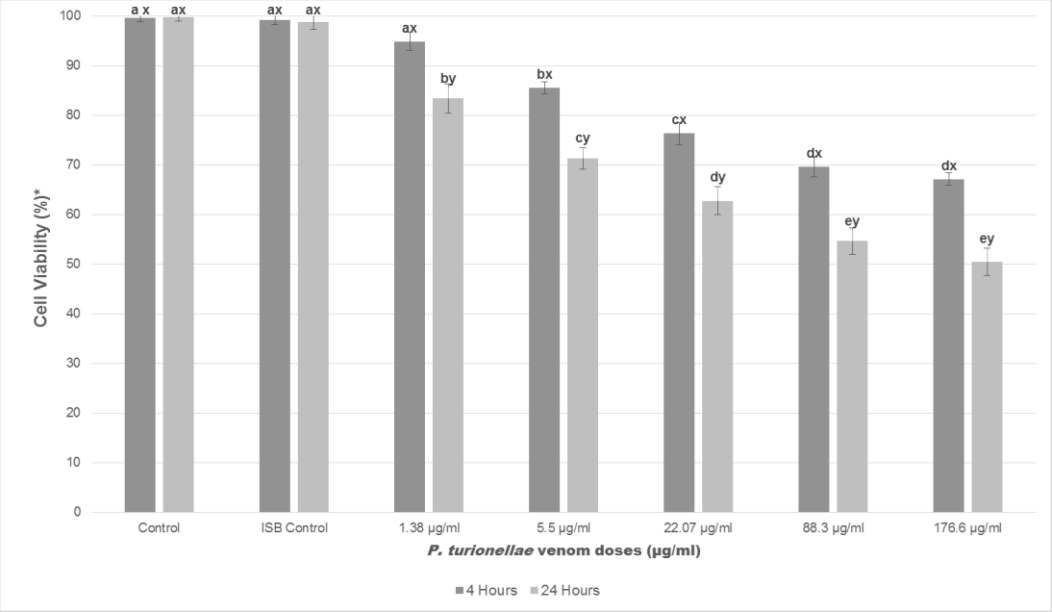

Figure 1. C6 glioblastoma cell viability (%) exposed to different venom concentrations of *P. turionellae* at different time intervals (4 and 24 h).

* All data are expressed as means ± standard error. Each column followed by the different letters (a-e, LSD test) or between black and grey column followed by the different letters (x-y, t-test) are statistically significant (P< 0.05).

4. DISCUSSION AND CONCLUSION

In recent studies conducted with the venom of various animals, it has been found that venom or its derivate has cytotoxic effects on many different cancer cells in vitro [2-7]. Sarfo-Poku et al. [3] pointed out that scorpion venom may be a candidate to a pharmacologic agent for the treatment of breast cancer due to its antibacterial properties. A recent study conducted with the spider species reported that venom of *Haplopelma hainanum* induces apoptosis by caspase activation and suppresses the proliferation of hepatic cancer cells [7]. Kerkkampa et al. [8] also reported that venom from snake species has anti-metastatic, antiangiogenic, and cytotoxic properties when applied at low concentrations on tumorigenic cells. Khamis et al. [6] also tested bee venom on MCF7 and T47D breast cancer cell lines in vitro and cytotoxic effects were observed. In particular, Rady et al. [4] suggested that melittin and its conjugates isolated from bee venom have the potential for cancer therapy. Moreover, a previous study showed that cell viability of human glioblastoma cells significantly decreased in a concentration-dependent manner after 24 and 48 h of incubation of bee venom at similar concentrations with our study [5]. Similar to

these previous findings, our results demonstrated that endoparasitoid *P. turionellae* venom has cytotoxic effects on rat glioblastoma cells depending on concentrations even at short exposure time. At the same time, the results obtained are in agreement with data of other authors who revealed that cytotoxic effects of endoparasitoid venom on glioma cells did not when exposure time increased even at the highest concentrations. Moreau & Asgari [15] suggested that parasitoid venom components could be used potentially in pharmacological studies and pest control.

A previous study determined that *P. turionellae* venom primarily contains various proteins of molecular weight between 20 and 106 kDa, and biomolecules such as melittin, noradrenaline, serotonin, peptide I, peptide II, phospholipase B, histamine and apamine [24]. Ergin et al. [24] also observed that P. turionellae venom-induced morphological changes in cultured cells from two orders of insects: BTI-TN-5B1-4 cells derived from the cabbage looper, *Trichoplusia ni* (Lepidoptera: Noctuidae) and Aag-2 cells derived from the larvae of the yellow fever mosquito, *Aedes aegypti* (Diptera: Culicidae). These morphological changes in cells may be associated with melittin. Because, unlike other neurotoxic active peptides and proteins in the venom, melittin lacks a specific receptor in the cell membrane and interacts directly with the cell membrane [11,25]. For this reason, melittin, which has a cytotoxic effect by activating phospholipase A2, is known to have a significant role in the anticancer activity mechanism of the bee venom [12]. Therefore, the results presented here indicate that the inhibitory effect of the endoparasitoid venom on the rat glioma cell viability may be related to melittin. Additionally, apamin, found in *P. turionellae* venom as a paralytic factor, inhibits Ca2+-activated K+ channels in neurons of the nervous system of mammals and can pass through the blood-brain barrier [9-10]. Voltage and current probe experiments showed that externally applied apamin (0.1 μM) even at low concentrations cause long-term hyperpolarization after blocked Ca2+ -dependent slow K+ channel conductance in cultured neuroblastoma and muscle cells in rat [26]. Therefore, venom-induced cytotoxicity in glioblastoma cells could be related to these structural and pharmacological properties of apamin.

The results of the present study could be useful to design fundamental research for developing a new pharmacological drug to treat cancer disease. In conclusion, further study is required to define which component of the venom affects glioblastoma cell proliferation, and the molecular pathway of cell death in vivo.

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