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RESEARCH ARTICLE ARAŞTIRMA MAKALESİ

Determination of anti-cancer and antioxidant properties of protein extracts obtained from aquatic *Helophorus* (Coleoptera: Helophoridae) insects

Sucul *Helophorus* (Coleoptera: Helophoridae) böceklerinden elde edilen protein ekstraktlarının anti-kanser ve antioksidan özelliklerinin belirlenmesi

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Abstract: In this study, protein extraction was performed from the aquatic insect *Helophorus aquaticus* (Linnaeus, 1758) and *Helophorus syriacus* (Kuwert, 1885) species belonging to the genus *Helophorus* (Coleoptera: Helophoridae). Then, these protein extracts were studied *in vitro*. These species were collected from the shallow parts of various streams, springs, creeks, ponds and hot springs from Bingöl city centre and its districts between May to June 2017. The protein amount was determined 34.78 mg/mL in the *H. aquaticus* and 35.14 mg/mL in the *H. syriacus*, after that he antioxidant capacity of protein extracts was examined. Metal chelating activity was determined as 90-88.5% and DPPH removal activity 53.19-61.7% for *H. aquaticus* and *H. syriacus* respectively. Protein samples belonging to both species were tested for cell vitality with WST-1 in PC-3 (prostate cancer) cells with *in vitro* cell culture. Upon examination of the test results, it has been found out that protein extracts from both of the studied species caused a decrease in cell inhibition. The highest cell inhibition was observed in samples with 1000 µg/mL insect protein extract added. In this study, protein expression providing apoptosis was examined with the Western blot technique after the effective dose was established. By looking at the proteins of Cyt-C and Caspase 3 with the Western blot technique, the efficacy of the protein extracts from both species was demonstrated effectively for the *in vitro* PC-3 line in non-apoptosis cell death. As a result of the study, insect proteins were shown to support the production of proteins that ensure cell death with the western blot technique.

Keywords: *Helophorus*, protein, Cyt-C, Caspase-3, antioxidant

Öz: Bu çalışmada, *Helophorus* (Coleoptera: Helophoridae) cinsine ait sucul böcek *Helophorus aquaticus* ve *Helophorus syriacus* türlerinden protein ekstraksiyonu yapılmıştır. Böcek türleri 2017 Mayıs-Haziran ayları arasında ve Bingöl il merkezi ve ilçelerinden çeşitli akarsu, kaynak, dere, birikinti ve sıcak su gözelerinin sığ kesimlerinden toplandı. Protein miktarı *H. aquaticus*'da 34,78 mg/mL, *H. syriacus*'da 35,14 mg/mL olarak belirlenmiştir. Daha sonra elde edilen protein ekstraktlarının antioksidan kapasitesi incelendi. Metal şelatlama aktivitesi *H. aquaticus* ve *H. syriacus* için sırasıyla %90-88,5, DPPH uzaklaştırma aktivitesi %53,19-61,7 olarak belirlendi. Her iki türe ait protein numuneleri, *in vitro* hücre kültürü ile PC-3 (prostat kanseri) hücrelerinde WST-1 ile hücre canlılığı açısından test edildi. Test sonuçlarının incelenmesi üzerine, çalışılan türlerin her ikisinden alınan protein ekstraktları, hücre inhibisyonunda bir azalmaya neden oldu. En yüksek hücre inhibisyonu, 1000 µg/mL böcek proteini ekstresi eklenen numunelerde gözlendi. Bu çalışmada, etkin doz belirlendikten sonra Western blot tekniği ile apoptozu sağlayan protein ekspresyonu incelenmiştir. Western blot tekniği ile Cyt-C ve Caspase-3 proteinlerine bakılarak, apoptoz dışı hücre ölümünde *in vitro* PC-3 hattı için her iki türden protein ekstraktlarının etkinliği etkili bir şekilde gösterildi. Çalışma sonucunda böcek proteinlerinin western blot tekniği ile hücre ölümünü sağlayan proteinlerin üretimini desteklediği gösterildi.

Anahtar kelimeler: *Helophorus*, protein, Cyt-C, Kaspaz-3, antioksidan

INTRODUCTION

Insects represent 55% of the biological diversity on the earth (Chernysh et al., 2002). Members of this highly diversified group have spread to almost all ecosystems around the world. Insects' ability to thrive in various ecosystems is due to their extremely strong adaptability. Insects have a very important place in terms of ecological and economic life of people and other living groups (Koç et al., 2011).

Coleoptera, containing 170 families, is the largest group of insects and is represented by more than 350,000 species worldwide. Globally, Hydrophilinae, which is sub-coleoptera is represented by approximately 57 genera and 1784 species, mostly consisting of species living in wetlands (Mart, 2009; Fikáček et al., 2010). Hydrophilidae is distributed in Europe, Asia and North Africa with a total of 34 genera. In Türkiye, 19 genera, 95 species and 4 subspecies belonging to two subfamilies have been identified living in certain regions (Polat et al., 2021). Helophoridae is a family of aquatic beetle (Coleoptera) with a single genus (*Helophorus*) and an average of 200 species (Darılmaz, 2010). *Helophorus* species, in general aquatic or semi-aquatic, spread over a wide area from the peaks of high mountains all the way to sea level. Many species prefer stagnant and shallow waters rich in organic matter as their habitat, for example, the edges of small puddles. Furthermore, sandy or muddy areas between water and soil, as well as very vital areas with moss or other

vegetation elements, can also be selected as habitats by the aforementioned species (Yılmaz, 2011).

Insects are healthy and sustainable sources of high-quality protein. Entomophagia is encountered in more than 90 developing countries (Defoliart, 1995). Many insect species are used in traditional and folk medicine in various parts of the world. (Koç et al., 2019).

Some edible insects contain pharmacologically active substances, while others contain toxic metabolites produced for self-defence or other purposes, and still others contain chemical compounds such as alkaloids. Other insects can separate secondary metabolites from host plants for their own defence mechanisms and store them by converting to substances such as aristocolitic acid and glucosinolates (Duffey, 1980; Berenbaum, 1993; Blum, 1994).

Use of insects and insect-derived from products for therapeutic purposes is called "entomotherapy" (Costa-Neto, 2002). Entomotherapy is practiced in traditional folk medicine by various societies in numerous parts of the world. It prefers bees and wasps, ants, grasshoppers, termites, crickets, cockroaches, dung beetles and caterpillars. These are used to treat a variety of diseases, including upset stomach, skin diseases, epilepsy, asthma, bronchitis, rheumatism, and infertility (Costa-Neto, 2002). Most multi-protein complexes are key regulators in the cellular process. The sizes of these complexes can vary from only two or three components to multimeric complexes (Charbonnier et al., 2008; Doucet and Hetzer 2010; Riccio, 2010). Recombinant protein technologies form the fundamentals of not only a lot of research but also biological drugs (McKenzie and Abbott, 2018). Recombinant proteins represent the largest class of new therapeutic products developed by the biopharmaceutical industry (Stuible et al., 2018). Various studies showed that antioxidant and anti-inflammatory peptides have protective effects against reactive oxygen species and may contribute to a significant reduction in the level of oxidative stress, which is the main risk factor for diseases in civilization (Torres-Fuentes et al., 2011; Karaś et al., 2015).

The most widely consumed insect groups globally are in the order of Coleoptrea with a rate of 31%. The species belonging to the Helophoridae family which are used in traditional medicine and widely consumed in Central Asian and African countries have spread over a wide geography. These beetle species are potential sources of antioxidants like plants, seafood, and mushrooms, but are relatively unexplored (Van Huis, 2013). More taxonomic studies on these insect groups are being conducted in Türkiye. At the same point, important unsaturated fatty acid groups (palmitoleic acid and vaccenic acid) such as omega-7 were determined in these aquatic insects (Caf et al., 2020). In insect organisms, these bioactive compounds play a vital role against oxidative damage (Suh et al., 2010). Insects can also quickly resolve microbial infections by producing most immune-induced molecules, including antibacterial or antifungal peptides and polypeptides (Chernysh et al., 2002). Zielińska et al. (2018) concluded that edible insects were a valuable source of bioactive peptides with antioxidant and anti-inflammatory properties, and demonstrated that peptide fractions isolated from edible insect hydrolysates had high antioxidant and lipoxygenase and cyclooxygenase-2 inhibitory activities. Moreover, they reported that the heat treatment process had a significant effect on improving these properties, and twelve antioxidant and anti-inflammatory peptides were also identified in their study.

This study was conducted due to the lack of previous research on the antioxidant and anti-cancer properties of protein extracts from helophorid species. This was supported by the perception that these protein extracts might exhibit antioxidant and anticancer properties owing to the belief that these insects had high antimicrobial efficiencies due to their living habitat of aquatic ecosystems.

MATERIALS AND METHODS

Chemicals

Trypsin-EDTA, trypan-blue, WST-1 viability and spreading agent, penicillin streptomycin solution, foetal bovine serum (FBS), SDS, 10% trichloroacetic acid (TCA), Dulbecco's Modified Eagle Media (DMEM), 70% and 95% ethyl alcohol, methanol, dimethyl sulfoxide (DMSO), phosphate buffer saline (PBS), antibody (GABDH, Caspase-3 and CYT-C), sample buffer, running buffer, transfer buffer, marker, 12% gel, RIPA lysis buffer, nitrocellulose membrane, blotting paper, skimmed milk powder, secondary antibody, ECL, peroxide solution, TBS, Tween20, Ac./Bis, 10% APS, TEMED, Tris-Cl, NaCl, KCl. Thoma slide, 25 cm and 75 cm flasks, serological pipettes of various sizes, 96-well plates, 15 mL centrifuge tubes, 50 mL centrifuge tubes, Eppendorf tubes of various sizes, automatic pipettes of various sizes, and homogenizer (Cell disruption tube).

The materials used comprised the following items and trans blot was completed; microbiological safety cabin, heated water bath, automatic pipettes, freezer, mortar, vortex, sonicator, precision scales, refrigerator (+ 4 ºC), autoclave, pure water device, oven 5% CO_2 incubator, vertical and horizontal electrophoresis and gel imaging.

Preparation of Protein Extract

Insect species were collected from Bingöl province and identified by Prof. Dr. Abdullah Mart. *Helophorus syriacus* and *Helophorus aquaticus* species stored at -80 °C were weighed to 2 g, and were thoroughly disintegrated in a homogenizer by adding 4 mL 10% TCA after being placed in flacons. Subsequently, another 1 mL of 10% TCA was added, and cooled in a cooled centrifuge for 50 minutes at 4,000 RPM. The supernatant portion was removed and the pellet portion was rotated at 3,500 RPM for 10 minutes with 3 repetitions, washed with 5 mL of 95% ethyl alcohol and kept in an oven at 37 °C for drying. The dried sample was properly crushed in a

mortar and 2 mL of purified water was poured on it. The sample was then incubated in an oven at 37 °C for 15 minutes and centrifuged at 4,500 RPM for 15 minutes. Then 100 µL of the sample was used for Bradford. At this point, 2 mL of 95% ethyl alcohol was added to the remaining sample, and this was left at 37 °C for 1 night. The following day, it was centrifuged at 4,500 RPM for 1 hour and left at 37 °C to completely removed alcohol and dried the protein extracts. The precipitate, which alcohol was removed, was poured into a tube with a thin spatula and pulverized into powder (Coşkun et al., 2009).

Total Protein Determination with Bradford Method

This method (5-100 µL) is based on the interaction of basic and acidic groups from proteins to form organic dyes. The protein's amino acid composition is important in the formation of blue colour. The dye has a strong affinity for basic amino acids like arginine and certain aromatic amino acids. The main phenomenon in this method is that while the dye has the highest absorbance at 465 nm under normal conditions, it has the highest absorbance at 595 nm wavelength when bound to protein. A standard calibration graph was prepared with bovine serum albumin (BSA) solution (Figure 1).

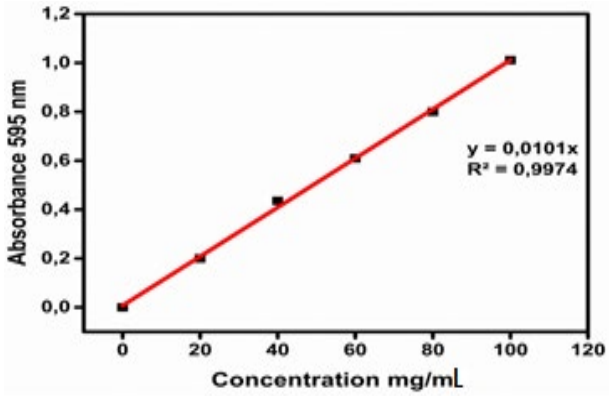

Figure 2. Bradford standard calibration chart for protein determination

The previously prepared insect samples, stored at +4 °C, were measured with the UV-VIS spectrophotometer (Shimadzu/Jasco V650). Protein amounts of the samples were calculated by placing the read absorbances in the regression equation. The absorbance value read for *Helophorus syriacus* at 595 nm was 0.3478. The total protein amount was 34.78 mg/mL after incorporating the absorbance into the regression equation. At 595 nm, the absorbance value for *Helophorus aquaticus* was 0.3514. Using the regression equation and the absorbance, the total protein amount was calculated to be 35.14 mg/mL.

Determination of DPPH Activity

First, 2.7 mL of methanolic solution containing DPPH radical prepared to 6x10 $^{-5}$ mol per litre was mixed into the insect protein extract (0.3 mL) prepared at a concentration of 1 mg/mL. This mixture was strongly mixed and kept in a dark place for 60 minutes. Removal of DPPH radical activity was determined by measuring the absorption with the spectrophotometer at 517 nm. Efforts to remove this radical were performed by following the method proposed by various researchers (Hatano et al., 1988). BHT was used as a positive control for the DPPH test.

Determination of Metal Chelating Activity

The metal chelating activity was examined through the iron chelating feature. Determination of the properties according to this method in brief, began by adding 1.6 mL of deionized water and 0.05 mL of 2 mM $FeCl_2$ to each 0.5 mL extract. After 30 seconds, 0.1 mL of 5 mM ferrozine was added. Ferrozine became very soluble in water after reactions with bivalent iron. Subsequently, the absorbance of the Fe^{2+} ferrozine complex was measured at 562 nm for 10 minutes at room temperature. Here, EDTA was used as a standard chelator, and the chelating activity was expressed according to the EDTA standard. Thus, the chelating activity of iron from the extract was calculated using the formula below (Wenli et al., 2004).

% Chelation Rate = $(A_0 - A_1)/ A_0 \times 100$;

$\mathbf{A_1}$ is the absorbance value measured in the presence of the extract, and $\mathbf{A_0}$ is the control or blind absorbance.

Cell Vitality Analysis with WST-1

In this study, the PC-3 cell line was utilized. The PC-3 cell line was procured from the Molecular Biology and Genetics department of Bingol University. Firstly, 500 mL of DMEM, 50 mL of foetal bovine serum, and 5 mL of penicillin-streptomycin medium were mixed in the medium container to grow these cells. PC-3 cells stored at -80 °C were propagated in DMEM growth medium, passaged at 37 °C in a 5% CO_2 oven every 2 or 3 days. The proliferated cells were examined with a reverse microscope in a 96-well plate. After establishing that the cells were proliferating sufficiently, insect protein extract concentration in the range of 5-1000 µg/mL was prepared and added to each well with 3 repetitions and incubated for 48 hours. PC-3 cells were seeded in 96 wells and incubated. Insect protein extracts (3.125, 6.25, 12.5, 25, 50 and 100 mg/mL) were diluted with cell culture medium, treated with cells and incubated for 48 hours. Only medium + cells were used as the control group. After 48 hours, 15 µl of WST-1 was added to each well. Cells were incubated at 37 °C for 4 hours in an incubator with 5% CO_2. After 4 hours of incubation, the 96-well plate was placed on an ELISA reader and absorbance values for each well were recorded at 450-630 nm.

Colour formation of cells and dead cells in the WST-1 toxicity test were spectrophotometrically measured at 450 nm, and the presence of inactive WST-1 was measured at 630 nm. Absorbance values were plotted on a graph.

Analysis of Target Proteins with the Western Blot Technique

PC-3 cells were grown in 75 cm^2 flasks to reach 3-4x10^6, and treated with protein extracts obtained from the insects at a concentration of 1 mg/mL, after which they were prepared for protein isolation by washing with PBS and centrifuging. Around 3-4x10^6 cells were homogenized in a cold environment with the aid of a protein isolation kit at a ratio of 1:5 (w/v). In order to prevent the proteins from degrading due to protease activity, both protease inhibitor cocktail (PIC) and PMSF were used during homogenization processes and all processes were performed on ice. The supernatants were placed in microcentrifuge tubes after centrifuging the homogenates in a cooled centrifuge at 14.000 RPM for 20 minutes at +4 °C were placed in microcentrifuge tubes. The Bradford method was used to determine the amount of protein in each sample. Samples were then stored at –80 °C, until Western blotting experiments were carried out. Protein lysates from the cell culture were processed with the 12% SDS-PAGE (Sodium dodecyl sulphate polyacrylamide gel electrophoresis) technique in concentration gel; then, caspase 3, cytochrome C and GABDH were used as housekeeping and transferred to the PVDF membrane. The blotting sequence with 5% BSA for 1 hour began. Subsequently, the bands of the proteins fixed on the membrane were incubated for 3 hours with suitable primary antibodies and washed with TBST (Tris Buffer Saline, 0.1% Tween 20) for 5x5 minutes, and incubated for 1.5 hours with the secondary suitable for the primary. Then, washing was done with 5x5 min TBS-T. The membrane was then incubated with ECL buffer for about 3-4 minutes and thanks to the radiation on the membrane, protein bands were fixed on X-Ray films in the medical X-Ray image stabilization device. Then, the synthesis quantity of these tapes was calculated using computerized software (Image Lab, Bio Rad). The calculation method normalized the target genes with the housekeeping used in the gene GABDH and changed the percentage based on the control.

RESULTS AND DISCUSSION

Natural antioxidant endogenous compounds play an important role in most living organisms' innate host defence mechanisms of, including plants, insects, amphibians and mammals (Koczulla et al., 2003). Insects are a significant source of potential be considered as a serious antioxidant and antimicrobial agents because they contain more antimicrobial and antioxidant compounds depending on the environment in which they live.

Due to the rapid resistance of microorganisms to existing antibiotics, insects are being considered as a potential source of new antibiotic agents. (Mittapalli et al., 2007). Compounds with antioxidant effects can be found in edible insect species, or antioxidant effects can be achieved with bioactive compounds obtained from these sources, for oxidant compounds, which human beings face more due to factors such as industrial development of and rapid eating habits, There are studies to determine the antioxidant activities of insect proteins (Liu et al., 2012; Suh et al., 2010; Zielińska et al., 2017; Zielińska et al., 2018). Peptide fractions formed as a result of enzymatic hydrolysis of edible insect species reduced the concentration of free radicals that cause oxidative stress. DPPH and metal chelation are assays commonly used to determine antioxidant activity. Figures 2 and 3 show the DPPH and metal chelation results for insect protein.

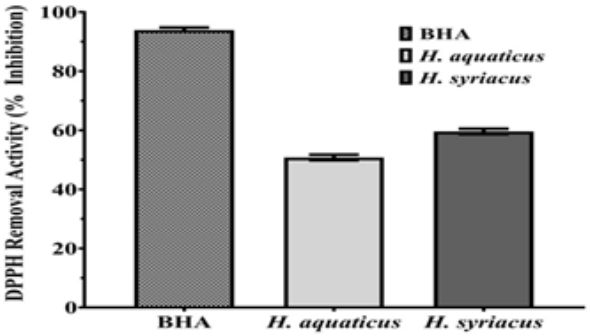

Figure 2. DPPH removal activity of insect protein

When DPPH removal activity is examined and species are compared according to the absorbance value of DPPH, it was close to 62% for *H. syriacus* and 48% for *H. aquatic*. This result shows that the protein extracts from the species have the capacity to remove DPPH oxidant**.** In a study investigating the *in vitro* antioxidant effect of water and oil-soluble extracts from edible insects, grasshopper, silkworm and cricket had antioxidant capacity 5 times higher than fresh orange juice (Di Mattia et al., 2019). The DPPH activity of *Allomyrina dichotoma* insect larvae was examined in different solvent media and the best activity was obtained in ethanol medium (Suh et al., 2010).

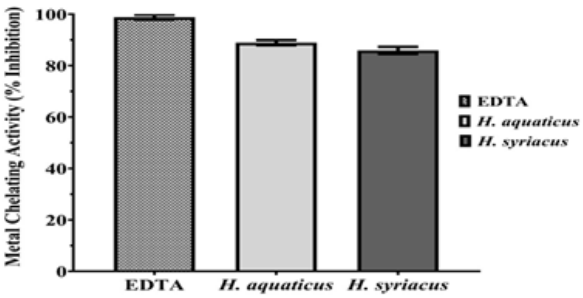

Figure 3. Metal chelating activity

Insects are constanty exposed to microbial infections, pathogens and oxidative stress on a daily basis. Antioxidant and antimicrobial products, in particular, play important roles in defence mechanisms in order to protect themselves against environmental attacks (Bulet et al., 2004). One of the ways to determine the antioxidant property of insects is with the metal chelating property. In this study, Metal chelating activity was examined through the iron-chelating feature. When metal

chelating activity is evaluated, it has been seen that around 90% of metal ions in the environment were removed with the metal chelation activity of *H. aquaticus* while *H. syriacus* removed about 89%. Studies on iron-metal chelation of the beetles extracts and insect protein lysates are limited. In a study determining the metal chelating activity of proteins from three edible insect species, insect proteins had iron chelating properties (Zieliǹska et al., 2018). In another study, silkworm protein hydrolysates were reported to have high iron chelation capacity (IC_{50} 2.03 mg/mL) (Wu et al., 2011). These beetles groups lived in swamps have developed their defence systems. The redox properties of phenolics and polyphenolic compounds are primarily responsible for their antioxidant activities (Gil et al., 2000). It is thought that proteins in insects may be responsible for the antioxidant activity of phenolic and polyphenolic compounds. Because of phenols are one of the important compounds as antioxidant (Suh et al., 2010).

Studies show that insect protein extracts can inhibit or promote cell proliferation. Protein hydrolysates can be administered as bioactive components that suppress inflammation, regulate the synthesis of extracellular matrix proteins, or stimulate the proliferation of skin cells (Zielińska et al., 2015). Peptides with antimicrobial effect are also isolated by hydrolysis of proteins contained in edible insects (Jantzen da Silva Lucas et al., 2020). As shown in Figure 4, the addition of insect hydrolysates is observed to significantly inhibit PC-3 cell proliferation. This effect occurs depending on the type of insect protein hydrolysate samples and their handling. Both insect protein hydrolysates showed the highest cytotoxic effect at 1000 µg/mL. In the study of cytotoxicity for human skin fibroblast, both stimulant and inhibitory effects were observed in a study with different insect groups. The presence of insect hydrolysates from *T. molitor* and G. *sigillatus* stimulated the growth of human skin fibroblasts, while *S. gregaria* protein hydrolysate showed cytotoxic effects (Zielińska et al., 2015). The highest inhibition by *H. aquaticus* and *H. syriacus* was seen at 1000 µg/mL. Subsequently, inhibition was observed at concentrations of 500 µg/mL, 250 µg/mL, and 62.5 µg/mL, in order. As a result of this study, 1000 µg/mL was determined to be the most effective concentration when all studies were compared.

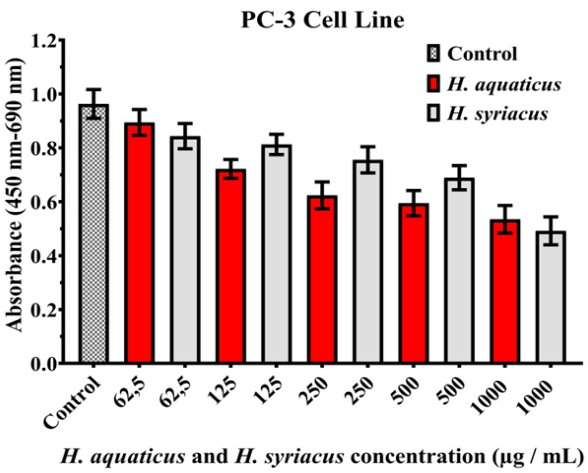

Figure 4. WST-1 viability test analysis showing the effect of different concentrations of *H. aquaticus* and *H. syriacus* protein extracts on cell inhibition. No agent was applied to control cells and only growth medium was added. Statistical analysis was performed with one-way ANOVA using the Dunnett's multiple comparison test as a post-test in the confidence interval of $p < 0.01$. Values are expressed as mean ± SEM (n = 3)

Analysis of Target Proteins with the Western Blot Technique

The antioxidant mechanisms influenced by peptide fractions formed as a result of insect protein hydrolysis are still unknown. The Western Blot technique was used to examine protein products that cause cell death. In this study, the amount of protein was compared in *H. aquaticus* and *H. syriacus* in accordance with the control group. GAPDH was utilized as housekeeping protein. Compared to this protein, Cyt-C and Cas-3 ratios were evaluated, which provide information about cell inhibition and cell death. As stated in Figure 5, Cyt-C levels were determined in both types compared to the control group. Cas-3 activity was also determined in both types.

The tendency for apoptosis in cells is followed by the infiltration of cytochrome-c into the cytoplasm by disruption of mitochondrial outer membrane permeability (MOMP), followed by activation of caspases. Caspases mediate the formation of apoptosis, but inhibiting caspases does not solve the problem of cell survival after the formation of MOMPs. In this case, "caspase-independent cell death" (CICD) occurs in cells.

Therefore, the disruption of mitochondrial outer membrane permeability, namely MOMP, and the release of Cyt-C may represent the main entry point to cell death. Increases in the amount of Cyt-C and leakage from mitochondria cause the cell to enter the apoptosis pathway (Colell et al., 2007).

Caspases are the most critical mediates in the formation of apoptosis. Within the caspase family, caspase-3 is the protease within the caspase family that catalyses apoptosis by stimulating the specific cleavage of multiple important cellular proteins and activates the most fundamental step of apoptosis, which is frequently activated. However, when the mechanism of apoptosis is studied, the specific requirements of a member of the caspase family are not known as a definitive diagnosis until now. Looking at the pathways leading to caspase-3 activity, they were identified as dependent on or independent of mitochondrial cytochrome-c release and caspase-9 function. Caspase-3 is important in other apoptotic scenarios, as it is required for normal brain development and exhibits remarkable activity specific to cell type, tissue, or death stimulus. However, caspase-3 is required in the presence of typical markers of apoptosis. It is essential for the condensation of apoptotic chromatin and

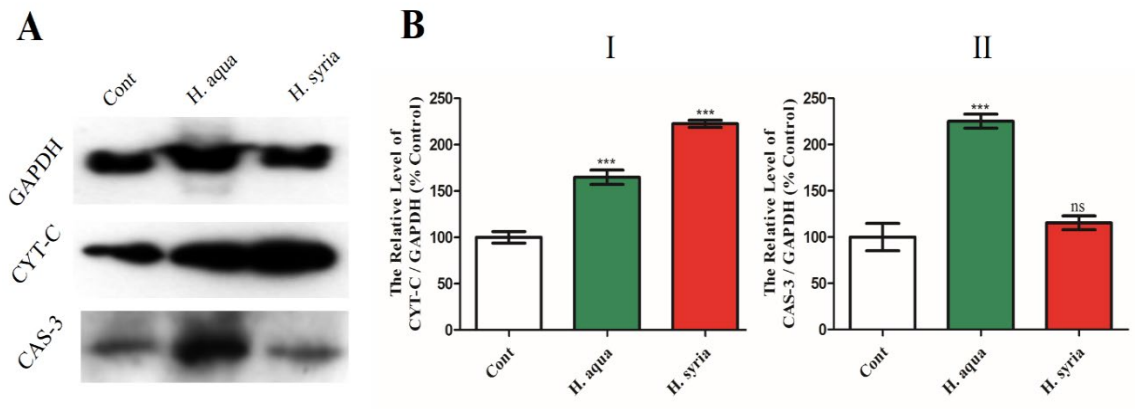

Figure 5. Effects of protein extracts on apoptosis-related protein expression in PC-3 cells. **(A)** Cytochrome-*c* (15 kDa) and caspase-3 protein levels were measured after western blotting. GAPDH was used as the loading control. **(B)** Data are shown as mean ± SEM (n=3). p <0.01(**) Control vs protein extracts.

subsequent DNA fragmentation in most cell types. In the light of these evaluations, caspase-3 is a necessity for its presence in the cell and subsequent formation of apoptotic bodies and the formation of pathways (Porter and Jänicke, 1999).

As seen from the graph, the ratio of Cyt-C to GAPDH is higher compared to the control group for *H. aquaticus*. This difference indicates that Cyt-C is released in high amounts when compared to the control group (i.e. cells replicated under the same conditions but without insect protein added). When we look at Cyt-C/GAPDH rate, it has been seen that there is a high Cyt-C release. Hence, it has induced the death of cell because of increasing the permeability of mitochondrial outer membranes and explained the setting of apoptosis. When the GAPDH ratio of the caspases is examined, it was also higher than the control group. This difference indicates that the amount of caspase-3 is higher than the control group. This excess shows the high amount and presence of precursor caspase-3 activity that leads to cell death; thus, showing that *H. aquaticus* proteins direct the cell toward cell death (Colell et al., 2007).

In *H. syriacus*, the ratio of Cyt-C to GAPDH is approximately 2.25 times higher than that of the control Cyt-C to GAPDH. This difference indicates that Cyt-C is released more and is more effective for *H. aquaticus* compared to the control group (i.e., cells replicated at the same time under the same conditions without insect protein added). However, when evaluated in terms of caspase-3compared to *H. aquaticus*, the amount of Caspase-3 was lower compared to the cell vitality test, and a different pathway supports cell death. As there is no study planned around the project, DNA fragmentation should be supported by methods such as real time PCR for definitive proof. However, the budget and scope of this study did not cover this.

These results show that when the data for both types studied are evaluated, the WST-1 cell vitality test showed that PC-3 cancer cell line had a higher level of cellular death than the control group. Attempts were made to explain how cell death occurs via the western blot test. The cause of cell death was also observed at the protein level by examining Cyt-C and Caspase-3 activities. In this study, when *H. aquaticus* and *H. syriacus*, which live in the aquatic environment, are regarded as the content of antioxidants, they have high antioxidant capacity. Similarly, they displayed activity on PC-3 (human prostate cancer) *in vitro* cell vitality, and the vitality of cancer cells diminished with the increased concentrations and increasing activity (62.5-1000 µg/mL). *H. aquaticus* and *H. syriacus* extracts may play an important role in ROS scavenging against oxidative stress. Given the high consumer demand for beneficial health effects, these groups of insects can be used to develop functional food, health promoting and pharmaceutical agents.

CONCLUSION

It is important for sustainability of the ecological system that insects consume much less water and produce fewer greenhouse gases compared to other animals. Insects are economical sources that can be used as an alternative to animal protein, especially when produced on an industrial scale. At the same time, as a good source of protein and antioxidants, they may be an alternative food that can eliminate anxiety about food shortages in the future.

This study is a first step towards determining the protein content of *Helophorus* (Coleoptera: Helophoridae), which is abundant in Türkiye, as an alternative to prevent anxiety about finding nutrients that may occur in the future. These proteins are also antioxidant and have a positive effect on cancer cells. Investigation of PC-3 cell line activity, antioxidant activity and apoptosis function of protein extracts from *H. syriacus*, and *H. aquaticus* may be an important step in this area. Further molecular and *in vivo* studies are needed on this beetle extracts.

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Authorship Contributions

This study is a part of Master Thesis by Tuba ELHAZAR (2019). Tuba Elhazar carried out the collection and storage conditions of the samples from the field for this study. Tuba Elhazar and Bülent Kaya performed the anticancer, antioxidant, and Western Blot. The evaluation of the study's data and the article's writing was done by Bülent Kaya and Fatma Caf.

Conflict Of Interest

There is no conflict of interest in this study

Ethical Approval

No specific ethical approval was required for this study.

Data Availability

All relevant data is inside the article

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