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The Effects of Squirting Cucumber (*Ecballium elaterium* l. 1758) on the Blood Cell Morphology of Common Carp (*Cyprinus carpio* L. 1758)

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Research Article

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Abstract

It is well-known that for centuries, humans have used plants and herbal remedies to treat many diseases, in addition to the treatment options brought and applied by modern medicine, which relies on scientific studies and actual evidence. One of these plants is the squirting cucumber (*Ecballium elaterium*), or the exploding cucumber. Despite its therapeutic properties, the squirting cucumber may also cause serious biological damage to humans, as demonstrated several studies. This study was conducted to determine whether the juice obtained from ripe fruits of squirting cucumber has any effect on the cell morphology of common carp (*Cyprinus carpio*). Juice application was carried out on 4 groups, composed of 1 control and 3 experimental groups. Squirting cucumber juice was added to three aquariums containing different experimental groups, in three different ppm (100, 200, and 300) levels, for 72 hours. Afterwards, blood smear samples of the fish were prepared, and the preparations were scanned under a microscope to determine the changes in blood cell morphology. The results demonstrated that squirting cucumber juice caused micronucleus formation during cell division in *C. carpio* blood cells, and several abnormalities in blood cell morphology including Lobed nucleus (LB), Bud nucleus (BL), Notched nucleus (NT) and Binucleated erythrocytes (BN).

Keywords: Ecballium elaterium, Cyprinus carpio, micronuclei, erythrocyte morphology

Acı Kavun (*Ecballıum Elaterıum* L. 1758)'un Sazan (*Cyprınus Carpıo* L. 1758) Balıkları Kan Hücre Morfolojilerine Etkileri

Özet

Yüzyıllardır birçok hastalığın tedavisinde modern tıp tarafından uygulanan, kanıta ve bilimsel çalışmalara dayalı tedavi seçeneklerinin yanı sıra bitkiler ve bitkisel ilaçların da kullanıldığı bilinmektedir. Tedavi amaçlı kullanılan bitkilerden bir tanesi acı kavun (*Ecballium elaterium*) bitkisidir. Acı kavun bitkinin tedavi edici özelliğinin yan ısıra, insanlar üzerinde ciddi biyolojik hasarlar da bıraktığı yapılan çalışmalarda görülmektedir. Bu çalışmada, acı kavun bitkisine ait olgun meyvelerden elde edilen özsuyun sazan balıkları (*Cyprinus carpio*)'nın hücre morfolojilerine etkisinin olup olmadığının belirlenmesi amaçlanmıştır. Uygulama 3 deney ve 1 kontrol grubu olmak üzere 4 grup üzerinde yapılmıştır. Üç farklı deney grubuna ait akvaryumlara 3 farklı ppm (100, 200 ve 300) değerinde acı kavun özsuyu 72 saat uygulanmıştır. Balıklardan kan yayma preparatları hazırlanmıştır ve hazırlanan preparatlar mikroskop altında taranarak kan hücrelerinin morfolojilerinde meydana gelen değişiklikler tespit edilmiştir. Sonuç olarak acı kavun özsuyunun *C. carpio* kan hücrelerinde bölünme esnasında mikronükleus oluşumuna neden olduğu ve kan hücrelerinin morfolojilerinde Loblu nükleus (LB), Tomurcuklu nükleus (BL), Çentikli nükleus (NT) ve Binükleat (BN) gibi anormalliklere neden olduğu tespit edilmiştir.

Anahtar Kelimeler: Ecballium elaterium, Cyprinus carpio, Mikronükleus, Eritrosit morfolojisi

INTRODUCTION

Since ancient times, medicinal plants and herbal remedies have been used for the treatment of certain diseases, besides the treatment options of medical doctors that are based on hard evidence brought by scientific studies. Diseases emerging as a result of changes in the dietary habits of humans, along with changes in environmental conditions, and the inadequacy of drugs used for treating these diseases cumulatively increased the human interest in traditional medicine (Baytop, 1999). For the last two thousand years, humans have been using the fruits, roots, and leaves of many known plants

against many diseases including tumors, skin spots, eczema, and sinusitis. One of these medicinal herbs is the squirting cucumber (*Ecballium elaterium*).

In scientific studies, the juice of this plant has been observed to show both positive and negative effects on organisms (Çelik, 2009). Specifically, the juice obtained from the ripened fruits of E. elaterium was determined to show certain destructive effects on living things (Mazokopakis, 2009). The effects of E. elaterium on living things were tested on mice, plants, and cultured human peripheral Lymphocytes (Rencüzoğullari, 2006; Shabbar and Maslat, 2007; Çelik, 2009). It is known that alterations in blood cells of living organisms, and usually the changes in the number and nucleus morphology of erythrocytes, adversely affect metabolism (Verhovsek, 2017).

In vertebrates, erythrocytes account for more than 99% of the cells found in the blood. Young erythrocytes, produced in the red bone marrow and added to the blood circulation, lose their nucleus along with many of their organelles when they mature in vertebrates except the fish (Aktümsek, 2002). Since mature fish erythrocytes have nuclei (Claver and Quaglia, 2009; Mumford, 2007), it is easier to observe the abnormalities and micronuclei (MN) that may develop in fish erythrocytes due to the effects of *E. elaterium* juice. Abnormalities seen in the erythrocytes are divided into two groups: 1) nucleus abnormalities, and 2) cytoplasm abnormalities (Verhovsek, 2004). Micronuclei (MN) can be seen in cells when cytoplasmic division is disrupted and cells end up containing either entire chromosomes that fail to travel to the poles of the cell during mitosis or chromosome fragments that do not have centromeres. During telophase, a nuclear membrane forms around these entire and/or broken chromosomes, resulting in a structure smaller than the cell's main nucleus, forming what is called a "micronucleus" (Fenech, 2000).

The effect of *E. elaterium* juice on blood cell morphology has been tested on many organisms including mice, rabbits, and plants (Uslu et al., 2006). However, there is no study on *C. carpio*, which has nucleated erythrocytes. In this context, this study was carried out to observe the effects on the nucleus morphology of erythrocytes more easily.

MATERIALS and METHODS

Fish specimens (*Cyprinus carpio*) used in the experiment were obtained from the Yedikır Aquaculture Station operated by the 73rd Branch Directorate of the 7th Regional Directorate under the General Directorate of State Hydraulic Works (DSI) of the Ministry of Forestry and Water Affairs, along with necessary legal permissions, and were left to rest in 173-liter aquariums filled with rested tap water to ensure their adaptation to the new environment. Fish were divided into 4 groups (1 control and 3 experimental groups), with 7 individuals in each group (Çiğremiş, 2003; Özkan, 2011). The fish used in the experiments had an average body weight of 100-120 grams, and an average length of 18-20 cm.

Ripe fruits of squirting cucumber (*Ecballium elaterium*) plants used in the experiment were collected from Manisa, Ankara, and Kırşehir provinces during May and September, and brought to the laboratory (Table 1). With the circular published in 2016by the Ministry of Health, the cultivation, and sale of this plant were prohibited. Therefore, the necessary legal permissions were taken from the Ministry of Agriculture and Forestry.

Table 1. Localities of *Ecballium elaterium* fruits collected.

Locality	Date	Coordinates	
		Latitude	Longitude
Manisa - Salihli	May - 2017	38.479625°	28.037181°
Ankara- Batıkent	September - 2017	39.967906°	32.713785°
Kırşehir	September - 2017	39.145961°	34.153838°

Ripe fruits brought to the laboratory were crushed in a sterile container. The fruit juice was obtained from the crushed fruit material by using filter paper. About 500 ml of fruit juice was obtained from approximately 7-8 kg of ripe fruit. This juice was added to all 3 aquariums in the experimental group, in volumes of 17.3, 34.6, and 51.9 ml (100, 200, and 300 ppm), respectively (Table 2). The application continued for 72 hours, as seen in Figure 1 below, and the water in all aquariums was changed every 24 hours.

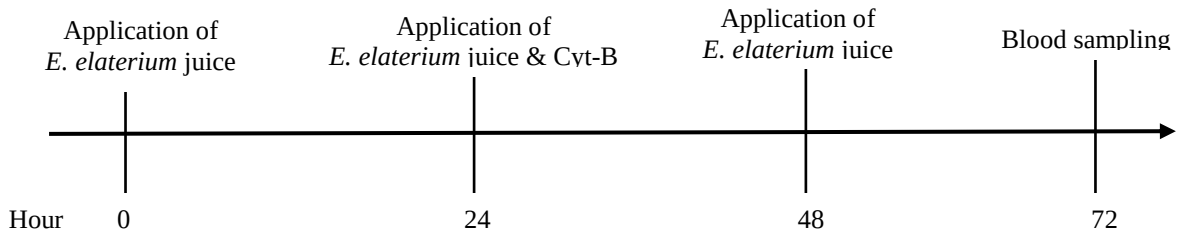

Figure 1. Working schedule (timetable).

On the 24th hour of the experiment, 1 mg of Cytochalasin B (Cyt-B) was injected into each fish, from an 80 ml solution prepared by dissolving 1 ml of Cyt-B (Sigma, 6762) in 79 ml of Dimethyl Sulfoxide (DMSO), to inhibit telophase (a division of the cytoplasm), which is the final stage of mitosis (Fenech, 1992; Fenech, 2007).

Table 2. Information on experimental and control groups

	Control Group	Experimental Groups		
		1	2	3
Number of fish	7	7	7	7
E. elaterium ppm value	0	100	200	300

At the end of the juice application period, on the 72nd hour, the fish in all 4 aquariums were knocked on the head to be rendered unconscious. The unconscious fish were then cut with a sharp knife at about 1-2 cm to the rear of their tail fin (Kocabatmaz and Ekingen, 1984). Blood samples about the size of lentil were taken directly from the caudal vein onto one side of slides, which were cleaned beforehand. Drops of blood were then treated with spreader slides, by bringing the short edges of these second slides towards the drops, held at an angle of 45° degrees. The blood was smeared thinly over the original slide by first keeping the second slide still until blood spread along its edge, and then moving it forward in the opposite direction by keeping the same angle between the two slides. With this method, blood smear preparations were obtained.

The preparations were air-dried for one day, and the dried preparations were then fixed by using methyl alcohol. Fixed preparations were stacked in shawls and stained by being suspended in 5% Giemsa solution for 20 minutes. The stained preparations were washed with distilled water until the last drop was clear, and air-dried once again. Final preparations were sealed with Entellan and made permanent. For every individual fish, 2 preparations were worked on with this method.

Samples prepared by the *in vitro* micronucleus (MN) test technique were examined under Leica DM 3000 research microscope to determine the erythrocytes displaying abnormal nuclei and micronuclei formations. For each preparation, a random count of 1,000 erythrocytes was conducted. Among these, the number of erythrocytes displaying MN formations and other abnormalities were determined, and the numbers were expressed in per thousand (‰) (Fenech, 2000; Şekeroğlu, 2011). In all examined preparations, abnormalities detected in cells were divided into 2 different groups: 1) Erythrocyte Nucleus Abnormalities and 2) Erythrocyte Cytoplasm Abnormalities.

Abnormalities detected in the nuclei of erythrocytes were divided into 5 groups according to the method used in the study conducted by Carrasco (1990), and the abnormalities detected in the cytoplasm of erythrocytes were grouped according to abnormality types identified in the study conducted by Gill (1985).

The numerical data obtained in the study were evaluated with one-way analysis of variance test to see whether the fruit juice of squirting cucumber added to the aquariums showed a normal distribution in terms of ppm values. Abnormal erythrocyte numbers detected in the cells were compared with multiple analysis of variance. Scheffe's method (multiple comparison test) was used for statistical evaluation of data between experimental groups. The differences at $P < .05$ level were referred to as significant, and the data for all groups were expressed as mean ± standard deviation.

RESULTS

In the study, the genotoxic effects of *Ecballium elaterium* juice on fish were investigated, and the results were evaluated regarding the relationship between dosage and the number of abnormalities. To detect MN formations and morphological disorders in erythrocytes, blood samples were taken from all of the fish specimens in all four groups, two blood sample preparations were made for each specimen, a random 1,000 cells were counted on each preparation, and a total of 14,000 cells were examined in each group. At the end of the study, it was observed that the *E. elaterium* juice added to the aquarium water in different doses resulted in an increased number of MN formations and other abnormalities in erythrocytes in line with the increased dose of application.

Erythrocyte Nucleus Abnormalities

When the abnormalities detected in all examined preparations are divided into groups according to the nomenclature by Carrasco (1990), 5 separate groups can be observed. These groups are formed according to the type of abnormality seen in the nuclei of erythrocytes. The groups are named as follows; Micronucleus (MN), Lobed nucleus (LB), Bud nucleus (BL), Notched nucleus (NT), and Binucleate (BN).

In the overall analysis of cells, the types of abnormalities detected in the nuclei of erythrocytes were found as shown in Figure 2, and the numerical values belonging to the detected abnormalities were found as given in Table 2 and Figure 3.

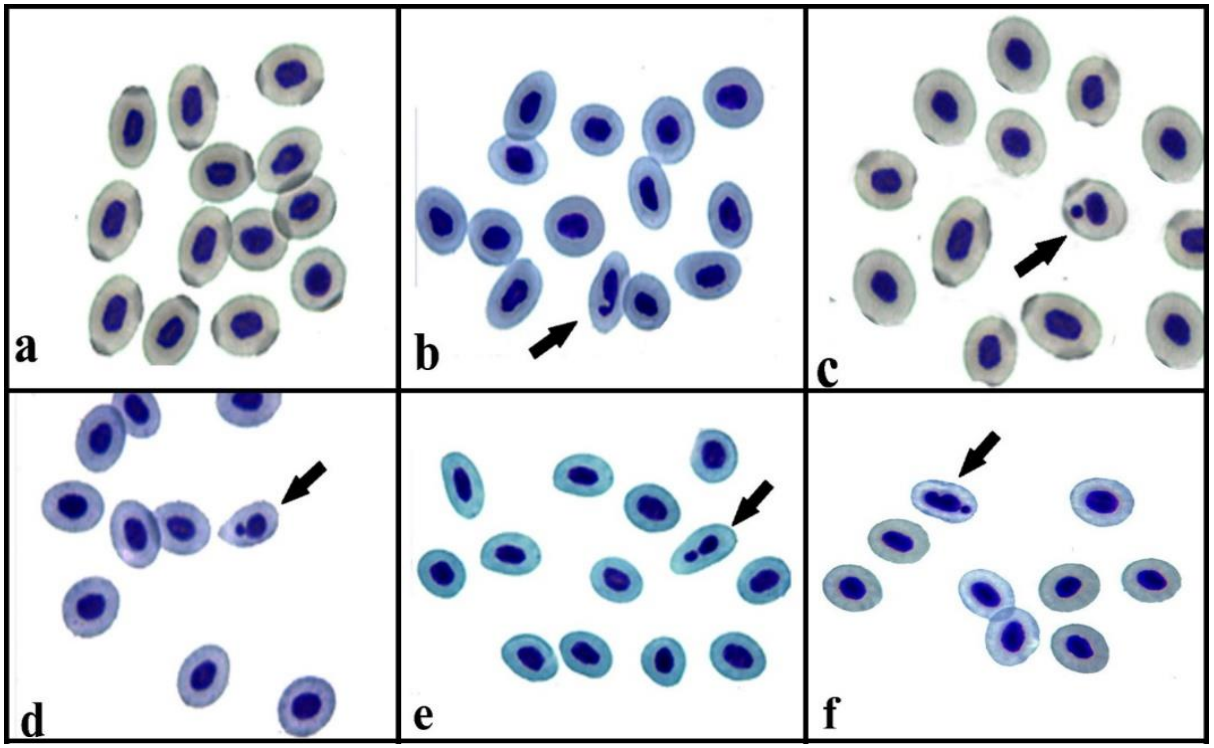

Figure 2. Types of abnormalities detected in the nuclei of *C. carpio* erythrocytes: Normal erythrocyte **(a),** Notched nucleus **(b)**, Micronucleus **(c)**, Lobed nucleus **(d)**, Binucleate **(e)**, Bud nucleus **(f)**.

Table 2. Frequencies of abnormalities detected in the nuclei of erythrocytes (**MN**: Micronucleus, **LB:** Lobed nucleus, **BL:** Bud nucleus, **NT:** Notched nucleus, **BN:** Binucleate).

Dose	No. of Specimens	Cells Counted	Abnormality Types									
			MN		LB		BL		NT		BN	
			f	‰	f	‰	f	‰	f	‰	f	‰
Control	7	14,000	0	0.00	8	0.57	12	0.86	14	1.00	4	0.29
100 ppm	7	14,000	8	0.57	10	0.71	18	1.29	20	1.43	8	0.57
200 ppm	7	14,000	14	1.00	20	1.43	28	2.00	34	2.43	8	0.57
300 ppm	7	14,000	24	1.71	34	2.43	46	3.29	58	4.14	17	1.21

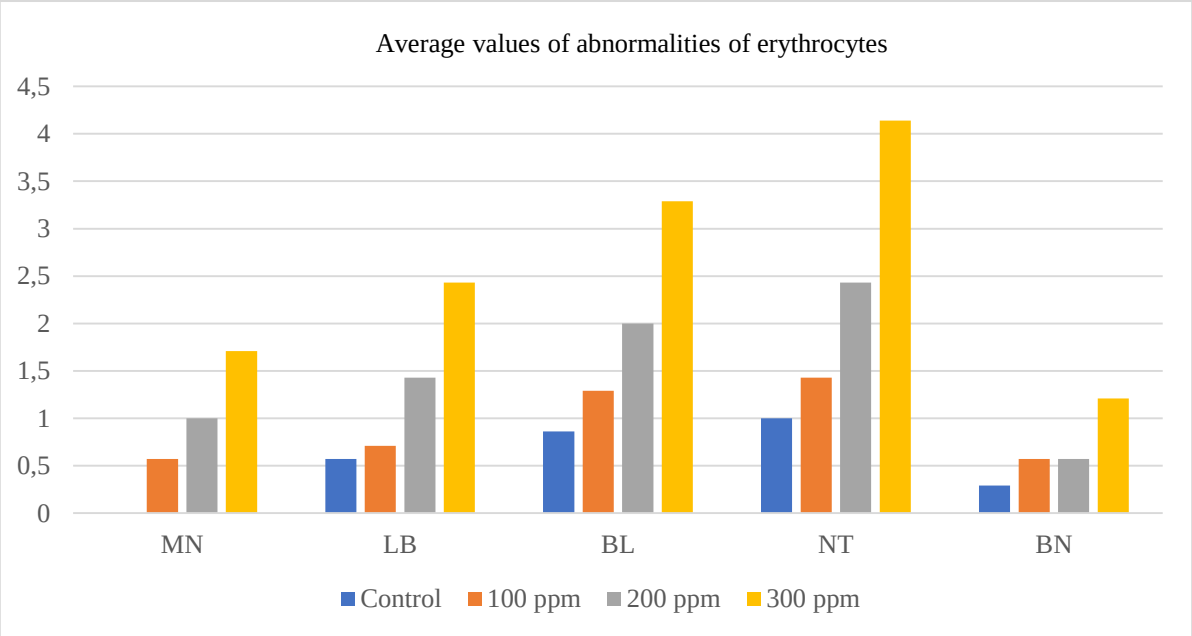

Figure 3. Average values of abnormalities detected in the nuclei of erythrocytes, (**MN**: Micronucleus, **LB:** Lobed nucleus, **BL:** Bud nucleus, **NT:** Notched nucleus, **BN:** Binucleate).

Regarding the table data, it was determined that the numerical values (‰) of abnormalities detected in the erythrocyte nuclei showed an increase in correlation with the increase in the dose of *E. elaterium* juice added to the aquarium environment.

Erythrocyte Cytoplasm Abnormalities

In the overall analysis of cells, the types of abnormalities detected in the cytoplasm of erythrocytes were found as shown in Figure 4, and the numerical values belonging to the detected abnormalities were found as given in Table 3 and Figure 5

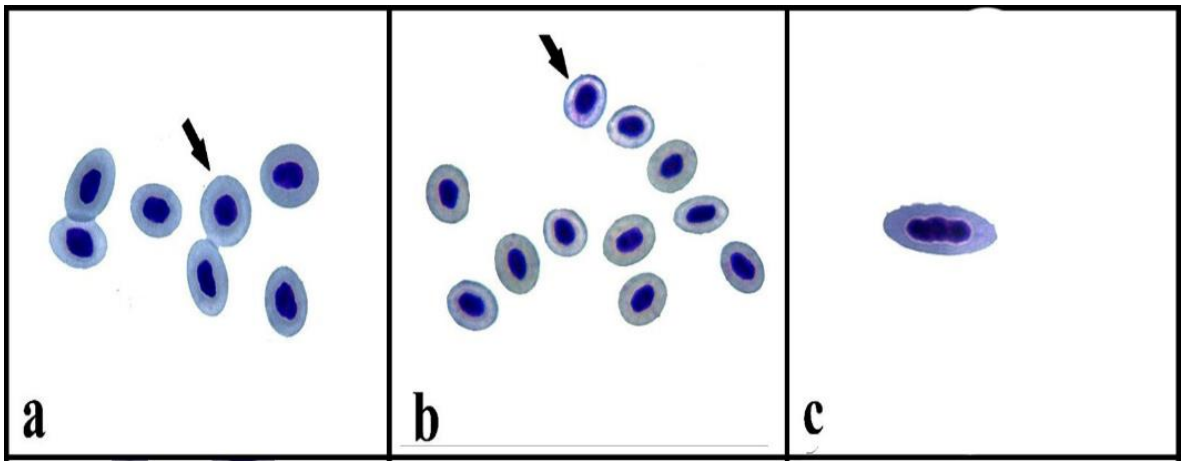

Figure 4. Types of cytoplasmic abnormalities detected in C. carpio erythrocytes: Spherocyte **(a)**, Hypochromia **(b)**, Elliptocyte **(c)**.

Upon the examination of erythrocytes with cytoplasm abnormalities detected during the scanning of preparations, three types of abnormalities were encountered: 1) spherocyte (full round shape of erythrocyte cytoplasm), 2) hypochromia (pale-colored stain due to haemoglobin deficiency in erythrocytes), and 3) elliptocyte (elliptical and elongated erythrocytes).

Table 3. Frequencies of cytoplasmic abnormalities detected in erythrocytes.

Dose (ppm)	No. of Specimens	Cells Counted	Abnormality Types					
			Spherocyte		Elliptocyte		Hypochromia	
			f	‰	f	‰	f	‰
Control	7	14,000	6	0.42	4	0.30	10	0.71
100	7	14,000	14	1	10	0.71	12	0.85
200	7	14,000	18	1.3	14	1.0	20	1.40
300	7	14,000	22	1.57	20	1.50	26	1.90

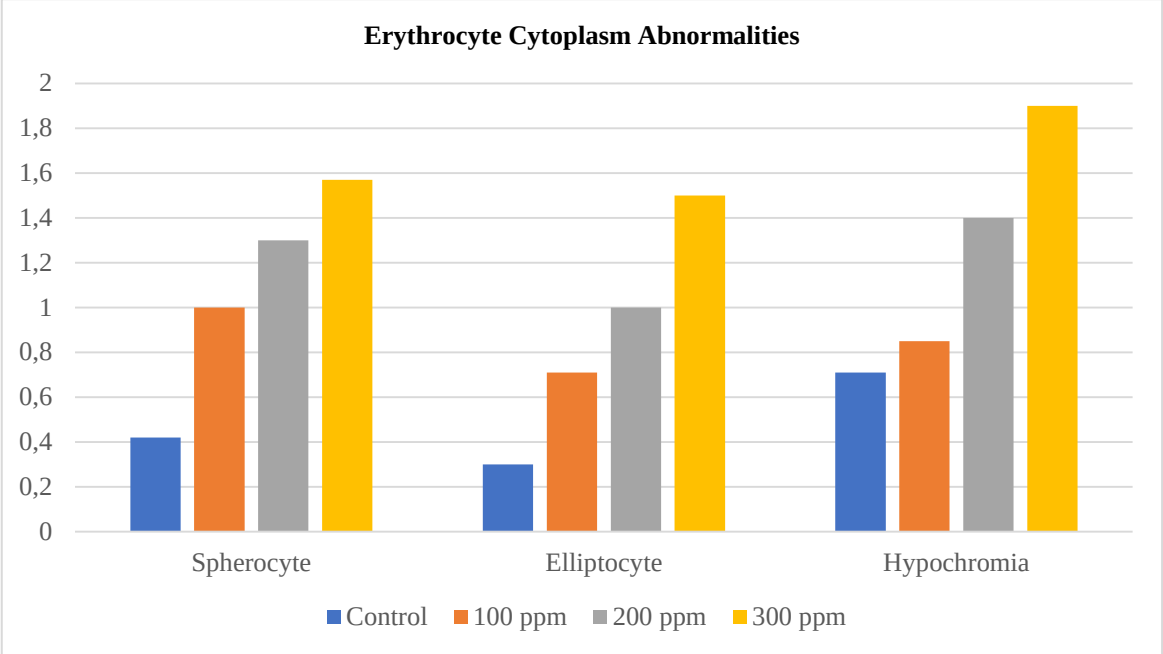

Figure 5. Average values of abnormalities detected in the cytoplasm of erythrocytes.

The numerical data given in Table 3 shows the frequency of cytoplasmic abnormalities detected in fish erythrocytes among 14,000 cells counted in each group. Regarding these data, the number of spherocyte (round-shaped) cells that formed as a result of cytoplasmic abnormalities in erythrocytes was found as 6, 4, and 10, respectively. The number of erythrocytes with elliptocyte nuclei was 14, 10, and 12, respectively; and the number of erythrocytes with hypochromia was 22, 20, and 26, respectively.

Scheffe's multiple comparison test results for the statistical significance levels between the numbers of abnormalities detected in the examination of experimental groups are given in Table 4.

Table 4. Significance data regarding the number of nucleus abnormalities detected in erythrocytes (MN: Micronucleus, LB: Lobed nucleus, BL: Bud nucleus, NT: Notched nucleus, BN: Binucleate).

Groups	Comparison (ppm)	Nucleus abnormalities					Cytoplasmic abnormalities		
		MN	LB	BL	NT	BN	Spherocyte	Elliptocyte	Hypochromia
Control	100 ppm	P<.05	P>.05	P<.05	P>.05	P>.05	P>.05	P>.05	P>.05
	200 ppm	P<.05	P<.05	P<.05	P<.05	P>.05	P>.05	P<.05	P<.05
	300 ppm	P<.05	P<.05	P<.05	P<.05	P<.05	P<.05	P<.05	P<.05
100 ppm	200 ppm	P<.05	P<.05	P<.05	P<.05	P<.05	P>.05	P>.05	P<.05
	300 ppm	P<.05	P<.05	P<.05	P<.05	P<.05	P<.05	P<.05	P<.05
200 ppm	300 ppm	P<.05	P<.05	P<.05	P<.05	P<.05	P<.05	P<.05	P<.05

The comparison of numerical values for nucleus abnormalities among all 4 groups showed the following results;

Values for micronucleus (MN) formation showed a statistically significant difference among all groups (P<.05).

Regarding the significance levels of the number of erythrocytes with lobed nuclei (LB), the control group showed no statistically significant difference from the experimental group that received 100 ppm of juice (P>.05), whereas a significant difference was found in the comparison of all other groups (P<.05).

Regarding the significance levels of the number of erythrocytes with buds (BL), the difference among all groups was statistically significant (P<.05).

Regarding the significance levels of the number of erythrocytes with notched nucleus (NT), there was no statistically significant difference between the control group and the experimental group that received 100 ppm of juice (P>.05), whereas a significant difference was found in the comparison of all other groups (P <.05).

When the significance levels of binucleate (BN) numbers were examined, there was no statistically significant difference between the control group and the experimental groups that received doses of 100 ppm and 200 ppm (P>.05), whereas a significant difference was found in the comparison of all other groups (P<.05).

The comparison of numerical values for cytoplasmic abnormalities among all 4 groups showed the following results;

The analysis of significance levels between the numbers of spherocytes showed no statistically significant difference between the control group and the experimental groups that received doses of 100 ppm and 200 ppm, as well as between the two experimental groups that were given 100 ppm and 200 ppm of juice (P>.05). The significance levels between other groups were determined to show differences (P <.05).

When the significance levels between the number of elliptocyte-shaped erythrocytes were examined, there was no statistically significant difference between the control group and the experimental group that received the dose of 100 ppm, as well as between the two experimental groups that received 100 ppm and 200 ppm of juice (P>.05). The significance levels between other groups were determined to show differences (P<.05).

When the significance levels between the number of erythrocytes with hypochromia were examined, no statistically significant difference was found between the control group and the experimental group that received the dose of 100 ppm (P>.05), while significance levels between other groups were different (P<.05).

CONCLUSION

In studies on fish blood cells, erythrocytes were found to be the most common cell type in the blood (Örün, 2000). Defects in the structure of erythrocytes are known to have a negative effect on the organisms. In accordance with this information, the effects of juice obtained from the ripe fruits of *Ecballium elaterium* plant on fish erythrocytes was investigated. In this study, MN formation and morphological abnormalities were detected in the blood cells of *Cyprinus carpio* individuals that were given 3 different doses of *E. elaterium* fruit juice in an aquarium environment.

The limited number of studies carried out to determine the genotoxic effects of *E. elaterium* fruit juice on living cells revealed that the juice of this plant caused toxic effects in living organisms. This study showed compliance with other genotoxicity studies conducted with *E. elaterium*, and the applications yielded findings that support the results of previous studies conducted on different organisms. Studies in which the toxic properties of *E. elaterium* fruit juice were demonstrated are as follows:

In a study by Çelik and Aslantürk (2009), the genotoxic effects of *E. elaterium* juice on onion stem cells were investigated. MN formation was observed in onion stem cells germinated in environments where different doses (10 ml/L, 20 ml/L, 50 ml/L) of *E. elaterium* juice was added to. It was found that the number of MN detected in onion stem cells germinating in environments with different doses increased in correlation with the amount of dose.

The doses of *E. elaterium* used in this study were determined in reference to the study conducted by Çelik and Aslantürk (2009).

In a different study by Shabbar and Maslat (2009), the effect of *E. elaterium* juice on mice (*Mus musculus*) was investigated. In the study, *E. elaterium juice* prepared in doses of 160, 113, 100, 87, 74,

61, 48, and 24 µl was administered orally to 32 mice. When blood samples taken from the mice were examined, MN formation was observed in cells due to the administration of *E. elaterium* fruit juice. As a result of the study, it was concluded that the amount of MN formation in the cells was dependent on the increase in the dose of fruit juice.

In a study by Bohlooli (2012), the cytotoxicity of *E. elaterium* juice on connective tissue cells was investigated. The study showed that Cucurbitacin in the *E. elaterium* plant may bind to the actin filaments of cells and disrupt the actin structure, which destroys the cytoskeleton, causing cell division.

The results of this study are similar to those obtained by Bohlooli (2012). In both studies, *E. elaterium* juice was observed to damage cellular structure. Furthermore, cellular damage increased proportionally with an increase in the *E. elaterium* dose. Increasing abnormalities in erythrocytes will result in decreased oxygen transport to tissues and organs.

In this study, when compared with the control group, a statistically significant increase in the number of MN formations in erythrocyte nuclei and abnormalities in blood cell morphologies of *C. carpio* individuals was found depending on the dose of *E. elaterium* juice added to the aquariums.

When compared to the previous studies in the literature investigating the effect of *E. elaterium* juice on other organisms, it was determined that the juice obtained from the ripe *E. elaterium* fruit had both positive and negative effects on living things. Upon examining the damage on *C. carpio* blood cells as a result of adding *E. elaterium* juice to the environment of *C. carpio* individuals, and by comparing the examined cellular damage with different studies in the literature, it was concluded that *E. elaterium* juice may cause adverse effects on the blood cells of *C. carpio*.

In the future, a different study can be carried out on fish by increasing their exposure times to *E. elaterium* juices and with increased dosages. Besides, a comparison study may also be conducted by examining the effects on two different fish species.

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