

## Determination of LC<sub>50</sub> Value of Borax (Na<sub>2</sub>O<sub>2</sub>B<sub>2</sub>O<sub>3</sub>.10H<sub>2</sub>O) on *Tenebrio mollitor* (Coleoptera: Tenebrionidae) and Determination Toxic Effects of Sublethal Doses of Borax on Mealworm Larvae

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### Abstract

In this study, lethal concentration 50 (LC<sub>50</sub>) value of borax on *Tenebrio mollitor* (Coleoptera: Tenebrionidae) was determined with testing 100, 1.000, 10.000, 100.000 ppm borax doses. The LC<sub>50</sub> value was determined after 20 days later as 3.402,15 ppm for larvae and 28.512,5 ppm for adults. Also sublethal 100, 1.000, 3.000 ppm borax doses were tested to determine the effects on the larval weight of insect and the adverse effects on the hemocytes. When at other doses except 100 ppm, a decrease was observed in the larval weight means compared to the control group, also a statistically significant decrease was observed in the 3.000 ppm test group compared to the control group. Finally, apoptosis, necrosis and micronucleus formations were determined by Giemsa staining technique in hemocytes of groups exposed to high doses (1.000 ppm and 3.000 ppm borax).

**Keywords:** Borax, Giemsa, Hemocyte, Larval weight, *Tenebrio mollitor*.

## Boraksın (Na<sub>2</sub>O<sub>2</sub>B<sub>2</sub>O<sub>3</sub>.10H<sub>2</sub>O) *Tenebrio mollitor* (Coleoptera: Tenebrionidae) Üzerindeki LC<sub>50</sub> Değerinin Belirlenmesi ve Boraksın Subletal Dozlarının Un Kurdu Larvaları Üzerindeki Toksik Etkilerinin Belirlenmesi

### Öz

Bu çalışmada, boraksın; 100, 1.000, 10.000, 100.000 ppm dozları *Tenebrio mollitor* (Coleoptera: Tenebrionidae)'un üzerinde test edilerek letal konsantrasyon 50 (LC<sub>50</sub>) değeri belirlendi. LC<sub>50</sub> değeri 20 gün sonra larvalar için 3.402,15 ppm, erginler için ise 28.512,5 ppm olarak tespit edildi. Ayrıca boraksın subletal dozları olan 100, 1.000, 3.000 ppm dozları, böceğin larval ağırlığına etkilerini ve hemositleri üzerinde meydana getirdiği olumsuz etkileri belirlemek için test edildi. 100 ppm hariç diğer dozlarda kontrol grubuna göre larval ağırlık ortalamalarında düşüş gözlemlenirken, 3.000 ppm deney grubunda kontrol grubuna göre istatistiksel açıdan önemli derecede düşüş görüldü. Son olarak yüksek dozlara maruz kalan grupların (1.000 ppm ve 3.000 ppm boraks) hemositlerinde; apoptoz, nekroz ve mikronükleus oluşumları Giemsa boyama tekniği ile belirlendi.

**Anahtar Kelimeler:** Boraks, Giemsa, Hemosit, Larval ağırlık, *Tenebrio mollitor*.

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## 1. Introduction

*Tenebrio molitor* (Coleoptera: Tenebrionidae) is a globally harmful insect that causing a high-loss commodity in stored grains [1]. But also mealworms are an excellent model organism in toxicology research because of similarities to the nervous system between humans and insects [1].

Circulating hemocytes (sometimes called “blood cells”) play important roles in defense mechanisms against microorganisms in the insect hemocoel. The most common types of hemocytes are prohemocytes, plasmatocytes, granulocytes, spherulocytes, adipocytes, and oenocytoids. Their characteristics slightly differ in various insect species [2, 3].

Borax ( $\text{Na}_2\text{O}_2\text{B}_2\text{O}_3 \cdot 10\text{H}_2\text{O}$ ), also known as sodium borate, has a molecular weight 327,75g [4] and it was used in the past as a cleaning material in the Ancient Greeks and Romans. Today, it uses in the plastic industry, in rocket fuels and agriculture, in industrial fiber production, in glass production, in detergent production [5]. Because of boric acid and borax are extensively consumed in various areas, they can enter the aquatic ecosystems with or without treatment especially with industrial wastes [6]. Lethal concentration ( $\text{LC}_{50}$ ) is the dose that kills 50% of the organisms exposed to the toxic substance [7].

Apoptosis is genetically programmed cell death to remove wastes and damaged cells around the tissue without deteriorating tissue balance [8]. Apoptotic cells are transformed into apoptotic bodies and they phagocytoses by the surrounding cells. Necrosis is a pathological cell death form and results in inflammation with the development of an uncontrolled process [9]. Micronucleus analyzes are also carried out to determine genotoxic effects. The causes of micronucleus formation can also originate from chromosomal fragments or the entire chromosome. Precisely, micronucleus is small, extra-nuclear constituents and it is surrounded by a nucleoplasm and locates near the main nucleus in the cell [10].

The first aim of this study is to determining of lethal concentration 50 ( $\text{LC}_{50}$ ) value of borax on *T. molitor* larvae and adult with testing different borax doses. Our second aim is to determine sublethal doses borax effects on the larval weight means of insect and observing of adverse effects in the hemocytes.

## 2. Materials and Methods

### Insect diet

Insect cultivation and experiment groups were reared at  $28 \pm 3^\circ\text{C}$ ,  $60 \pm 5\%$  relative humidity and dark conditions at laboratory conditionals. 2,60 gram thick bran, 1,30 gram corn starch was mixed and then put into the plastic boxes as Figure 1. 100, 1.000, 3.000, 10.000, 100.000 ppm borax suspensions were prepared and then 1,5 ml of each of these suspensions were added into insect diet. For the control group, 1,5 ml pure water was added to the diet. Diet was kept one day at room temperature to dry. 10 mg of grated carrots were added every two days to ensure insects water needs for all experiment groups and dried carrot parts were removed two days later.



Figure 1. Insect diet in plastic box (20ml).

**Determination of lethal concentration 50 (LC<sub>50</sub>) value and sublethal doses for *T. mollitor***

100, 1.000, 10.000, 100.000 (this dose didn't apply on larvae, it was applied on adults) ppm doses borax suspensions (experiment groups) were prepared and then 1,5 ml of each of these suspensions added into insect diet for determination of LC<sub>50</sub> value of borax. 1,5 ml pure water was added into the diet for the control group. Adult and larval experiments were created separately. One *T. mollitor* second instar larva (15 days old) was placed into each boxes with a brush for larval experiments and also same process was applied for adults (30 days old). In one replicate; 5 larvae/adult were used and four replicates were created in the experiments. Each larva and adult was checked daily for 20 days until the all larvae/adult died in any concentration. After LC<sub>50</sub> value was determined as 3.402,15 ppm for larvae, we decided to work for all experiments with 100, 1.000, 3.000 ppm doses as sublethal doses. We didn't create another experiment for adults because providing insect culture continuation in our lab for the future studies. We only obtained LC<sub>50</sub> value of borax for adults.

**Determination of effects of sublethal doses on larval weights of *T. mollitor***

Larval weights were measured after 70 days as milligram (mg) on digital precision scale (0.001-10) grams for determination of effects of sublethal doses on larval weights on *T. mollitor*. The SPSS 20.0 version (IBM) computer program was used for statistical analysis of our larval weight mean results. Larval weight data were analyzed by nonparametric statistic (Mann-Whitney U test).

**Determination of adverse effects of sublethal doses on larval hemocytes of *T. mollitor* with Giemsa staining technique**

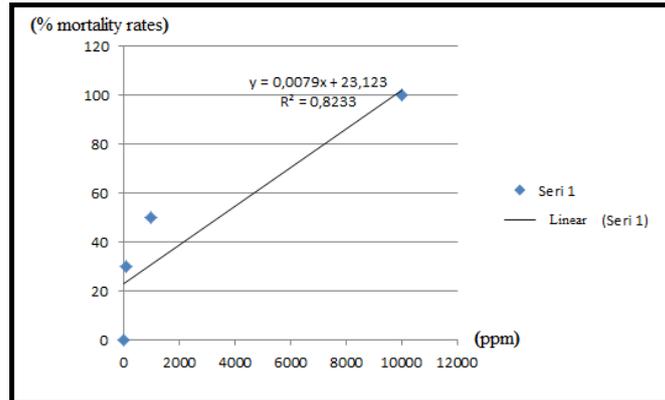
Firstly, larvae were cleaned with ethanol (70%)-impregnated moist sterile piece of cloth for preparing slides. Then larvae were drilled from first segment on the back of the head by fine needle and 3 µl hemolymph was taken by micropipet. Hemolymph was smeared on to clean and dry slide immediately. This smear was air-dried for 10 minutes. Then it was fixed in ethanol 5 minutes. Finally, smear was stained with Giemsa for 20 minutes. The slide was rinsed with distilled water. After 15 minutes, finally slides were observed under light microscope at 1600X magnification with using cedarwood oil.

**3. Results**

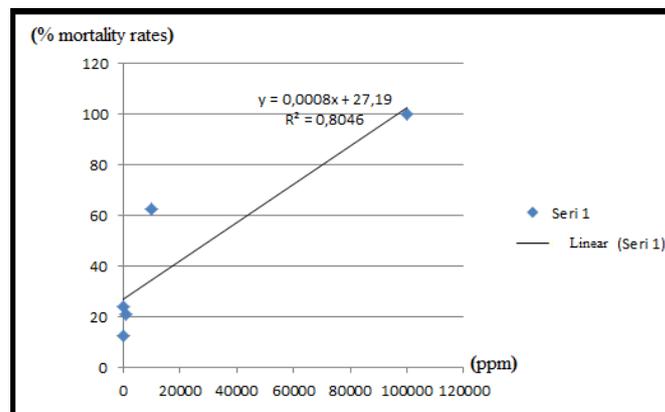
After one *T. mollitor* second instar larvae/adult was placed into each boxes with a brush, each larva and adult were checked daily for 20 days until all larvae and all adults died in any concentration. The number of individuals who died and survived was recorded every day. Twenty days later, % mortality rates were calculated for larvae and adults (Table 1.) According to these data, LC<sub>50</sub> value was calculated in Microsoft Excell 2010 program with dose-response data were transformed into a straight line by means of a trendline fit linear regression analysis. The LC<sub>50</sub> was derived from the best-fit line obtained (Fig. 2 and 3) [11]. LC<sub>50</sub> value was determined as 3.402,15 ppm for larvae and 28.512,5 ppm for adults (Fig. 2 and 3). In the next experiment, larval weights were measured after 70 days as mg on digital precision scale (0.001-10 g) and the results were given in Table 2.

**Table 1.** % Mortality rates for larvae and adults according to applied doses.

Applied Doses	% Mortality rates for larvae	% Mortality rates for adults
Control (0)	0	24
100	30	12,5
1.000	50	20,83
10.000	100	62,5
100.000	This dose did not apply on larvae.	100



**Figure 2.** Mortality rates (%) of larvae according to applied doses.



**Figure 3.** Mortality rates (%) of adults according to applied doses.

**Table 2.** Effects of Sublethal Doses of Borax on larval weights of *T. mollitor*

Dose (ppm)	Minimum-Maximum Larval Weights (mg)	Larval Weights (mg) (Mean <sup>a</sup> ± SE) <sup>b</sup>
Control (0)	8-129	47,60 ± 7,70b
100	5-138	67,00 ± 3,57a
1.000	9-138	40,00 ± 2,82bc
3.000	5-91	25,46 ± 2,96c

<sup>a</sup>Data are means ± standart errors of four replicates using five larvae per replicate,

<sup>b</sup>Values followed by the same letter in the same vertical column are not significantly different from each other Man Whitney U Test, P= 0,001<0,05.

According to the (Table 2), the highest larval weight mean (67,00 mg) was calculated in larvae fed the diet with 100 ppm borax. The observed mean value at this dose was found statistically significant according to the control group. The lowest larval weight mean (25,46 mg) was obtained in larvae fed the diet with 3.000 ppm borax dose. This mean value was found statistically significant according to control group (Man Whitney U Test,  $P= 0,001<0,05$ ). At other doses except 100 ppm, a decrease was observed in larval mean weights according to the control group.

According to the Giemsa staining technique, we did not observe any abnormal change in control group and 100 ppm group's hemocytes (Fig. 4; 1 and 2, ). But we observed micronucleus containing cells and apoptotic cells in 1.000 ppm (Fig. 4; 3a, b, 4b, c) and also excessive vacuolization in granulocytes that exposed to 3.000 ppm borax (Fig. 4; 4a. and Fig. 5). Finally, we observed necrotic cell in 3.000 ppm dose exposed group whose small nucleus part hurtled out to the cell (Fig. 4; 4d). Control group's hemocytes and all borax group's hemocyte samples were given in Figure 6.

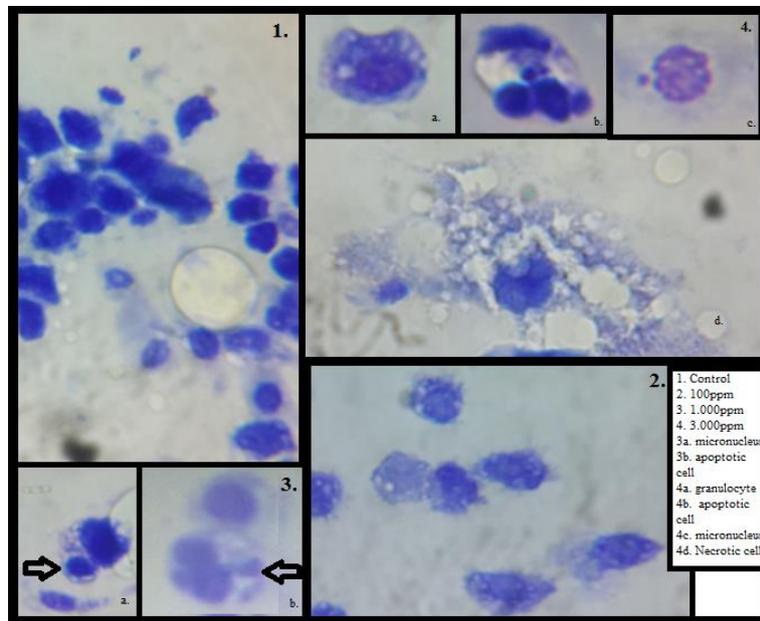


Figure 4. Abnormal changes in hemocytes due to borax doses (1600X).

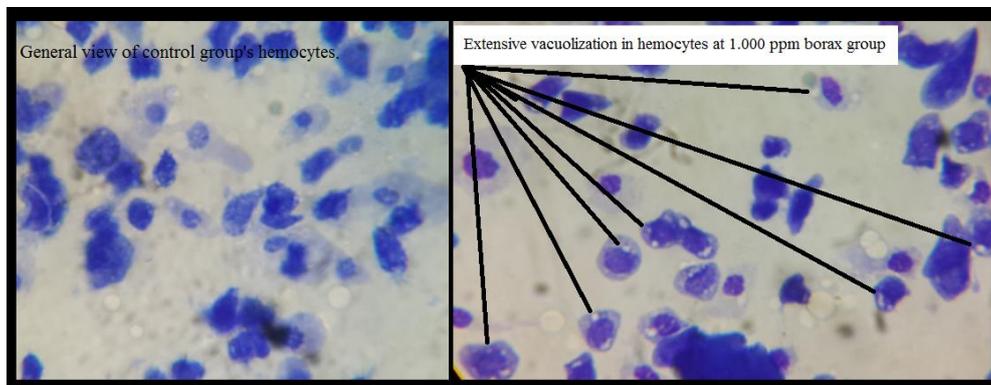


Figure 5. Control group's hemocytes (left) and 1.000 borax group's extensive and big vacuolated hemocytes (right) (1600X).

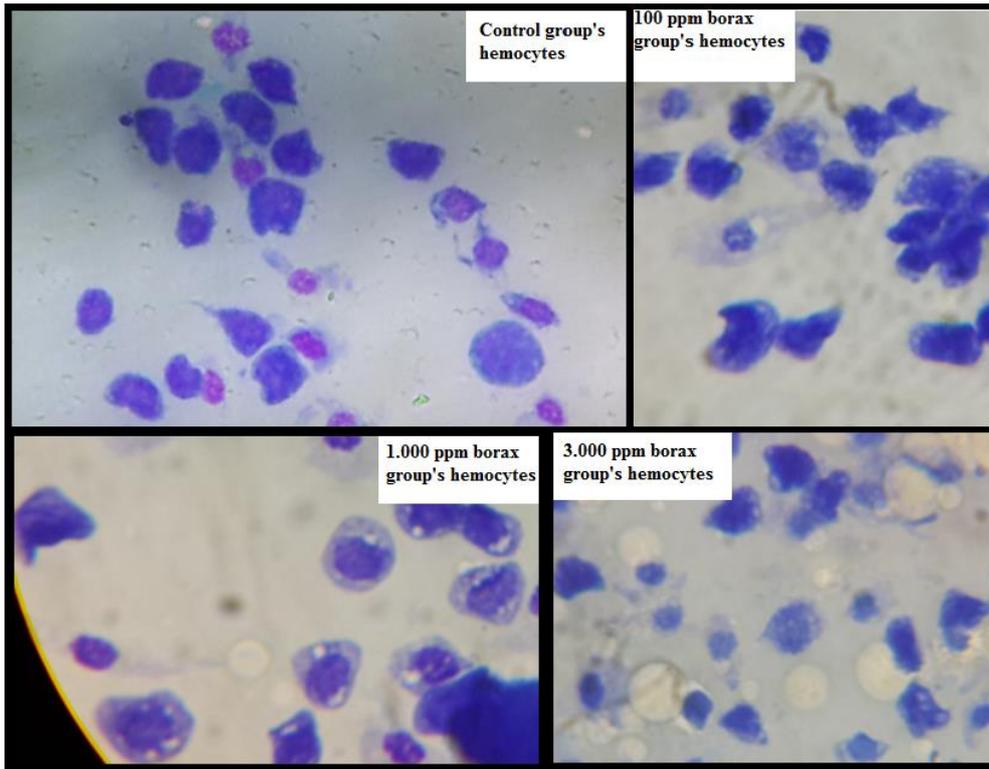


Figure 6. Control group's hemocytes and all borax groups hemocyte samples (1600X).

#### 4. Discussion and Conclusion

In this study, mealworms were exposed to borax by oral ways through insect diet. So, the  $LC_{50}$  (20<sup>th</sup> day) value of borax was determined as 3.402,15 ppm for larvae and 28.512,5 ppm for adults. According to these values, borax has relatively low acute toxicity characteristic for this insect species. Because, these values are in category 4 and 5 respectively according to the toxicological rankings [12]. With this feature, borax is a low toxicity mineral for this species in terms of insecticidal property [13]. We observed some adverse effects on larval weight means of larvae and larval hemocytes in high doses. It is quite difficult to find studies about borax adverse effects on insects today. However, there is a little information about boric acid that damages to the midgut of insects. It causes in insect to die and prevent the insect from digesting the food. As an insecticide, boric acid acts like a stomach poison on the metabolism of bugs. At the same time, when boric acid is mixed with the food, it stimulates the food intake of insects. Thus, the amount of boric acid increases with the increase of the amount of nutrients consumed by insect [14]. According to the our larval weight mean results, there is a drastically decreasing in larval weight means of mealworm larvae in high level doses of borax (Table 2). [15], applied cypermethrin to *Galleria mellonella* (Lepidoptera: Pyralidae)'s diet. They observed a decrease in pupal weights of the insect. They explained reason of this position as "due to insecticide-induced toxic effects". Stress occurs in the living body after insecticide taken by food by insect. If stress conditions continues, high energy will be required. Various repair mechanisms also cause energy expenditure in this process with leading to a decrease in the energy stores of *G. mellonella* (glycogen deposits and fatty tissues). In another study, [16] reported such kind of situation in their boron based study as "observed effects could be formed by decreased ATP level or suppression of the engine of energy production". So, the reason of

decreasing in larval weight means in 1.000 and 3.000 ppm borax doses may be similar to explained by [15, 16]. Another reason for the reduces in larval weight of mealworm larvae can be explained as follows “the insect may refuse to feed on borax containing diet”. [17], applied alkaloids to the *Phormia regina* (Meigen) (Diptera: Calliphoridae) larval diet and they explained reduces in larval weight as “reduced consumption of alkaloid treated diets by larvae”. All these explanations may be a valid explanation for the reduction of larval weights.

[18], emphasized that some boron-derived chemicals such as boric acid causes to molecular oxygen and superoxide radicals by transferring electron. [19], reported that free radicals can give damage to protein, lipid, DNA and coenzymes. Moreover, [20] clearly stated that chemical pollutants can cause apoptosis and necrosis in the cells. [21, 22], observed vacuolization of the apical cytoplasm, nuclear picnosis and boric acid accumulation in lumen where Malpighi tubes were attached to the intestines after application of boric acid to adult bees. When the above-mentioned results are evaluated we can explain the reason of observing apoptosis, necrosis, micronucleus formations and vacuolization in hemocytes (Fig. 4; 3a-b, 4a-d, and Fig.5). In our study, we observed extensive vacuoles in granulocytes and plasmatocytes (Fig. 5). These vacuoles probably contains sodium or boron compounds. They may accumulate toxic substances in their vesicles to prevent toxicity at the cellular level. In a study supporting this view, [23], were detected membrane-bound iron-rich granules in fat cells and midgut cells of the adult honeybee (*Apis mellifera* L.). [24], suggested that borax induced apoptosis in HepG2 (a human hepatoblastoma cell line) cells in a concentration-dependent manner. Apoptotic lesions in rat thymocytes were detected when rats received 2.000 ppm of borax in their food for 16 days [13, 25]. So we can explain why we observe normal cell density in the control group hemocytes and 100ppm borax exposed group hemocytes in our study (Figure 4; 1-2, Fig. 5) as well as other adverse effects on same figure.

As a result of cell and organelle breakdown in necrosis, the cytoplasm and nucleus contents are released into the intercellular space [25]. So we can observe this situation in Figure 4; 4d. Finally, high level borax caused to micronucleus formation in hemocytes (Fig. 4; 3a, Fig. 4; 4c). [13], suggested that cells are under stress at borax concentrations of 0,15, 0,2 and 0,3 mg/ml. So this data is related to chromosome abnormalities and also suggests borax causes to genetic toxicity at these doses. The toxicity of borax affects the chromosomes and leads to genetic defects. As we know, micronucleus are whole lagging chromosome or an acentric chromosome fragment and indicates genomic instability. And they use for determine the genotoxic effects of chemical and physical agents on somatic cells [26]. So we can say that high level borax doses may be arise the micronucleus formation in hemocytes as we showed in (Fig. 4; 3a and 4c). Also [27] reported micronucleus formation on *Allium cepa* root meristematic cells after they used boron to this plant.

Finally, if we compare the oral toxicity of borax on developmental stages of *T. molitor*, the larvae is significantly more susceptible to borax than adults. Because we determined the LC<sub>50</sub> value of borax of larvae as 3.402,15 ppm and 28.512,5 ppm for adults. Our results showed compatibility with [28]’s study. They studied insecticidal activity of garlic essential oil and their constituents against to *T. molitor*. According to their study, the larva was significantly more susceptible followed by pupa and adult that similar to our study results. They explained this situation as follows “these results indicate that

small quantities of the garlic essential oil are toxic in this insect, being more tolerant with age". So we strongly agree to these researchers explanation.

We think that the present results have added new scientific knowledges to the literature about insecticidal property of borax for mealworm and adverse effects on mealworm hemocytes caused by high level borax doses.

## 5. Acknowledgement

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## 6. References

- [1] Collins, L., Edwards, L., "Toxicity of moist snuff and impact on various stages of darkling beetles (*Tenebrio molitor*)", *Explorations, Biology and Biotechnology*, 2017.
- [2] Berger, J., Slavickova, K., "Morphological characterization of hemocytes in the adult Linden Bug, *Pyrrhocoris apterus* (L.) (Heteroptera)", *Zoological Studies*, 47(4), 466-472, 2008.
- [3] Gupta, A.P., "Hemocyte types: their structures, synonymies, interrelationships, and taxonomic significance", In AP Gupta, ed. *Insect hemocytes*, New York: *Cambridge University Press*, 85-127, 1979.
- [4] Anonima, "Kimya Borsası", <http://www.kimyaborsasi.com.tr/b/boraks-27.html>, 2017.
- [5] Sağlam, M., Köseoğlu, S., Enhoş, Ş., "Periodontolojide bor", *Sağlık Bilimleri Dergisi (Journal of Health Sciences)*, 22(1), 70-75, 2013.
- [6] Anonimb, "Boric acid, human and environmental risk assessment on ingredients of household cleaning products", *HERA*, 2-81, 2005.
- [7] Parlak, H., Arslan Çakal, Ö., Boyacıoğlu, M., Karaarslan, A.M., "Ekotoksikoloji", *Ege Üniversitesi Basımevi*, 63, 2009.
- [8] Qiang, X., Hongxia, S., Yinghua, S., Dexiang, U.G., Zhang, G., "Apoptosis of *Spodoptera litura* larval hemocytes induced by heavy metal zinc", *Chinese Science Bulletin*, 50(24), 2856-2860, 2005.
- [9] Coşkun, G., Özgür, H., "Molecular mechanism of apoptosis and necrosis", *Archives Medical Review Journal*, 20(3), 145-158, 2011.
- [10] Üstüner, D., "Kromozom kırıkları ve mikronükleus-apoptoz bağlantısı", *Tünav Bilim Dergisi*, 4, 64-69, 2011.
- [11] Patil, B.S., Magdum, S.C., "Determination of LC<sub>50</sub> values of extracts of *Euphorbia hirta* Linn and *Euphorbia nerifolia* Linn using brine shrimp lethality assay", *Asian Journal of Pharmaceutical Sciences*, 1, 2, 42-43, 2011.
- [12] Anonime, "Acute toxicity,", <http://www.ilo.org/legacy/english/protection/safework/ghs/ghsfinal/ghsc05.pdf>, 2017.
- [13] Pongsavee, M., "Genotoxic effects of borax on human lymphocytes", *Southeast Asian Journal of Tropical Medicine and Public Health*, Bangkok, 40, 2, 411-8, 2009.

- [14] Büyükgüzel, K., Büyükgüzel, E., “İnsan çevre ve tarım açısından borik asitin zararlı böceklerin ve diğer artropodların mücadelesindeki önemi”, *Zonguldak Karaelmas Üniversitesi, Avrupa Birliği 6. Çerçeve Programı kapsamında II. Bor Çalıştayı*, Zonguldak, 2004
- [15] Sak, O., Uçkan, F., “Cypermethrinin *Galleria mellonella* L. (Lepidoptera: Pyralidae)’nın puplaşma ve ölüm oranına etkisi”, *Uludağ Arıcılık Dergisi*, 9(3), 88-96, 2009.
- [16] Konuk, M., Liman, R., Ciğerci, H.İ., “Determination of genotoxic effect of boron on *Allium cepa* root meristematic cell”, *Pakistan Journal of Botanic*, 39(1), 73-79, 2007.
- [17] Green, C.W.P., Simmonds, J.S., Blaney, M.W., Toxicity and behavioural effects of diet-borne alkaloids on larvae of the black blowfly, *Phormia regina*, *Medical and Veterinary Entomology*, 16, 157-160, 2002.
- [18] Jolly, W.L., “Modern inorganic chemistry”, *2nd Ed. McGraw-Hill*, New York, 1991.
- [19] Diplock, A., “Healthy lifestyles nutrition and physical activity: antioxidant nutrients”, *ILSI Europe Concise Monograph Series*, 59, 1998.
- [20] Palupi, M.R., Andayani, S., Fadjar, M., “Bioaccumulation of heavy metal zinc (Zn) and copper (Cu) and histology of grouper (*Epinephelus Suius*) in coastal panceng waters Of Gresik regency. *Journal of Scientific & Technology Research*, 5(1), 2277-8616, 2016.
- [21] Jesus D., Silva-Zacarin, E.C.M., Malaspina, O., “Histological studies in the midgut and malpighian tubules of *Apis mellifera* workers treated with boric acid”, *Brazilian Journal of Morphological Sciences*, 22, 168, 2005.
- [22] Malaspina, O., Mathias, C.E., Zacarin, S., “Cell markers for ecotoxicological studies in target organs bees”, *Brazilian Journal of Morphological Sciences*, 23(3-4), 303-309, 2006.
- [23] Raes, H., Bohyn, W., Rycke, De H.P., Jacobs, F., Membrane-bound Iron-rich Granules in Fat Cells and Midgut Cells of the Adult Honeybee (*Apis mellifera* L.), *Apidologie*, 20, 327-337, 1989.
- [24] Wei, Y., Yuan, J.F., Zhou, B.W., Wu, L., Chen, L., Wang, J.J., Zhang, S.Y., “Borax-induced apoptosis in HepG2 cells involves p53, Bcl-2, and Bax”, *Genetics and Molecular Research*, 15 (2), 1-10, 2016.
- [25] Sylvain, I.C., Berry J.P., Galle P., “Ultrastructural apoptotic lesions induced in rat thymocytes after borax ingestion”, *Anticancer Res*, 18, 2455-61, 1998.
- [26] Altunkaynak, Z.B., Özbek, E., “Programlanmış Hücre Ölümü: Apoptoz Nedir?”, *Tıp Araştırmaları Dergisi*”, 6 (2), 93 -104, 2008.
- [27] Şekeroğlu, V., Atlı-Şekeroğlu, Z., “Genotoksik hasarın belirlenmesinde mikronükleus testi”, *Türk Hijyen ve Deneysel Biyoloji Dergisi*, 68(4), 241 – 252, 2011.
- [28] Plata-Rueada, A., Martinez, C.L., Santos, D.H.M., Fernandes, L.F., Wilcken, F.C., Soares, A.M., Serrao, E.C., Zanunciob, C.J., “Insecticidal activity of garlic essential oil and their constituents against the mealworm beetle, *Tenebrio molitor* Linnaeus (Coleoptera: Tenebrionidae), *Scientific Reports*, 7, 46406, 2017.