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Effects of the High Doses of *Urginea maritima* (L.) Baker Extract on Chromosomes[1]

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Abstract — The effects of high doses of extracts of *Urginea maritima* bulbs which can be used as bio-pesticides were investigated with the Allium test method. The bulbs of *Allium cepa* were rooted in solutions including 8%, 10%, 12%, 14%, 16% and 100% (v/v) *U. maritima* extracts for 48, 72 and 144 hours. Moreover, the bulbs were rooted in a solution including 2% of a chemical pesticide (Vydate®) and in tap water (as control). *A. cepa* root tip meristem cells were prepared according to Feulgen squash procedure and the chromosomal aberrations and cell anomalies were determined. The data were evaluated with statistical analyses. Chromosome aberrations occurring in all solutions including *U. maritima* extract and vydate solution were found to be significantly related to the increasing dose and period of the treatment. Moreover, a statistically significant decrease was observed in the mitotic index (MI) when compared to the control. It was found that even the high doses of *U. maritima* extract are less genotoxic and cytotoxic than vydate.

Key Words: Allium test, Biopesticide, Cell anomalies, Chromosomal aberrations, *Urginea maritima* extract.

INTRODUCTION

Rapid population growth and irregular migration-related movements coupled by unplanned urbanization have led to the destruction of agriculture fields and every type of natural resource (Anonymous 2001; Ünver *et al*. 2004; Delen *et al.* 2005). Various activities performed on the earth to provide foods and other basic needs of human beings result in global environmental problems. The chemical controls used in agricultural are one of these problem sources. Pesticides, used unconsciously, intensely and erroneously in fields to increase the yields may create tolerance in harmful organisms, kill the natural predators, and thus cause pollution of the nature equilibrium. The residues of pesticides mix into the soil, into the air and into the underground waters in various ways. Many of pesticides have high toxicity and pesticides and their constituents accumulate in the living organisms and environment. These pesticide residues can accumulate in humans and can cause important health problems as cancer or genetic disorder (Anonymous 2001; Madanlar *et al.* 2002; Özmen and Sümer 2004; Ünver *et al*. 2004; Delen *et al.* 2005).

Because of the risks and damage from synthetic pesticides, in recent years there has been a great increase in the number of the studies carried out to examine the effects of biopesticides in the agricultural context as an alternative to chemicals (Civelek and Weintraub 2004). Plant extracts, especially terpenoids, alkaloids and phenolic compounds have been examined with respect to their effects on the growth and development of harmful insects (Ertürk *et al* 2004). *Urginea maritima* is one of this plant extracts used as medicinal plant and biopesticide. *Urginea maritima* has been used as a medicinal plant through centuries over the world, believed to have certain traditional actions. The squill bulb was used by herbalists traditionally for the treatment of injury, haemorrhoids, warts (skin

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problems), cardiac failure, pneumonia, cough, chronic bronchitis, asthma, abortive, vipers bites, aphrodisiac, jaundice, diuretic, negative kidney performance, internal tumours and purgative effects. Raphides of calcium oxalate (3%) have an irritant action on mucosa therefore used treatment of rheumatic disorders (SHARAF *et al*. 2006; ADAMSA *et al.* 2009). *U. maritima* contains 1-3% cardiac glycosides. Novel cardiac glycosides recently have been isolated from squill known as bufodienolides such as glucoscillarene A, proscillaridine A, scillarene A, scilliglaucoside and scilliphaeoside, anthocyanins, fatty acids, flavonoids and polysaccharides (ADAMSA *et al.* 2009; NAWAL *et al*. 2009; BENÍTEZ *et al.* 2010; KAWA and BADR-ALDIN 2010). The cardiac glycosides (scillaren and scillarenin) are used as a cardiotonic diuretic for the treatment of cardiac marasmus and edema. Scilliroside, the major toxic glycoside, occurs in all plant parts including the leaves, flowers, stalks, scales, and especially the roots and the core of the bulbous part (SHARAF *et al*. 2006). Poisoning occurs frequently in autumn; there are marked differences in the susceptibility of livestock to the *U. maritima.* Young calves are more susceptible, while goats and wild boars are less. The scillioside is further used as a rodenticide, mouse repellent (PAOLA *et al.* 2005), while the bulb extract is a strong insecticide (PASCUAL-VILLALOBOS and FERNANDEZ 1999). *U. maritima* bulbs have been planted in some cases touching the roots of fruit trees in Spain to avoid ant infestations (SHARAF *et al*. 2006).

Most natural plant products do not leave harmful residues, and they have hardly any adverse effects on nature. In fact, they have hardly any harmful effects on plant-animal relationships in nature. However, the biological activity of many of these natural products is still not well known. Some of the questions that remain regarding alkaloids, terpenoids and phenolic compounds include: how long does it take for these natural products to disintegrate or disappear from nature (i.e. underground water, soil)? What is their role in the food cycle as a whole? Are there significant reactions with other compounds in the water and soil? With which chemicals do they possess synergistic or antagonistic interactions? Investigation of traditionally medicinal plants is thus valuable on two levels: firstly, as a source of potential chemotherapeutic drugs, and secondly, as a measure of safety for the continued use of medicinal plants. Plant species represent a great source of biologically active compounds whose effects on heritable material are mostly unknown. However, the present widespread use of essential oils in pharmacy, biopesticides and industry (antiseptics, soaps, deodorants, flavours and dentistry products) as well as of aqueous extracts in traditional medicine would seem to necessitate research on their cytotoxicity and genotoxicity (ÇELIK and ASLANTÜRK 2007; ASLANTÜRK and ÇELIK 2009).

Therefore, the purpose of this study was to investigate the genotoxic and cytotoxic effects of *Urginea maritima* bulb extracts, especially at high doses. These extract properties for potential use as a biopesticide in comparison with the effects of vydate, a chemical pesticide, by using the Allium test method. *Allium* test has been extremely useful in biological monitoring and determination of toxicity and pollution. It has been widely used for the evaluation of cytotoxic and anti-mitotic activity of various compounds (SEHGAL *et al.* 2006; SOUSO *et al*., 2009). The most important advantage of this test is that it is a low budget method, which besides being fast and easy to handle, it also yields reliable results (RANK 2003; ÇELIK and ASLANTÜRK 2006, 2007; ASLANTÜRK and ÇELIK 2009; SOUSA *et al*., 2009).

MATERIALS AND METHODS

Collection of the Plants and their Extraction - Extract of *Urginea maritima* (L.) Baker (DAVIS 1984) bulbs which have medicinal and insecticidal activity properties belonging to Liliaceae family were used (PASCUAL-VILLALOBOS and FERNANDEZ 1999).

Urginea maritima bulbs were collected from the stony surroundings of Ortaca Vocational School of Muğla University, in February-March, 2004. The bulbs were broken into smaller parts then dried at room temperature in an environment with no direct sunlight. Extract was obtained from *U. maritima* bulbs in a soxhalet mechanism through a hot water (100°C) extraction method. *U. maritima* ground bulb sample (2 g) was slowly boiled in distilled water (100 ml) for 4 h (CIVELEK and WEINTRAUB 2004; EROĞLU *et al*., 2009). The extract obtained was stored at 4ºC.

Allium Test - 8%, 10%, 12%, 14%, 16% (v/v) solutions of *U. maritima* extract were prepared with distilled water and 2% vydate solution (v/v) was prepared (recommended dose). Vydate® is a commercially available pesticide whose active ingredient is oxamyl ($C_7H_{13}N_3O_3S$) and which has contact and systemic insecticide, acaricide and nematocide properties (ÖNCÜER 2004). The

effects of the extract on cells and chromosomes were investigated through Allium test (FISKESJÖ 1981). To conduct the Allium test, *Allium cepa* bulbs were placed in beakers including 0 (control), 8, 10, 12, 14, 16 and 100 percent solutions of *Urginea maritima* extract (v/v) and 2% (v/v) vydate solution for rooting. The experiment was conducted with four replicates for each dose at room temperature (20±2°C). At the end of 48, 72 and 144 h, the root number and length of *Allium cepa* were determined and at least ten root tips from each treatment were dipped in Carnoy solution (absolute alcohol: chloroform: glacial acetic acid, 6:3:1) for four h. Thereafter, roots tips were applied to cold hydrolysis in 5 N HCl for 15 min. At the end of the hydrolysis process, they were rinsed with 70% ethyl alcohol and then with distilled water. The root tips were kept at least 24 h in feulgene and 4°C in darkness. They were stored in the same conditions. After removal from the feulgene, root tips were prepared according to squashing technique and examined under the microscope (ELÇI 1994). From these squashed root tips, ten random areas were selected for observation (approximately 2000 cells were counted in an area), minimum 20.000 mitotic cells were counted from each the slides. The following positions were recorded for each scanned area: the number of total cells, the number of divided cells and mitotic phases, the fragments and bridges in divided cells, chromosome stickiness, irregular metaphase and anaphase, pole deviations and aberrant cells. Aberrant cell ratios were calculated and photographs were taken. Moreover, the mitotic index (MI) was calculated for each treatment dose and period as a number of dividing cells/100 cells.

Statistical Analysis - For statistical analyses, One-Way ANOVA and LSD (Least Significant Difference) tests from SPSS 14.0 Software Package Program were used.

RESULTS AND DISCUSSION

In this study, the effects of high doses of the extract of *U. maritima*, a natural pesticide were compared with vydate, a chemical pesticide. Aqueous extracts of *U. maritima* bulbs (8%, 10%, 12%, 14%, 16% and 100% *U. maritima* extracts) and 2% vydate were observed to cause the occurrence of aberrant cells and considerably decrease cell division, depending on the increase in dose and the period of treatment.

The Number and Lengths of Root according to Extract Treatment Dose and Period - The effects of *U. maritima* extract were investigated through *Allium* test. At the end of the 48 h treatment period, the roots of *Allium cepa* in the control were well developed and healthy, while the roots of *Allium cepa* in *U. maritima* extracts and vydate were undeveloped and the root number and root length were divided into statistically different groups depending on the treatment dose and period (Table 1). After 72 h treatment, the effects of vydate and 100% *U. maritima* extract on the number of roots were the same, yet, they had statistically different effects with regards to the length of the root. In vydate solution, at the end of the 72 and 144 hour treatment period, it

TABLE 1 — Mean root number and length (mm) of *Allium cepa* depending on the treatment dose and period of *U. maritima* extract

Doses	48 h treatment		72 h treatment		144 h treatment	
	Root number ± SD	Root length (mm) ± SD	Root number ± SD	Root length (mm) ± SD	Root number ± SD	Root length (mm) ± SD
Control	22 ± 0.6853 e	25 ± 0.6396 e	17 ± 0.9342 c	45 ± 0.6467 d	16 ± 1.0219 c	70 ± 4.4621 b
8%	8 ± 0.6324 ab	11.5 ± 0.4472 d	10 ± 0.4741 b	13.5 ± 0.9098 c	15 ± 0.8697 c	14.5 ± 0.9163 a
10%	13 ± 0.7071 c	10.5 ± 0.5669 bcd	11 ± 1.4981 b	13.5 ± 1.1105 c	10 ± 0.9660 b	14.5 ± 2.0453 a
12%	8 ± 0.3944 ab	11 ± 0.3651 cd	7 ± 0.9128 a	12.5 ± 0.6455 bc	11 ± 0.7348 b	13.5 ± 0.9219 a
14%	12 ± 0.6146 c	10.5 ± 1.0247 bc	12 ± 0.9660 b	12.5 ± 0.7637bc	9 ± 0.5773 b	14.5 ± 1.4776 a
16%	7 ± 0.7071 a	10.5 ± 0.5000 bcd	12 ± 0.8165 b	11.5 ± 0.4965 bc	9 ± 0.4629 b	13.5 ± 0.9819 a
100%	10 ± 0.5773 b	9 ± 0.3944 b	11 ± 0.7601 b	11 ± 0.5773 b	2 ± 0.2357 a	11 ± 0.6236 a
Vydate	15 ±0.9309 d	6.5 ± 0.3415 a	10 ± 0.9660 b	7.5 ± 0.5000 a	10 ± 0.5773 b	7.5 ± 0.5000 a

Variability around the mean was represented as ± SD (Standart Deviation). Data having the same letter in a column were not significantly differed by LSD's multipli comparison test ($P<0.05$)

was found that the number of roots (10 number) and their length (7.5 mm) remained the same. In all the treatments, the highest root elongation was observed in the control and the lowest was observed in the vydate treatment (Table 1). This is because the toxic effect of the chemical hinders mitosis of the meristematic cells of *A. cepa* (ÇELIK and ASLANTÜRK 2006, 2007; SOUSA *et al*., 2009). At the end of the 72 and 144 hour treatment periods, due to their toxic effects, the high doses of *U. maritima* extract caused the occurrence of lumps on the root tips and a brownish coloring. The end of the 144 hour treatment period in 100% *U. maritima* solution, the number of roots was decreased due to the putrefaction (Figure 1a, b, c).

Effects on the Mitotic Index (MI) of the Extract Treatment Dose and Period - At the end of the 48 h treatment period, the highest level of MI (4.9%) was observed in the 2% vydate solution, and this was followed by the 14% and 16% *U. maritima* extracts and control. Consequently, *A. cepa* meristem cells are not affected by the level of toxicity of these solutions, in contrast, some chemicals involved in vydate and the *U. maritima* extracts promote the cells into mitosis. However, by increasing the treatment period, the toxic impact prevents cell division, and, as a result MI decreases (Table 2). According to ÇELIK and ASLANTÜRK (2007) cytotoxicity level can be determined by the decreased rate of the MI. The reduction in number of the dividing cells in the roots shows the cytotoxic effects of the substances that are found in the aqueous extracts (ASLANTÜRK and ÇELIK 2009). MI and number of aberrant cells were high in *Allium cepa* meristematic tissue cells which were exposed vydate solution and the 14%, 16% and 100% *U. mar-*

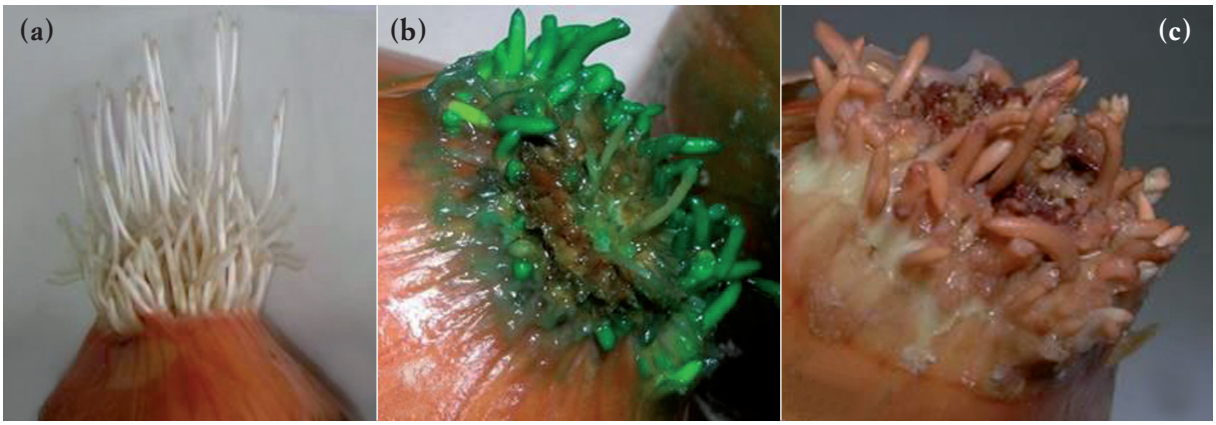

Fig. 1 — At the end of 144 h treatment, state and length of bulbs rooted in Control **(a)**, Vydate **(b)** and 100% *U. maritima* extract **(c)**

TABLE 2 — Mitotic index (MI) values according to the treatment dose and period of *U. maritima* extract

Doses	48 h treatment		72 h treatment		144 h treatment	
	Average total divided Cells ± SD	MI (Mean ±SD)	Average total divided Cells ± SD	MI (Mean ±SD)	Average total divided Cells ± SD	MI (Mean ±SD)
Control	34 ± 8.0322 bc	1.7 ± 2.4403 ab	66 ± 15.0828 b	3.3 ± 0.7541 a	216 ± 21.7911 c	10.8 ± 1.0895 d
8%	10 ± 3.1271 a	0.5 ± 0.1563 a	7 ± 6.6674 a	0.35 ± 0.3333 a	32 ± 12.9964 b	1.6 ± 0.6677 b
10%	9 ± 2.3371 a	0.45 ± 0.1168 a	8 ± 1.9564 a	0.4 ± 0.0978 a	32 ± 4.4227 b	1.6 ± 0.2211 b
12%	10 ± 1.4333 a	0.5 ± 0.0682 a	32 ± 13.3759 a	1.6 ± 0.5462 a	44 ± 22.9643 b	2.2 ± 1.1482 c
14%	49 ± 11.7269 c	2.45 ± 0.5863 ab	24 ± 12.0933 a	1.2 ± 2.5748 a	54 ± 17.6345 bc	2.7 ± 4.5580 cd
16%	44 ± 8.4947 c	2.2 ± 0.4247 ab	6 ± 1.8442 a	0.3 ± 0.0922 a	19 ± 8.1469 a	0.95 ± 0.3865 a
100%	16 ± 1.5779 ab	0.8 ±0.0789 a	20 ± 2.7221 a	1 ± 0.1361 a	19 ± 5.4161 a	0.96 ± 0.0968 a
Vydate	98 ± 6.8888 d	4.9 ± 0.3444 b	29 ± 6.6433 a	1.45 ± 6.3671 a	30 ± 5.4161 a	1.5 ± 0.2708 a

Variability around the mean was represented as ± SD (Standart Deviation). Data having the same letter in a column were not significantly differed by LSD's multipli comparison test ($P<0.05$)

itima extracts for 48 hours. This indicates that 48 h treatment does not have enough influence on to stop mitosis. On the other hand, in 72 and 144 h treatments, the defense systems preventing mitosis become active and this results in the decrease of MI. The decline in mitotic index value shows interference in the cell cycle (OLOYEDE *et al.* 2009). The high ratio of MI exposed to 14% and 12% *U. maritima* extracts together with control indicates that the damage on living cells from these extracts can be recoverable and tolerable. Individual plant components like sulfhydryl and flavonoid compounds, gallic acid, ellagic acid, mucic acid, citric acid, reducing sugars and tannins can modulate effect of many genotoxicants (JAFFEREY and RATHORE 2007). The same result was not observed for vydate solution and it was thought that the damage from this chemical on living cells were irrecoverable and much more destructive when compared to *U. maritima* extract. According to YÜZBAŞIOĞLU *et al.* (2003), the decrease in MI is caused by pressure on DNA synthesis or the complete halt of metabolic activities to prevent the cell entering mitosis (ASLANTÜRK and ÇELIK 2009).

Formation Aberrant Cells According to Treatment Dose and Period of the Extract - In all of the doses and periods of treatment, various abnormal cells were observed at different levels. At the end of the 48 h treatment, the highest number of aberrant cells was observed in vydate treatment with 32 aberrant cells, followed by the 14% *U. maritima* extract with 17 aberrant cells. The rate of the aberrant cells observed in the other treatments was statistically found to be same as that of control. At the end of the 72 h treatment, the highest rate of aberrant cells was observed in vydate treatment with 7 cells, followed by 100% *U. maritima* treatment with 6.2 aberrant cells, and they were found statistically in the same group. At the end of the 144 h treatment, the highest rate of aberrant cells was observed in the control with 11.6 cells (Table 3). Because the increase of aberrant cells paralleled the dose increase in the 48 hour treatment, in the 72 and 144 hour treatments, cells exposed to high doses remained in one of the G_1, S or G_2 phases of the cell cycle as mentioned by AKPINAR *et al.* (2001). During the late G_1 phase the restriction point gate opens in the presence of a complex molecule at promoters of essential cell cycle genes and unreplicated and/or DNA does not allow cells to go beyond G_1 state (JAFFEREY and RATHORE 2007). It was known that the chemicals were effective on the cell energy production centers, and so by the decrease of ATP synthesis the cell division gets slower and cytotoxic effects were observed (ÇAVUŞOĞLU 2008). YÜZBAŞIOĞLU (2003) reported that inhibition of the cell cycle results from the fact that pesticides target areas including special proteins. The reason behind this inhibition is the lack of DNA polymerase enzyme, which is necessary for the synthesis of DNA. In addition to lack of DNA polymerase enzyme, the lack of other enzymes enabling the formation of spindle fibers and their healthy functioning directly leads to the halt of the cell cycle. Cadmium affects both gene transcription and translation, and modulates the signal transduction pathway. All such known toxic effects of cadmium chloride can be held responsible for causing low mitosis i.e. mitodepression in *Allium* root tip cells (JAFFEREY and RATHORE 2007).

Anomalies that were observed in the study are as follows: The highest anomaly was chromosome stickiness, fragmentation in the metaphase,

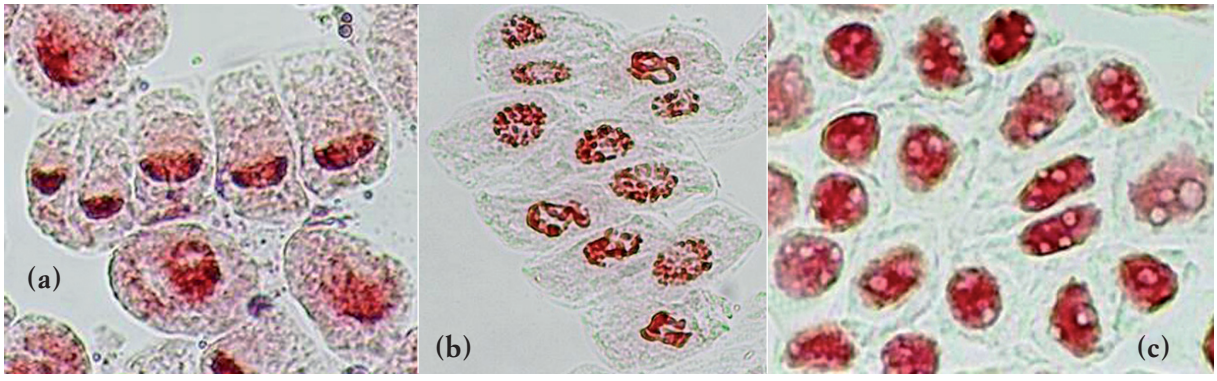

Fig. 2 — Some anomalies **a)** Nucleus erosion in interphase **b)** Granulation of nucleus in interphase **c)** Increase in the number of nucleolus in the nucleus (×400)

an irregular metaphase, bridge in the anaphase, pole deviations in the anaphase and an irregular anaphase (Table 3). In addition to these anomalies, in the 14%, 16% and 100% *U. maritima* extracts and vydate solution, anomalies that can be generally regarded as nucleus deformations such as erosion in the interphase nucleus (Figure 2a) and in the prophase nucleus, granulation in the interphase nucleus (Figure 2b), split in the interphase nucleus, granulation in the prophase nucleus. Nucleus vacuolization (only in vydate and 100% *U. maritima*) and micronuclei formation were rarely observed and were statistically not significant. The induction of spindle disturbances in the cell of *Allium cepa* by extracts may lead to aneuploidy and or micronucleus formation at the next stage of cell division. The lagging chromosome(s) may be lost or form nuclear membrane around itself thereby forming micronucleus (GRANT 1978). C-mitosis, binucleate cells and increases in the number of nucleolus in the nucleus (Figure 2c) were also observed. Mitotic toxicity affects spindle fibers and causes their irregular distribution or completely prevents their formation, and as a result C-mitosis is observed (YÜZBAŞIOĞLU 2003; GRISOLIA *et al.* 2004). According to RANK (2003), laggards or vagrant chromosomes are considered indicators of spindle poisoning. Even a weak C-mitotic effect prevents spindle fibers from reaching the chromosome and as a result, laggard chromosomes appear. The lagging chromosome(s) usually arises from irregular separation of chromosomes at anaphase thereby making some chromosomes to reach the poles before the other (GRANT 1978). DANE and DALGIÇ (2003), report that the fungicide benomyl inhibits microtubules and thus cytokinesis. The *Allium cepa* root cells also possess mixed function oxidases like that of mammalian hepatocytes which can activate promutagens to mutagens according to JAFFEREY and RATHORE (2007). Disorders in the stability of cytoskeleton proteins under oxidative stress cause the inhibition of mitotic events and result in cell death. KAYMAK (2005) states that chromosome splits, breakages and inhibition of spindle treads result from various chemicals which affect the basic proteins required for spindle fibers. ÇAVUŞOĞLU (2008) reported that the cytotoxic effects of some chemicals were observed from the occurrence of fragments on DNA doubled spindles. KHORA *et al.* (1997) reported that sister chromatid exchanges (SCEs) are widely believed to represent the interchanges of DNA replication products at apparently homologous loci, and involve DNA breakage and reunion. It is claimed that the stickiness, bridges and fragments which are scored as indicators of clastogenicity in chromosomes are induced by chemicals regarded as clastogenic agents (RANK 2003; KAYMAK 2005). Stickiness in the chromosomes is an indication that chemical substance has a high toxicity, and may cause the death of cells by inducing unrecoverable damages (FISKESJÖ 1985).

When stickiness in the chromosomes is seen in vydate and high doses of *U. maritima* extract compared with literature, it was seen that *U. maritima* extract is less cytotoxic than vydate, however, when it was compared to the control, it has a more cytotoxic influence. In this study, nucleus deformation increase depending on the increase in dose and the period of the treatment, which indicates that the cells are cytotoxically and genotoxically affected and as a result DNA synthesis is pressured. Vacuolizations observed in vydate and 100% *U. maritima* extract treatment indicated that the chemical pesticide is a more destructive and comprehensive mutagen, and those high concentrations of *U. maritima* extract, gives the same results.

The anomalies occurring in the 100%, 16% and 14% *U. maritima* extracts are usually observed in the long treatment periods (72 and 144 h treatments). When vydate is routinely used against root tumor nematode (at the commercially suggested dose of 2%) compared to the treatment doses of *U. maritima* extract, it was seen that even the high doses of *U. maritima* extracts are less mutagenic than vydate. *U. maritima* extract that can be used as a biopesticide against root tumor nematode was found to be effective at 4% dose in green house conditions (CIVELEK and WEINTRAUB 2004). METIN and BÜRÜN (2008) found that the 6% *U. maritima* dose is less cytotoxic and genotoxic when compared to 2% vydate.

Chemicals used for agricultural control are an important source of environmental pollution. It is therefore necessary to find alternatives to these chemicals which are used widely and might be an indispensable way to fight for agricultural uses in today's conditions in order to minimize their harms. Consequently, it is necessary to investigate the natural pesticides obtained from different plants. This study establishes that *U. maritima* extract can be used more safety than chemical pesticides. In order to reach more information and certain conclusions about this subject, however, further researches should be performed whit different test system.

TABLE 3 — Cell anomalies according to the extract treatments dose and periods

Doses	Periods (hour)	Average total divided Cell ± SD	Stickiness ± SD	Fragment ± SD	Irregular Metaphase ± SD	Pole Deviation ± SD	Bridge ± SD	Irregular Anaphase ± SD	% Aberrant Cell ± SD
Control	48	34 ± 8.0322 bc	1 ± 0.2132 a	0.7 ± 0.1880 a	0.1 ± 0.0833 a	0.2 ± 0.1666 a	0.6 ± 0.2289 a	0.09 ± 0.0833 a	0.1 ± 0.0257 a
8%	48	10 ± 3.1271 a	1.8 ± 1.0413 a	0 ± 0.000 a	0 ± 0.0000 a	0.7 ± 0.2134 a	0.3 ± 0.1527 a	0 ± 0.0000 a	0.2 ± 0.0653 a
10%	48	9 ± 2.3371 a	0.7 ± 0.1829 a	0.4 ± 0.2630 a	0 ± 0.0000 a	0.5 ± 0.1889 a	0 ± 0.0000 a	0.1 ± 0.1250 a	0.1 ± 0.0181 a
12%	48	10 ± 1.4333 a	1.2 ± 0.2000 a	0.3 ± 0.1527 a	0 ± 0.0000 a	0.8 ± 0.1333 a	0.5 ± 0.1666 a	0 ± 0.0000 a	0.1 ± 0.0256 a
14%	48	49 ± 11.7269 c	7.2 ± 3.1686 bc	3 ± 0.7000 b	0.9 ± 0.4818 ab	3.5 ± 1.4925 b	1.6 ± 0.8516 ab	1.3 ± 0.5385 b	0.9 ± 0.2501 b
16%	48	44 ± 8.4947 c	3.2 ± 2.1771 ab	0.6 ± 0.4000 a	0 ± 0.0000 a	1.4 ± 0.9798 a	0.2 ± 0.2000 a	0.4 ± 0.4000 ab	0.3 ± 0.2051 a
100%	48	16 ± 1.5779 ab	1 ± 0.2582 a	0.3 ± 0.1527 a	1.6 ± 0.2666 b	0.2 ± 0.1333 a	0.3 ± 0.1469 a	0.7 ± 0.2603 ab	0.2 ± 0.0280 a
Vydate	48	98 ± 6.8888 d	10.4 ± 1.4544 c	4 ± 0.5783 b	6.2 ± 1.0306 c	2 ± 0.2108 ab	6.3 ± 4.3257 b	2.6 ± 0.5206 c	1.6 ± 0.1766 c
Control	72	66 ± 15.0828 b	0.1 ± 0.0909 a	0.5 ± 0.2816 a	0.1 ± 0.0000 a	0 ± 0.0000 a	0.3 ± 0.1408 a	0 ± 0.0000 a	0.05 ± 0.1548 a
8%	72	7 ± 6.6674 a	0.1 ± 0.1000 a	0 ± 0.0000 a	0 ± 0.0000 a	0 ± 0.0000 a	0.2 ± 0.2000 a	0 ± 0.0000 a	0.02 ± 0.0000 a
10%	72	8 ± 1.9564 a	0 ± 0.0000 a	0 ± 0.0000 a	0 ± 0.0000 a	0 ± 0.0000 a	0 ± 0.0000 a	0 ± 0.0000 a	0 ± 0.0000 a
12%	72	32 ± 13.3759 a	0 ± 0.0000 a	0 ± 0.0000 a	0 ± 0.0000 a	0 ± 0.0000 a	0 ± 0.0000 a	0 ± 0.0000 a	0 ± 0.0000 a
14%	72	24 ± 12.0933 a	0.2 ± 0.1666 a	0 ± 0.0000 a	0 ± 0.0000 a	1.2 ± 0.1666 c	0.4 ± 0.3333 a	0 ± 0.0000 a	0.1 ± 0.0000 a
16%	72	6 ± 1.8442 a	0 ± 0.0000 a	0 ± 0.0000 a	0 ± 0.0000 a	0 ± 0.0000 a	0 ± 0.0000 a	0 ± 0.0000 a	0 ± 0.0000 a
100%	72	20 ± 2.7221 a	1.6 ± 0.3399 b	0.1 ± 0.1000 a	1.6 ± 0.4268 b	0.7 ± 0.2134 b	0.2 ± 0.1333 a	1.8 ± 0.2494 c	0.3 ± 0.0371 b
Vydate	72	29 ± 6.6433 a	1.5 ± 0.3415 b	1.1 ± 0.2768 b	1.5 ± 0.2687 b	0.8 ± 0.2905 b	0.5 ± 0.1666 a	0.9 ± 0.20 b	0.3 ± 0.0581 b
Control	144	216 ± 21.7911 c	4.4 ± 1.0241 c	4 ± 0.8491 b	0 ± 0.0000 a	1.7 ± 0.2603 c	1.8 ± 0.4163 b	0 ± 0.0000 a	0.6 ± 0.0760 c
8%	144	32 ± 12.9964 b	0 ± 0.0000 a	0 ± 0.0000 a	0 ± 0.0000 a	0.5 ±0.1831 ab	0.7 ± 0.3076 a	0 ± 0.0000 a	0.06 ± 0.0174 a
10%	144	32 ± 4.4227 b	0 ± 0.0000 a	0 ± 0.0000 a	0 ± 0.0000 a	0 ± 0.0000 a	0 ± 0.0000 a	0 ± 0.0000 a	0 ± 0.0000 a
12%	144	44 ± 22.9643 b	0 ± 0.0000 a	0 ± 0.0000 a	0 ± 0.0000 a	0.2 ± 0.2000 ab	0 ± 0.0000 a	0 ± 0.0000 a	0.01 ± 0.0100 a
14%	144	54 ± 17.6345 bc	0 ± 0.0000 a	0 ± 0.0000 a	0 ± 0.0000 a	0.4 ± 0.2108 ab	0 ± 0.0000 a	0 ± 0.0000 a	0.02 ± 0.000 a
16%	144	19 ± 8.1469 a	0 ± 0.0000 a	0 ± 0.0000 a	0 ± 0.0000 a	0 ± 0.0000 a	1 ± 0.4406 a	0 ± 0.0000 a	0.04 ± 0.0210 a
100%	144	19 ± 5.4161 a	2 ± 0.3379 ab	0.4 ± 0.1111 a	2 ± 0.4339 b	0.4 ± 0.1469 ab	0.1 ± 0.1111 a	1.2 ± 0.2605 b	0.3 ±0.0286 b
Vydate	144	30 ± 5.4161 a	3 ± 1.1742 bc	1 ± 0.1795 a	3 ± 0.6324 c	0.7 ± 0.2134 b	0.6 ± 0.1633 a	2 ± 0.3333 c	0.5 ± 0.1214 c

Variability around the mean was represented as ± SD (Standart Deviation). Data having the same letter in a column were not significantly differed by LSD's multipli comparison test ($P < 0.05$)

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