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CYTOLOGICAL EFFECTS OF COUMARIN ON THE MITOSIS OF *LENS CULINARIS MEDIK*

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ABSTRACT

This study examines the cytological effects of coumarin (2H-1-benzopyran-2-one) on *Lens culinaris* Medik. cv. Sultan in terms of cell response, mitotic index, mitotic abnormalities and chromosome aberrations. Effective concentration values were calculated based on a probit model, which is a type of regression where the dependent variable can take only two values, i.e., life or death, after 72 hours of treatment. The effective concentration value of EC50 was determined via probit analyses, approximately 278 µM, and was then adjusted to 300 µM. The bulb roots were treated with the following concentrations depending on the root growth inhibition test: 300 µM (EC50), 600 µM (2X EC50). The control group was treated with Hoagland. Germination percentages of *L.culinaris* roots decreased with increasing coumarin concentrations. Cytological observations demonstrated that the mitotic frequency in root meristematic cells lessened and that abnormality frequency decrease parallel to the increase in concentrations for coumarin. The obtained results indicated that this active ingredient had the ability to cause a reduction in the seed germination percentage in the various phases of mitosis and increased chromosomal aberrations in *L.culinaris* meristematic cells.

KEYWORDS:
L.culinaris, coumarin, cytological effects, chromosomal abnormalities, mitotic index.

INTRODUCTION

Coumarins (2H-1-benzopyran-2-one) consist of a large class of phenolic substances found in plants and are made up of fused benzene and α-pyrone rings [1]. More than 1300 coumarins have been identified as secondary metabolites from plants, bacteria and fungi [2]. Coumarin is a plant-derived natural product known for its pharmacological properties such as anti-inflammatory, anticoagulant, antibacterial, antifungal, antiviral, anticancer, antihypertensive, antitubercular, anticonvulsant, antiadipogenic, antihyperglycemic, antioxidant, and neuroprotective qualities. Dietary exposure to benzopyrones is considered relevant as these compounds are found in vegetables, fruits, seeds, nuts, coffee, tea and wine. Considering the established low toxicity, relative cheapness, presence in the diet and occurrence in various herbal remedies of coumarins, it appears prudent to evaluate their properties and applications further. The functions of coumarins are far from clear, although suggestions may include plant growth regulators, bacteriosid, fungicid and even waste products [3]. The effects of root exudates and decayed plant component residues, mainly allelochemicals such as phenolic acids on plant growth, have been reported in crops such as lettuce, cucumber, watermelon and sunflowers. In these cases, root exudates of plants and decayed plant residues have been reported to be toxic to the plants themselves which is the result of secondary metabolic products or decomposing plant substrates [4]. Some coumarins have been identified as allelochemicals. For example, Furanocoumarins (psoralen, in particular) also severely inhibit seed germination [5].

The discovery of the physiological activity of unsaturated lactones in plants has greatly increased biologists' interest in the general activity of coumarin derivatives [6]. Coumarin has the capacity either to promote or to inhibit plant growth, the precise response being species-specific and concentration-dependent [7]. In addition, coumarin decreases respiration and photosynthesis in intact plants by inhibiting electron transport. This affects the activity of enzymes responsible for nitrogen metabolism in carrot cell suspension culture [8], and inhibits elongation, cell division, differentiation, rhizogenesis and germination. It also promotes rooting in the leaf explants of petunias and in the inhypocotyl explants of soybean [9] and chromosome breaking in an allium test [6]. A study reports that Coumarin in a concentration range of 10–100 ppm has inhibited the growth of rice, mung bean, lettuce and clover seedlings in durum wheat (*Triticum durum* Desf., cv Simeto) [5]. Furthermore, coumarin concentrations beyond 1000 µM have prevented the net nitrate uptake [10]. Also, coumarin in a concentration range from of 10–100 ppm reduced the growth of rice, mung bean, lettuce and clover seedlings [11]. The aim of this study is to contribute to the recognition of primary biochemical

targets of coumarin and to understand its dynamics in a more detailed way.

Sources regarding the phytotoxic effects of coumarin in plant biosystem such as *L.culinaris* are nonexistent. In this study, we have investigated the cytological effects of coumarin, one of the fenolic compounds that emerge as a secondary compound in plants, on *L. culinaris* which is widely cultivated for its high economic and nutritional value. We also calculated the germination ratios and conducted probit analysis with EC50 values. The mitotic index test, which has been used as an important parameter in the identification of growth development ratio and the frequency of cell division, was carried out to identify cytological measurements and chromosomal abnormalities [12]. As biomarkers give information about the development and growth of the plants, the results may suggest a tolerance value of *L.culinaris* according to its growth.

MATERIALS AND METHODS

Plant Material and Coumarin. The selected plant material *Lens culinaris* Medik. cv. Sultan as commercial seeds were obtained from the Central Research Institute of Turkey. The coumarin was commercially (Sigma) purchased. The chemical structure of coumarin can be seen in Figure 1:

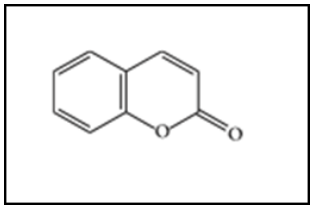


FIGURE 1
Coumarin (2H-1-benzopyran-2-one) chemical structure

Cytology. Determination of EC50 values. *Lens culinaris* seeds were pretreated with 20% ethanol solution for 5 minutes then were allowed to stand for 5 minutes in a 5% sodium hypochlorite solution using the surface sterilization method [13]. Later, the seeds were placed under clean bench conditions in Petri dishes and filled with boiled tap water at room temperature for soaking and germination. At the beginning of the study, six different coumarin concentrations (i.e., 100, 150, 200, 250, 300, 350 µM) were prepared in modified Hoagland nutrient solution dilutions as recommended by the US EPA[14]. The control groups were treated only with modified Hoagland nutrient solution. The EC50 values (the effective concentration for 50% growth inhibition) were calculated according to seed germination percentages with Probit analysis for 48 and 72 hours periods. The root tips that were 0.5 mm or higher were regarded as *germinated*. All experiments were carried out in three repetitions. The germination of seeds was observed in laboratory conditions in petri dishes for 48 and 72 hour time periods.

Mitotic analysis. The root tips were fixed first in Carnoy solution (3:1 alcohol-asetic acid) and hydrolysed in 1 N HCl at 60°C for 5–10 minutes. They were, then, crushed in a 2% orcein stain in 45% acetic acid and the slides were examined immediately. The mitotic abnormalities were determined by the examination of 500 cells per slide and calculated as approximately 5,000 mitotic cells. Three replicates were prepared for each concentration.

Detection of chromosomal abnormalities. Cells in different phases of mitosis were analysed. The types of chromosomal abnormalities that were investigated were: micronucleus, lagging chromosome, chromosome bridges, c-mitosis, polarity and frequency. Chromosomal abnormalities were evaluated separately in all phases of mitosis and photographic data was obtained using an Olympus BX51 photomicroscope.

Statistical Analysis. The statistical analyses of data were carried out using SPSS for Windows version 16.0 statistical software (SPSS Inc, Chicago, USA). Statistically significant differences between the groups were compared using both one-way analysis of variance (ANOVA) and the Probit test. The data were displayed by mean ± standard deviation (SD), and p-values less than 0.05 were considered *statistically significant*.

RESULTS

While treating seeds with different concentrations of coumarin, we observed that seed germination decreased while the concentrations increased for each concentration of coumarin over a 72 hour period. A significant decrease in live *L.culinaris'* seeds was shown at 300 µM (Table 1), and after 3 days a considerable decrease in seedling growth at concentration of 350 µM was observed (Figure 2). As a control, *L.culinaris* seeds were germinated in Hoagland solution. The different extracts concentrations and the control were arranged in a completely random design spread over three different repetitions (each repetition corresponding to 60 seeds of *L.culinaris* placed in a Petri dish).

The effects of coumarin on the seedling growth of *L.culinaris* were evaluated by probit analysis where the dependent variable can take only two values, for example life or death, after treatment for 72 hours. Probit analysis is commonly used in toxicology to determine the relative toxicity of chemicals to living organisms. This is achieved by testing the response of an organism under various concentrations of each of the chemicals in question and then comparing the concentrations at which one encounters a

response. The response is always binomial (e.g. death/no death) and the relationship between the response and the various concentrations is always sigmoid [15]. EC50 values were found at 278 µM for coumarin (Table 2) and then graphed (Figure 3). This was later adapted to a 300 µM coumarin concentration. Therefore, all experimental procedures were carried out using coumarin concentrations of 300 µM (EC50) and 600 µM (2xEC50).

TABLE 1
The effects of different concentrations of coumarin on germinated *L.culinaris* seed counts and residuals (72 hour time frame).

Number		Used Dose (µM)	Number of Subjects	Observed Responses	Expected Responses	Residual	Probability
PROBIT	1	,000	60	59	57,545	1,455	,959
	2	100,000	60	45	52,069	-7,069	,868
	3	150,000	60	46	47,361	-1,361	,789
	4	200,000	60	44	41,321	2,679	,689
	5	250,000	60	44	34,289	9,711	,571
	6	300,000	60	32	26,853	5,147	,448
	7	350,000	60	10	19,714	-9,714	,329

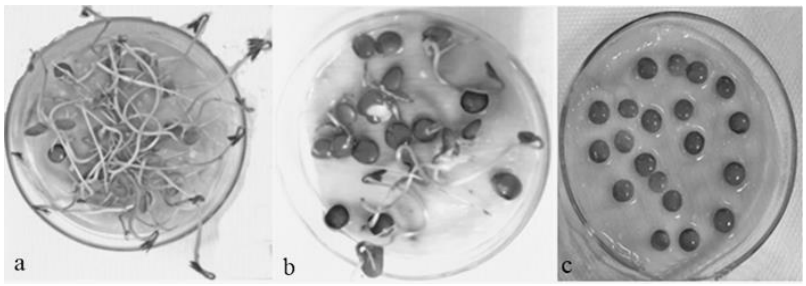


FIGURE 2
The germinated *L. culinaris* seeds treated with coumarin concentrations after 3 days: a) control (Hoagland solution), b) 300 µM coumarin, c) 600 µM coumarin.

TABLE 2
EC50 values (µM) of tested coumarin for lower and upper bounds and their corresponding 95 % confidence limits.

Confidence Limits				
Probability		95% Confidence Limits for Coumarin		
		Estimate	Lower Bound	Upper Bound
PROBIT	,050	542,460	406,023	1159,692
	,250	386,957	304,041	718,558
	,400	319,467	253,504	533,381
	,450	299,005	235,882	479,536
	,500	278,868	216,517	428,569
	,700	194,831	88,459	263,118
	,800	143,995	-32,884	206,912
	,900	73,495	-222,873	150,671
	,990	-93,937	-696,604	39,626

TABLE 3
The effect of coumarin concentrations on mitotic frequency percentage. (Values are presented as mean ± standard deviation. The exposure time of different coumarin concentrations was 72 hours.

Time (Hour)	Concentration (ppm)	Cell numbers	Divided Number of Cells	Mitotic Frequency Percentage +SD (%)
48	0	5154	595	11,54 ± ,220
	300	5031	405	8,05 ± ,368
	600	5105	281	5,50 ± 021
72	0	5043	527	10,45 ± ,468
	300	5127	244	4,67 ± ,119
	600	5170	205	3,96 ± ,181

The probit analysis also revealed the observed and expected responses of the cells and their residuals (Figure 4). As we can see in this figure, for the smallest concentration used (i.e., 100 µM), the obtained and predicted responses were very similar. However, for the largest concentration (i.e., 350 µM), there was a significant difference between the detected and anticipated responses. On the other hand, in the responses that were observed in the lower concentrations, i.e., 150 µM, 200 µM, 250 µM and 300 µM, there was almost no or very little variation, but, the response rate decreased solely in the largest concentration (350 µM).

The used concentrations of coumarin caused a decrease in the mitotic index when compared with the control (Table 3), indicating mitotic inhibition. The coumarin concentrations used had a negative effect on mitotic division. The mitotic frequency reduced as concentration increased when compared with the control group (Table 2). The mitotic frequency was 11,54% in the control group and decreased to 8,05% at 300µM concentration and 5,50% at 600µM concentration in 48 hours. In the control group, the mitotic frequency decreased from 10,45% to 4,67% at 300µM concentration and 3,96% at 600µM concentration over a 72 hour period. During our 3-day observation of seed germination, measurements were not calculated after 24 hours as there were no seedlings at that stage. In high coumarin concentration, mitotic frequency reduced to almost half of that of the control.

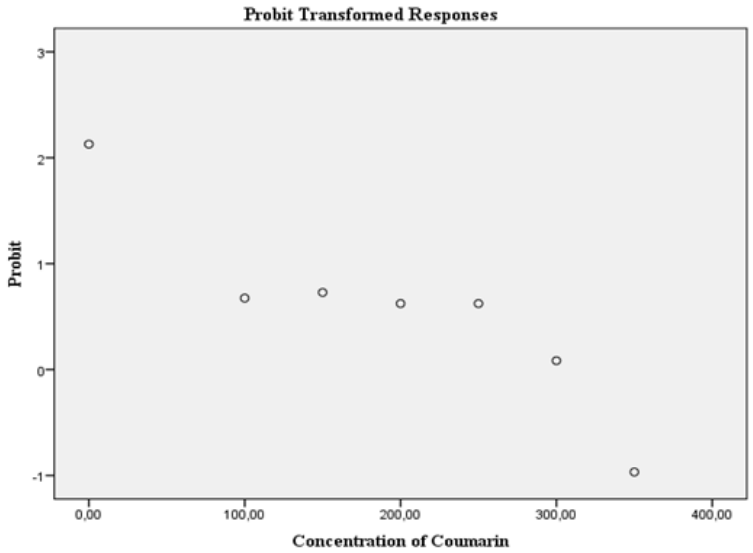


FIGURE 3
Probit values and regression graph of the effect of coumarin on *L.culinaris* seeds (72h)

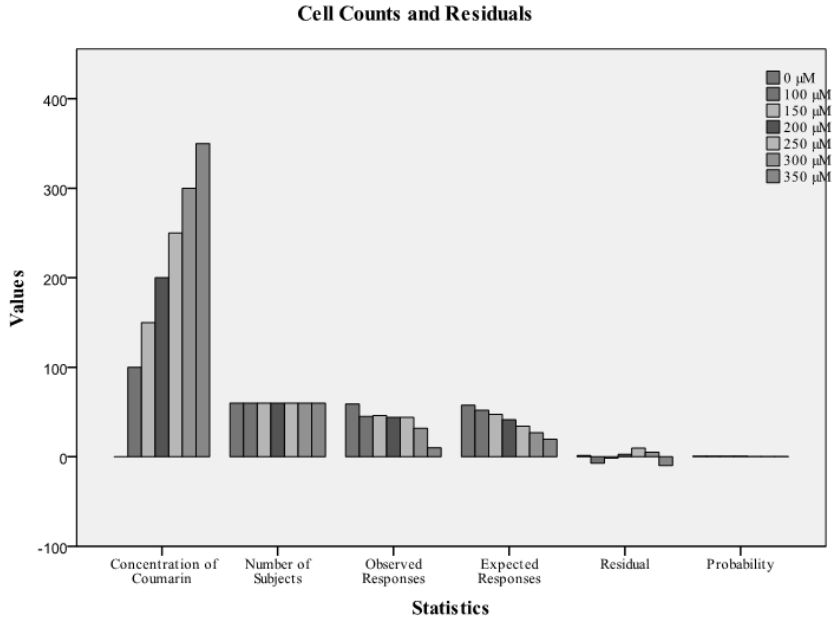


FIGURE 4
Cell counts and residuals for different concentrations of coumarin

TABLE 4
The effect of coumarin concentrations on the mitotic abnormality percentage and frequencies.

Time (Hour)	Concentration (µM)	Prophase N	Prophase A	Prophase %	Metaphase N	Metaphase A	Metaphase %	Anaphase N	Anaphase A	Anaphase %	Telophase N	Telophase A	Telophase %
	0	232	3	**1,27**	133	5	**3,6**	143	5	**3,37**	95	4	**4,04**
48	300	119	20	**14,38**	98	16	**14,03**	84	13	**13,40**	86	14	**14**
	600	80	33,3	**24**	51	23	**31,08**	57	14	**19,71**	52	14	**21,21**
	0	206	4	**1,9**	111	6	**5,1**	107	4	**3,66**	105	5	**5,6**
	300	113	26	**18,7**	73	21	**22,34**	63	14	**18,18**	92	13	**12,38**
72	600	87	25	**22,32**	32	22	**40,74**	29	26	**48,14**	57	14	**19,71**

(N; Normal cell, A;abnormal cell,)

TABLE 5
Abnormality counts and percentages for different times and concentrations.

	Time (hour)	Concentration (ppm)	Cell numbers	Cell deformation	Irregular Prophase	Irregular Metaphase	Sticky Choromosome	Sticky Prophase	Uunequal division	Choromosome Bridge	Laggard Chromosome	C-Mitosis	Binucleus	Multipolarity	Fragments at metaphase	Fragmentation of Amylo-plasts	Irregular Telophase	Nucleus deformation	Chromosome granulation	Polar Deviation	Irregular Anapahase
Coumarin	48	0	500	0	3	4	2	0	0	2	4	1	0	0	1	0	0	0	1	0	
		300	500	0	12	16	3	0	1	0	8	5	1	0	0	0	3	4	1	6	1
		600	500	7	25	19	4	7	1	2	7	5	6	1	3	29	5	15	3	6	0
	72	0	500	0	1	6	2	0	0	2	3	1	0	1	2	0	0	0	1	2	1
		300	500	12	21	11	2	2	1	0	12	1	2	2	2	5	2	9	1	5	2
		600	500	32	17	9	3	5	3	4	18	3	4	1	4	35	3	19	1	6	1
		Total		51	79	65	16	14	6	10	52	16	13	5	12	69	51	65	16	14	10
		abnormality percentage.%		11,5	17,9	14,7	3,6	3,2	1,4	2,3	11,7	3,6	2,9	1,1	2,7	15,6	11,5	14,7	3,6	3,2	2,3

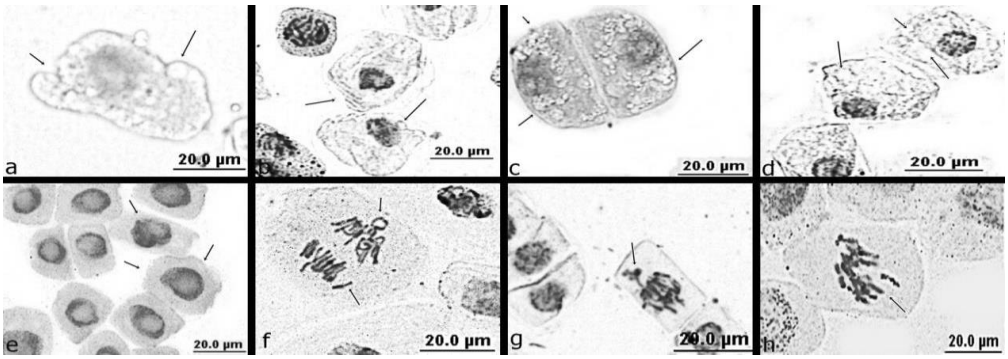


FIGURE 5
The following common abnormalities were observed: cell wall deformation in (a) and (b) cells, (c): fragmentation of amyloplasts, (d): necrotic cell, (e): nucleus and cell wall deformation, (f): laggard, ring chromosome in metaphase, (g): laggard chromosome in anaphase, (h): irregular metaphase. The abnormalities present are indicated by arrows.

We also examined the mitotic abnormality percentages and frequencies for prophase, metaphase, anaphase and telophase for different concentrations at different hours (Table 4). We observed that mitotic abnormality increased for all measurements as the concentration amount increased. For example, for prophase, the abnormality percentage was 1,27% in the control but increased to 14,38% at 300µM and 24% at 600µM at 48 hours. Similarly, at 72 hours, the abnormality percentage was 1,9 % in the control and increased to 18,7% at 300µM and 22,32% at 600µM. Similar increases were observed in other measurements for metaphase, anaphase and telophase (Table 4).

Another analysis included the counts and percentages of different abnormalities for different times and concenrations in 500 cells. The highest percentages were observed for irregular prophase (17,9 %), fragmentation of amyloplasts (15,6 %), irregular metaphase (14,7%) and nucleus deformation

(14,7 %). Multipolarity (1,1 %), unequal division (1,4%), irregular anaphase (2,3 %), polar deviation (3,2 %) and sticky prophase (3,2 %) yielded the lowest percentages. Coumarin increased the percentage of abnormal cells in *L.culinaris* meristematic cells (Table 5). This increase was significant in all concentrations applied when compared to the control and was dose dependent. The common abnormalities found were as follows: cell wall deformation in cells, fragmentation of amyloplasts, necrotic cell, nucleus and cell wall deformation, laggard, ring chromosome in metaphase, laggard chromosome in anaphase, irregular metaphase (Fig. 5). All abnormalities observed in different concentrations at different times are shown in Table 5.

DISCUSSION

Plant compounds affect different populations in their habitats. The determination of a plant's habitat is impacted to a great degree by allelophatic compounds. The impact mechanisms of these compounds that have inhibitory effects may cause some extent of physiological, cytological or genetic changes. They may weaken or diminish the populations of organisms that they compete with by causing genetic variations in their DNAs [16]. If these compounds are taken dietically, they might cause similar harms to the organisms. In this study, the effects of coumarin, which has been used in agriculture, medicine and industry as impact material, on the model organism *L.culinaris* were explored by measuring germination ratios and determining EC50 values for mitotic index and chromosome abnormalities [17]. *L. culinaris* was seleceted for this particular study due to its economic and nutritional values. A 50% growth inhibition was observed when *L.culinaris* seeds were treated with the EC value 300µM. If the dose is doubled (EC50x2), the lack of germination in most of the seeds can be observed.

To the best of our knowledge, this study is one the first that investigates the cytological effects of coumarin on *L.culinaris* in terms of response, mitotic index, mitotic abnormalities and chromosome aberrations and may provide some valuable data for further reseach. Many secondary compounds, such as coumarin, demonstrate different allelophatic effects on many plants [18, 19]. It has been also shown that these compounds have antioxidant [20, 21], antibacterial [21, 22] and nemetidisal [23] effects on some organisms. However, their impact on the genetic structure of the organisms is unknown.

Some coumarins have been identified as allelochemicals and they are potent inhibitors of seed germination [24]. Previous research has shown that a number of coumarin derivatives have the capacity to inhibit root elongation [25, 26, 27, 28, 29]. Moreover, it has been observed that coumarin has been much more effective than scopoletin in bringing about 50% inhibition of the overall growth rate in Phleum roots. It appears that coumarin is 3-4 times more effective than scopoletin in producing comparable reduction of the relative elemental growth rate [30].

Mitotic index (MI), which reflects the frequency of cell division, is an acceptable measure of cytotoxicity for all living organisms [31]. If the mitotic index decreases below 22% of the control, it can cause lethal effects on the test organism [32]. This study demonstrated the use of higher concentrations of each coumarin concentration in the various phases of mitosis. It was observed that the MI significantly decreased over 48 and 72 hours. Similarly, two coumarins (i.e., umbelliferone and daphnoretin) induced a decrease in the MI in lettuce in another study [33]. In addition, exogenous application of coumarin caused severe inhibition of carrot cell growth in suspension culture [34]. Similar inhibitory effects were also observed in Yang et al. [35] and Guo et al.'s [36] studies on the growth of *Festuca rubra* and *Medicago sativa* and *Arabidopsis*, respectively. Umbelliferone was also found to display highly inhibitory activity on the growth of lettuce and Chinese cabbage in the medicinal plants *Diplostephium foliosissimum* and *Heracleum lanat*um [37].

The most commonly observed damages in the mitosis of the meristematic cells in *L.culinaris* where coumarin was applied were: irregular prophase (17,9 %), regular prophase (14,7 %) and nucleus deformation (14,7%). The least common damage was multipolarity at just 1,1% . In developing an analytic technique for choromosomes, a study showed that pretreatment in a coumarin solution for short periods resulted in the clarification of plant choromosomes, but that prolonged treatment caused choromosome fragmentation and shrinkage [38]. In high concentrations, coumarin altered cell mitosis in plants by causing a choromosome breakage and a disorganisational effect on spindle formation. Choromosome breakage caused by coumarin was also found in resting and dividing nuclei of *Allium sp.* Action on choromosomal behaviour was also demonstrated during mitosis, presumably by blocking the synyhesis of nucleic acids in the nucleus of the cell. It was estimated that the choromosome breaking action of coumarin in the plant cells was connected to a general increase of DNA content [39]. Similar to coumarin, flavones also altered the permeability of mitochrondrial and chloroplast membranes [5].

Using compounds such as coumarin definitely have some benefits, however, one should not disregard their potential harm on people and animals. Therefore, it is one of the duties of the researchers to examine how compounds such as coumarin affect the plants they are applied to so that they can be actively used in agriculture, food, cosmetics and medicine. Detailed analyses of these compounds will yield useful results on its benefits and harms on

plants, animals and people.

CONCLUSION

The current research examined the cytological effects of coumarin (2H-1-benzopyran-2-one) on *Lens culinaris* Medik. cv. Sultan with regards to cell response, mitotic index, mitotic abnormalities and chromosome aberrations. First of all, the researchers calculated the effective concentration values based on a probit model (EC50 was calculated as approximately 278 µM, and then adjusted to 300 µM). 300 µM (EC50), 600 µM (2X EC50) and a control group (where Hoagland was used) were utilized as different concentrations in the treatment of the bulb roots. The results of our study demonstrated that germination percentages of *L.culinaris* roots decreased with increasing coumarin concentrations. Moreover, cytological observations showed that the mitotic frequency in root meristematic cells went down and that abnormality frequency decreased parallel to the increase in concentrations for coumarin. The results of our study imply that coumarin has the potential to cause a reduction in the seed germination percentage in the various phases of mitosis and can increase chromosomal aberrations in *L.culinaris* meristematic cells.

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The authors have declared no conflict of interest.

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