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Molecular and Spectroscopic Evaluation of the Effects of Coumarin on Lentil

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Abstract—This study examines the genotoxic effects of coumarin (2H-1-benzopyran-2-one) on *Lens culinaris* Medik cv Sultan in terms of DNA polymorphism and protein quantification. Effective concentration values were calculated according to a probit model, which is a type of regression where the dependent variable can take only two values, i.e., life or death, after 48 or 72 h of treatment. Based on this analysis, the effective concentration value of EC50 identified as approximately 278 μM, and then adjusted to 300 μM. The bulb roots were treated with 300 μM (EC50), 600 μM (2X EC50) concentrations and the Hoagland was used in the control group.10 RAPD primers were used and as a result of the analysis, it was observed that 2 monomorphic and 8 polymorphic band profile primers for treatment of 600 and 300 μM, coumarin concentration according to the control group. Total of 39 polymorphic bands were detected from total of 97 bands and the percentage of polymorphism was detected as 48.75%. SDS-PAGE analysis for total protein profile showed that there were differences between the treatment groups. In addition to PCR analysis, the root samples were examined by Fourier transform infrared (FT-IR) spectroscopy in order to determine the effects of coumarin on the quantity of biomolecules structure of *L. culinaris* One-way analysis of variance (ANOVA) was used in the calculation of statistical differences between the groups ($P < 0.05$).

Keywords: Lentil, Coumarin, RAPD-PCR, SDS-PAGE, FT-IR

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INTRODUCTION

Coumarin is a natural product generated from plants famous for its pharmacological qualities such as being anti-inflammatory, anticoagulant, antibacterial, antifungal, antiviral, and anticancer, and neuroprotective (Shinde et al., 2014; Venugopala et al., 2013). In additional to these potential qualities, extra application fields such as the treatment of multiple sclerosis (Wulff et al., 1998), treatment of T cell lymphoma via photochemotherapy (Plumas et al., 2003), utilization of coumarin in chemotherapy of some malignant tumors (Kawase et al., 2005), use of coumarin in successful transplantation of organs (Damjanovich et al., 2004), or assisting addicts of nicotine to overcome their smoking problems (Malaiyandi et al., 2005) are also listed in previous studies (Bourgaud et al., 2006) Coumarin also assists substantially to the persistence of plants in various defense mechanisms against phytopathogens, in the presence of abiotic stresses, control and manipulation of oxidative stress, and other hormonal adaptation (Borgaoud et al., 2006; Xu et al., 2016). Previous studies show that coumarin has impact at organ, tissue and cellular levels in plants (Gnonlonfin et al, 2012). These studies highlight that coumarin functions as a plant hormone (Yuksel and Aksoy, 2019). However, previous studies did not focus on the physiological function of the coumarin. The studies that focus on coumarin's molecular mode of action in plant tissues are also very rare (Bourgaud et al., 2006).

To our best knowledge, there were no sources regarding the phytotoxic effects of coumarin in plant bio-system such as *L. culinaris*. Motivated from this gap in literature, the current study, examines the genotoxic and biochemical effects of coumarin on *L. culinaris*, which is widely cultivated for its high economic and nutritional value. The EC50 values for the treatment were calculated by probit analysis. This study focused on the effects of coumarin on the genetic material of lentil seeds and utilized RAPD assay by evaluating the variations in RAPD profiles when compared to some population parameters such as SDS-PAGE band profiles of whole seed proteins. By means of FT-IR analysis, the increase in the amounts of coumarin and the aliphatic C=C vibrations which might cause the increase in C-0 groups and C-0 vibrations were investigated. With the fact that we can obtain information from biomarkers about the development and growth of the plants, the findings of our study may provide a tolerance value of *L. culinaris* based on its growth.

Table 1. The reaction mixture used in RAPD PCR

RAPD-PCR Components	Amount	Concentration
Buffer (1X One Taq)	12.5 μL	1X
Distilled water	9.5 μL	–
$MgCl_2$	0.5 μL	25 mM
Primer	1 μL	20 ng/mL
Taq DNA Polymerase	0.5 μL	1 U
gDNA	1 μL	20 ng/mL
Total reaction volume	25 μL	

MATERIALS AND METHODS

Plant Material and Coumarin

The Central Research Institute of Turkey was the source for *Lens culinaris* Medik. cv. Sultan and the coumarin was purchased from the seller (Sigma).

Determination of EC50 Values

The seedling growth effects of coumarin on *L. culinaris* were calculated by probit analysis. In this analysis, the dependent variable can take only two values, i.e., life or death, after the treatment for a specific time period.

DNA Extraction and RAPD PCR Technique

Germinated roots were collected after 72 h of treatment with coumarin, grounded in liquid nitrogen and total genomic DNA was extracted with DNeasy Plant Mini Kit (Qiagen). The quantity and purity of the isolated gDNA molecules was determined using a Thermo Scientific NanoDrop™ 1000 Spectrophotometer and the quality confirmed via electrophoresis on 1% agarose gel. Ten primers (OPA1, OPA2, OPA3, OPA4, OPA5, OPA6, OPA7, OPA8, OPA9, and OPA10, Operon Technologies (Alameda, California, USA) were utilized for RAPD analysis; PCR settings were optimized according to (Williams et al., 1990). RAPD banding patterns were visualized using a UV trans illuminator and photo documentation was performed under UV light using a photo imaging system.

The amplifications were carried out in triplicate. The molecular weights of the amplification products were estimated utilizing 50-bp DNA ladder standards. RAPD banding patterns were envisioned by utilizing a UV trans illuminator and the size of each amplification product was automatically calculated via the Vision Works LS Version 6.8.

Electropherograms of DNA native control is given in the attachment. The quantity and purity of the isolated gDNA molecules was determined using a Thermo Scientific NanoDrop™ 1000 spectrophotometer and the quality verified by electrophoresis on 1% agarose gel. The amount of DNA in the slots was the same (see Table 1 for details).

Determination of Genomic Template Stability

Genomic template stability (GST) was calculated as follows: GST, % = (1 – a/n) X 100. Where (a) was RAPD polymorphic profiles detected in each sample treated and (n) the number of total bands in the control (Atienzar et al., 1999; Liu et al., 2007).

RAPD Data Analysis

Amplicons (bands) were estimated as 1 (presence) or 0 (absence). Only strong bands were calculated for analysis. The size of each amplification product was automatically calculated via the Vision Works LS Version 6.8. Images were captured using a high-resolution scan and digitalized images were counted directly for RAPD analysis. The degree of DNA fragmentation (phenetic numerical analysis) among the roots of untreated control and treated roots were calculated based on Nei's unbiased measure (Nei, 1978) in POP GENE version 1.31.

SDS-PAGE Analyzes

The SDS-PAGE of total seed protein was performed in the discontinuous buffer system according to the method of Laemmli (Laemmli, 1970). Obtained gel after SDS-PAGE was visualized using a photo imaging system. After staining and destaining the gels, based on the presence or absence of polypeptide bands, dendrogram was automatically estimated using the Vision Works LS Version 6.8 for all possible pairs of protein type. The molecular weights of the protein bands of the control group and samples were determined using the Vision Works LS Version 6.8 program.

FT-IR spectroscopic Analyzes

For the FT-IR studies, there each sample of the maining powder was assorted with dried potassium bromide (KBr) at a ratio of 1 : 100 (m/m). This mixture was later dried using overnight freeze drying in

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

order to get rid of all remaining traces of water. With the help of a hydraulic pellet press, each sample was compressed at a pressure of ~100 kg cm^{-2} (1300 psi) to obtain a thin KBr disk for the analysis. A Perkin-Elmer Spectrum One FT-IR spectrometer (Perkin-Elmer Inc., Norwalk, CT, USA) was used to obtain infrared spectra using equipped with a Deuterated Triglycine Sulfate (DTGS) detector. Due to the fact that water and carbon dioxide molecules in the air may affect IR spectrum, both spectra were logged together as background and subtracted automatically via the utilization of suitable software (Perkin Elmer Spectrum One software). At room temperature, the spectra of samples were documented in the 4000–400 cm^{-1} area. Each interferogram was compiled with 100 scans at 4 cm^{-1} resolution. Each sample was scanned under the same conditions with three different pellets, with identical spectra. Three replicates were averaged and the final spectra were considered in statistical analysis. The data analysis and manipulations were conducted using the same software. In order to remove the noise, the spectra were first smoothed with nineteen-point Savitsky-Golay smooth function (Turker et al., 2007). The band positions were calculated via the frequency corresponding to the center of weight (80%) (Ozek et al., 2014).

Statistical Analysis

The statistical analyses of data were carried out using SPSS for Windows version 18.0 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 is displayed by mean ± standard deviation (SD), and p-values less than 0.05 were considered statistically significant.

RESULTS

Determination of EC50 Values

EC50 values were found at 278 μM for coumarin (Table 2). 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 (2X EC50).

DNA Extraction and RAPD PCR Technique

The gel images of RAPD profiles obtained from the genomic DNAs obtained from roots of treated and untreated seeds by using 10 primers are given in Fig. 1. In the first line of each agarose gel a 50 bp DNA ladder marker was used to determine the molecular weight of the DNA bands of the RAPD profiles.

A total of 97 DNA bands were amplified with 10 primers being worked on, and the weights of these bands varied between 170 (OPA05) and 1785 (OPA07) base pairs (bp). The total number of DNA bands obtained in all treatments was 97. Each primer produced a DNA bands between 3 (OPA04) to 16 (OPA05). The average number of DNA bands amplified per primer was calculated to be 9.7. Of the 97 DNA bands obtained in total, 41 are monomorphic bands and the remaining 39 are polymorphic DNA bands. The percentages of polymorphism and monomorphism determined for all treatment groups were 48.75 and 51.25%, respectively (Table 3).

RAPD profiles showed significant differences in the number and size of duplicated DNA bands (loss of a normal band and/or formation of a new band) between *L. culinaris* roots, untreated control and treatment (EC50 and 2 × EC50). These changes, which are determined in the RAPD profiles of the treated *L. culinaris* roots, are given in Table 4 together with all the details. It was observed that the total number of newly formed DNA bands increased markedly with increasing concentrations of coumarin. The

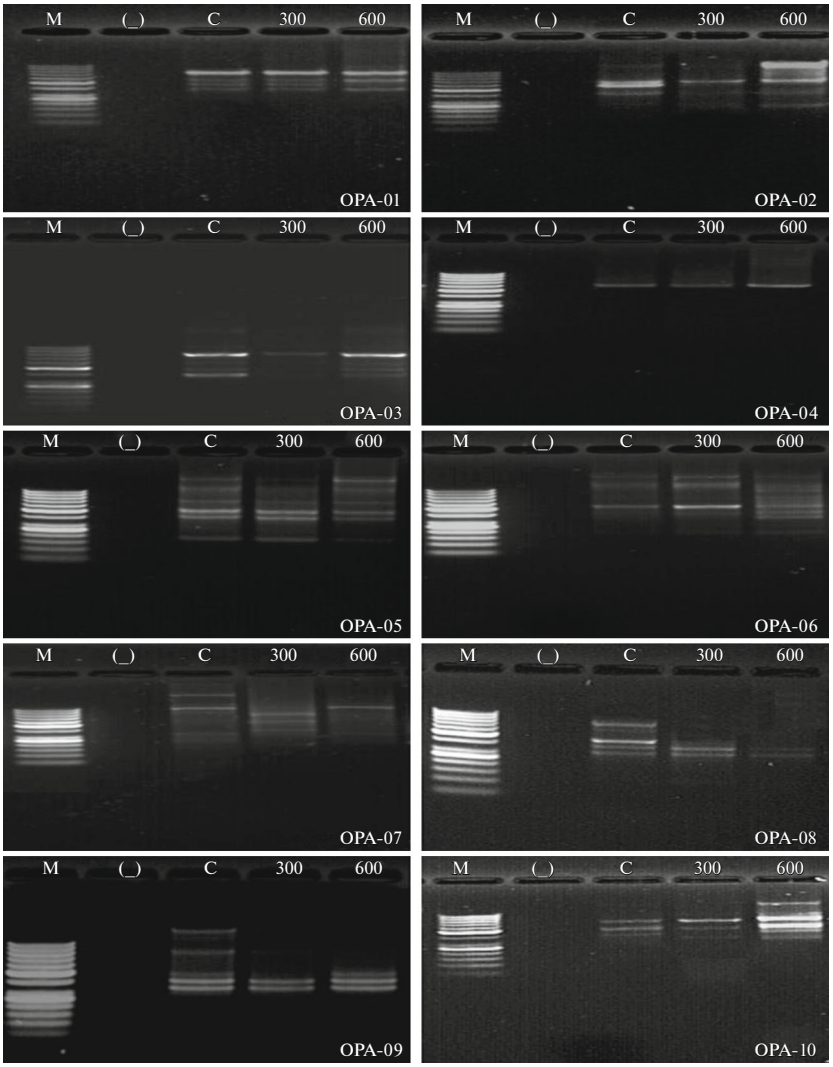


Fig. 1. RAPD profiles obtained via primers of OPA01, OPA02, OPA03, OPA04, OPA05, OPA06, OPA07, OPA08, OPA09, OPA10 from the control group and Coumarin treated *L. culinaris* genomic DNA. [M: Gene Ruler 50 bp Thermo Fischer, C: control (Hoagland), 300; 300 μM (EC50) coumarin, 600: 600 μM (2 × EC50).

highest loss of RAPD band among the treatment groups was determined at 300 μM (EC50) in coumarin administration. New band formations were detected at maximum (10 bands) 600 μM (2 × EC50) in coumarin treatment. In summary, the maximum number of RAPD band changes (lost and new bands) (EC50) (21 bands) was determined in total.

The degree of DNA fragmentation coefficients between all the treatment groups calculated using the PAST program are given in Fig. 2. According to the control group, the treatment of 300 μM coumarin demonstrated 0.59 proximity, while the 2 × EC50 value showed a proximity of 0.48 at 600 μM. The increase in the concentration of coumarin increased at the DNA fragmentation according to the control group.

Genomic template DNA stability (GTS, %) values, a qualitative representative of changes in RAPD profiles were calculated for 8 primers, which were tested. The decrease in GTS values was 45.8% in 600 μM treatment and while it was 48.5% in 300 μM treatment. According to the results obtained at the end of this study; a decrease in the average genomic template stability was observed after treatment with coumarin.

The 48 and 72 h samples with protein markers loaded on 12% removal and 4% loading gel prepared for SDS-PAGE were imaged and photographed after being run for approximately 2 h (Fig. 5).

SDS-PAGE analyzes of soluble protein samples were performed to observe the change in structural and regulatory proteins. In SDS-PAGE analysis of the samples obtained at 48 and 72 h; 300 and 600 μM coumarin treatment resulted in an increase in the protein band density of the samples. Figure 4 shows the mean molecular weight data of the total proteins formed by

Table 3. Primers used in RAPD studies, primer sequences, DNA molecular weights obtained from each primer, total number of amplified bands, number of monomorphic and polymorphic DNA bands

Primer Name	Primer 5' → 3'	Sequence	Biggest DNA Band	Smallest DNA Band	Total Amplified Band	Polymorphic DNA Number	Monomorphic DNA Number
OPA 01	CAGGCCCTTC		748	372	11	1	7
OPA 02	TGCCGAGCTG		1484	250	8	5	3
OPA 03	AGTCAGCCAC		832	392	9	3	5
OPA 04	AATCGGGCTG		635	635	3	–	2
OPA 05	AGGGGTCTTG		1524	170	16	3	9
OPA 06	GGTCCCTGAC		1754	187	14	7	1
OPA 07	GAAACGGGTG		1785	317	8	7	1
OPA 08	GTGACGTAGG		673	254	8	4	4
OPA 09	GGGTAACGCC		1324	346	10	5	5
OPA 10	GTGATCGCAG		1400	400	11	4	4
Total					97	39	41
Polymorphism					48.75%		
Monomorphism					51.25%		

SDS-PAGE at 48 and 72 h after administration in the control group and treated *L. culinaris* seeds. Coumarin treated *L. culinaris* seeds. Each sample subjected to separate electrophoresis was evaluated by comparing the band profiles with the controls. It was observed that increased concentrations of coumarin caused both polymorphic bands and increased or decreased band densities in protein profiles of *L. culinaris* seeds. An increase was found in proportion to the increasing concentration in the mean molecular weight data according to the obtained data. The data found were statistically significant.

The distance values between the dendrogram and the treatment groups obtained by separate evaluation of the protein band profiles obtained by SDS-PAGE analysis after 48 and 72 h in the control group and Coumarin treated *L. culinaris* seeds were shown in Fig. 5. Seeds treated and untreated in Dendrograms were composed of two main clusters. For both time treatments; The treatment of 600 μM of coumarin showed separate branching while the control group was in the same branch.

From incorporation coumarin on *L. culinaris*, FTIR analyzes were carried out with the aim of specifying the characteristic bonds of products (see Fig. 6 for details (300 μm coumarin and 600 μm coumarin). From the FTIR spectra, there were characteristic peaks of functional groups from lactone (C=O) in the band 1630–1780 cm^{-1} and CH_2 and CH_3 vibrations between 2910 and 1980–2170 cm^{-1}, aromatic and aliphatic respectively. Characteristic aliphatic C=C double bonds from lactone ring around 1524 cm^{-1} were clearly visible. The light swelling characteristic of –OH vibrations could also be as seen in the spectrum from 3290 to 3310 cm^{-1}. Sharp peaks at 1140–1415 cm^{-1} were characteristic peaks due to C–O vibration. Finally, the presence of C–H groups in the structure was evident straight-out from around the 150–950 cm^{-1} absorber bands. It is clear from the Fig. 6 that increasing the amount of coumarin, caused an increase in aliphatic C=C vibrations. At the same time, the C-0 groups and C–O vibration were also sharpened. Total spectra of metabolites are shown in Fig. 6.

DISCUSSION

The results of genotoxicity can be revealed by qualitative and quantitative evaluation of the samples that were treated and untreated. Reduced or increased

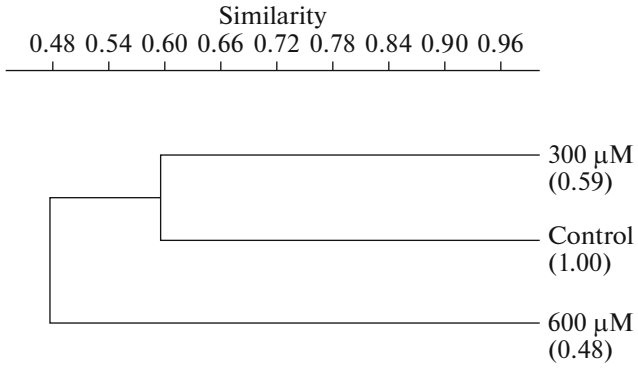


Fig. 2. Dendogram showing the degree of DNA fragmentation on *L. culinaris* roots of 300 and 600 μM treatments. Note: the numbers in brackets indicate the degree of DNA fragmentation calculated for each treatment group relative to the control.

Table 4. The number of bands in control and molecular sizes (base pair, bp) of disappearance (–) and/or appearance (+) of DNA bands for all primers of pesticide-treated germinated root tips of lentil using UVI soft image analyzer software

Primer	Control		300 μM	600 μM
OPA 01	4	+	0	0
		–	0	484
OPA 02	3	+	0	1484; 250
		–	589; 447	589
OPA 03	4	+	0	0
		–	611; 474; 406	0
OPA 04	1	+	0	0
		–	0	0
OPA 05	6	+	0	0
		–	1524; 1134	170
OPA 06	3	+	447	1035;786;669;447;382;187
		–	0	0
OPA 07	3	+	873; 658; 430	0
		–	1785; 1111; 317	1785
OPA 08	4	+	0	0
		–	673;400	673; 400
OPA 09	5	+	0	0
		–	1324; 848; 489	1324; 848
OPA 10	3	+	418	1400; 418
		–	457	0
Total	37		16(–) 5(+)	7(–) 10(+)
Grand Total			21	17

PCR band density is an important parameter in molecular systematic studies of the evaluation of lost and emerging PCR bands and analysis of the general structure of the population. Polymorphism in RAPD bands may be explained as a marker of genomic DNA chain damages, such as point mutations, genetic and chromosomal rearrangements, deletion and insertion. (Atienzar et al., 2002; De Wolf et al., 2004). It was found that the total number of new DNA bands increased significantly with increasing concentrations of coumarin. Loss of bands can be caused by DNA damages (single strand breaks, double strand breaks, modified bases, baseless strands, oxidase bases, DNA bridges, DNA-protein cross links) stemming from coumarin or by mutations involving dot and/or large chromosomal regions in genomic DNA (Atienzar et al., 1999). During the PCR reaction, the loss of the band may have occurred due to the absence of primer binding to the damaged regions of the genomic DNA or the abandonment of the Taq DNA polymerase enzyme reaction encountered with the damaged DNA region (Atienzar and Jha, 2006). The formation of new bands can be attributed to the formation of new primer binding sites by point mutation or the presence of homologous recombination and/or deletions/ insertions (Atienzar et al., 1999, Atienzar and Jha, 2006). It was determined that the increase in the concentration of coumarin caused an increase in genetic distance compared to the control group. According to the degree of DNA fragmentation, an increase in the level of DNA damage was also determined according to coumarin concentrations. According to the PAST analysis result 600 μM coumarin treatment causing the most destruction was clustered farthest from the control group. Changes in the RAPD profiles relative to the control group of the samples exposed to toxic agents were determined as the genomic template stability (GTS, %) for each primer (Atienzar et al., 1999). According to the results obtained from this study; there was a decrease in genomic template stability in increasing concentrations of coumarin. The departure of the GTS values from the control group due to increased dosing can be explained by the fact that doses of the administered (EC50 and 2 × EC50) coumarin may cause mutations. Genomic instability can be attributed to increased reactive oxygen species caused by the stress and forming genomic DNA damages such as point mutations, genetic and chromosomal regulation, deletion and insertion.

It is done to determine the possible protein profile changes that occur in SDS-PAGE protein control group and in coumarin-administered *L. culinaris* seeds and to determine total soluble protein quantities. When the average molecular weight data of the total proteins formed by SDS-PAGE were compared after 48 and 72 h in the control group and in the seeds of

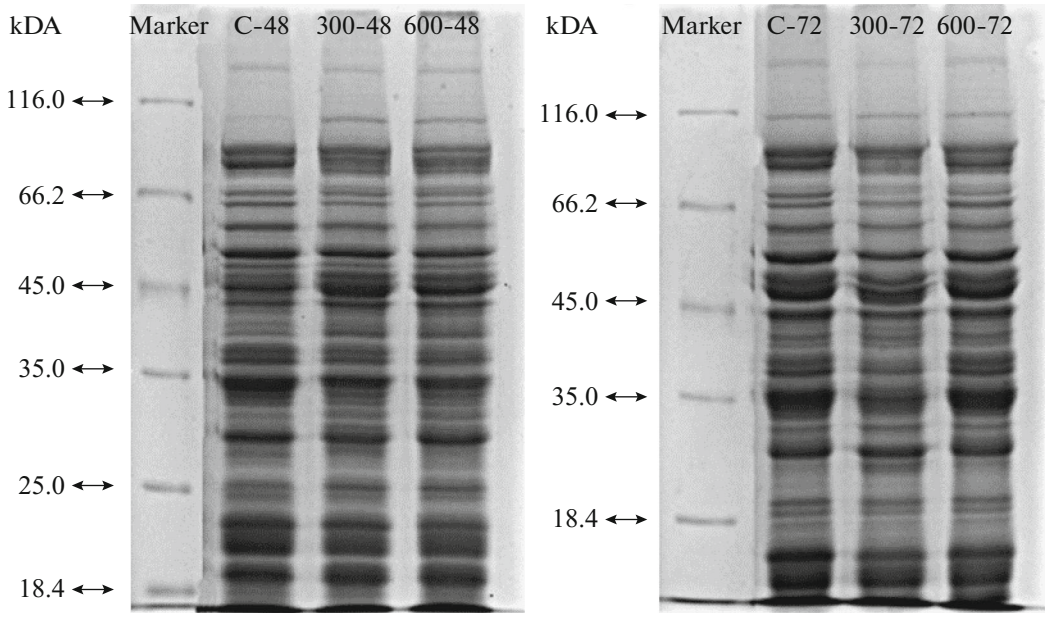


Fig. 3. Protein band profiles formed in the control group and coumarin treated *L. culinaris* seeds after 48 and 72 h in SDS-PAGE (C-48; Control 48 h, 300 to 48, 300 μM coumarin 48 h, 600-48; 600 μM coumarin 48 h, C-72, Control 72 h, 300-72, 300 μ coumarin 72 h, 600-72, 600 μM coumarin 72 h).

L. culinaris treated with Coumarin, it was determined that increased concentrations of coumarin caused both polymorphic bands and increased or decreased band densities in the protein profiles of *L. culinaris* seeds. The increase in the average molecular weight was found to be increased in proportion to the concentration obtained. When the distance between the dendrogram and the treatment groups obtained by separate evaluation of the protein band profiles obtained as a result of SDS-PAGE analysis is checked after 48 and 72 h coumarin treated *L. culinaris* seeds and the control group were in the same branch at 300 μM while the 600 μM coumarin administration showed separate branching. The change in the SDS-PAGE profiles in very small quantities and the observation of different band intensities in the produced protein bands can be expressed as the amount of protein may have changed. The changes in soluble protein quantities and protein profiles can be attributed to the stress of seeds, and thus to physiological, biochemical genetic changes and to the effects stress proteins that occur.

The accumulation of lipid peroxidation end-products, such as lipid aldehydes (Barun et al., 2015; Yuksel and Aksoy, 2017) may have caused the addition of coumarin. The presence of such products may be signs of changes in the structure and function of cell membrane because of readily undergoing peroxidation of unsaturated lipids. This result may be due to the production of free radicals that stem from dysfunction of both mitochondria and chloroplast. These two organelles that encompass electron transport chain significantly produce reactive oxygen species (ROS) and nitric oxide (NO) in plants. The attack of ROS to all lipids in the chloroplast and mitochondrial membrane may cause weakened oxidative phosphorylation. The inhibition of oxidative phosphorylation together with the use of energy during the development of the plant hinders the cell's capability to maintain energy levels, which in turn may lead to mitochondrial and chloroplast damage. This finding might support the claim that, when compared to other techniques, FT-IR spectroscopy may present a more valuable approach to investigate the presence of lipid peroxidation in a sim-

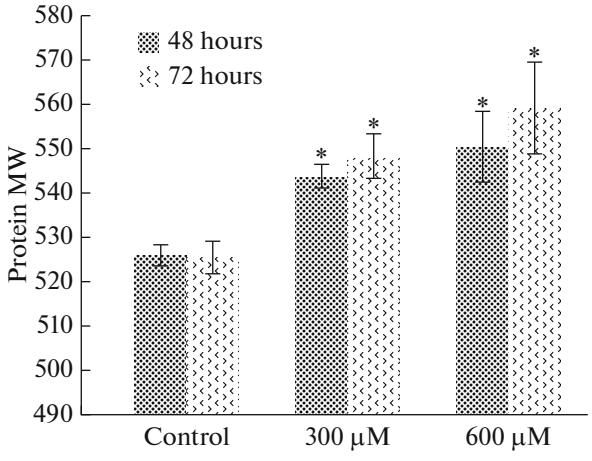


Fig. 4. Mean Molecular weight (MW) data for the total proteins produced by SDS-PAGE after 48 and 72 h in the control group and in the seeds of *L. culinaris* treated with Coumarin. (* The values are significantly different at $p < 0.05$ compared with the control group.)

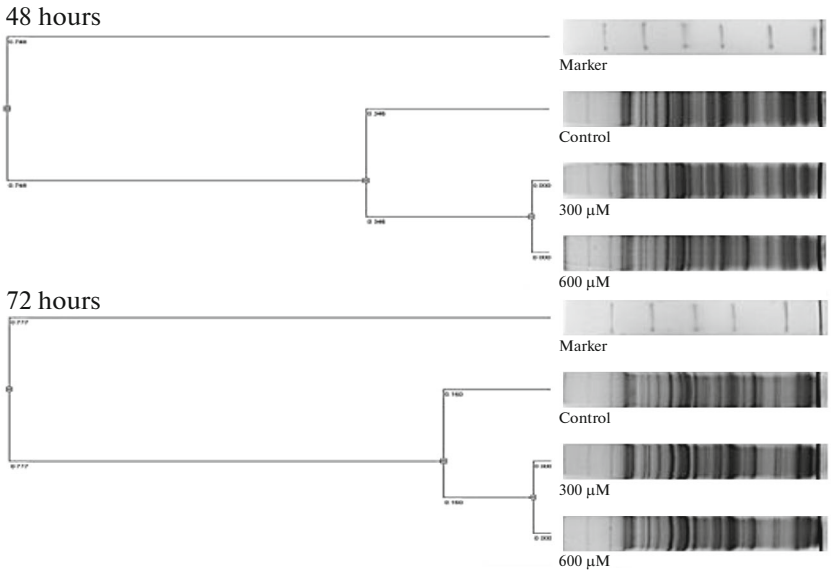


Fig. 5. Dendrogram obtained by separate evaluation of protein band profiles which were obtained by SDS-PAGE at 48 and 72 h after seeding in control group and Coumarin treated *L. culinaris* seeds.

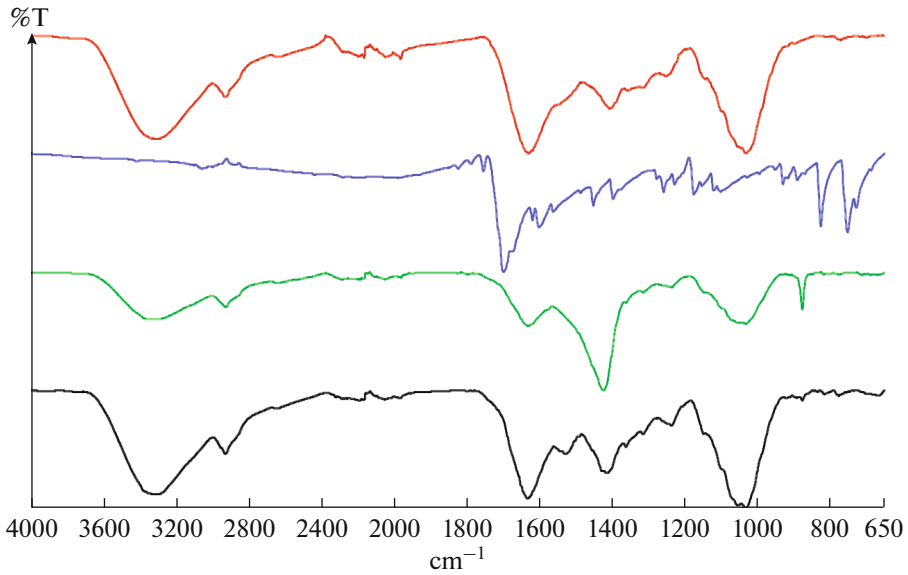


Fig. 6. The spectral investigation of the variations in the frequencies and peak areas of the vibrational modes from primary metabolites of *L. culinaris.* Redline Control; blueline 0.5; coumarin; greenline 300 μM coumarin; blackline 600 μM coumarin

ple and sensitive way (Ana et al., 2011). Abiotic and biotic stress factors may lead to changes in due to the physiological signals, their effects on protein synthesis and the direct toxic effects of some certain chemicals.

CONCLUSIONS

Most of the studies with cytotoxic and genotoxic focus examine the plants in terms of the effects of environmental pollutants. The results of the studies that utilize eukaryotic organisms such as plants are very important for the determination of possible pollutant effects, especially for humans. The results of this study, based on the data collected by examining the cytotoxic, genotoxic and biochemical effects of coumarin on *L. culinaris*, demonstrate that coumarin might have some allopathic effects. This study will motivate further research to examine the effects of plant secondary metabolites. It is believed that the biochemical and genetic changes of *L. culinaris* caused by coumarin can be used as a convenient and useful biomarker in the toxicology of environmental pollutants.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

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