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Analysis of the effects of coumarin on *Lens culinaris* Medik by some biochemical parameters using real-time polymerase chain reaction

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ABSTRACT

This study aimed to reveal the effects of coumarin on *Lens culinaris* Medik. The germinated and ungerminated seed counts of the experimental groups were determined and the EC_{50} value was calculated by probit analysis. In biochemical studies, catalase and superoxide dismutase enzyme activities were investigated together with lipid peroxidation and hydrogen peroxide quantities. The results demonstrated an increase in the amount of malondialdehyde, a measure of lipid peroxidation, a decrease in the amount of H_2O_2 and EC_{50} value in the CAT activity and an increase in the $EC_{50\,x\,2}$ value. In real-time PCR analysis, three different genetic expressions related to abiotic stress (CAT, Cu / Zn SOD and Mn SOD gene expression) were examined. It was determined that coumarin caused genotoxic and biochemical damage on *L. culinaris*.

Key words: Catalase, *L. culinaris*, Coumarin, RT PCR, Superoxide dismutase.

INTRODUCTION

Coumarin and derivatives are widely available as free or glycosides in plants and are natural chemical molecules that have gained importance in recent years due to their various biological activities. Their pharmacological properties and antioxidant properties have been investigated for years. Important effects of coumarin in plant biochemistry and physiology such as antioxidant action, enzyme inhibitor and toxic substances prevention have been investigated (Kostova, 2005). The effects of antibiotics, anticoagulants, anticancer, anti-inflammatory agents and bacteriostatic agents in coumarins and their derivatives make them an important substance in biology and medicine research El-Khatib*et al.* (2007). Coumarin is used in the perfume industry due to its pleasant smell and to mask unpleasant odors. Although it has been used for some time in the food industry due to its pleasant smell, this field has been abandoned due to its toxic character in research on animals (Guenther, 1950). Coumarins on the surface of plant organs play two important ecological roles in terms of protecting plants and communicating with plants' circles. Depending on the species and concentration, the coumarins in plants promote or inhibit plant growth, affecting cell differentiation and auxin metabolism (Yuksel and Aksoy, 2017).

In order to determine some biochemical and genetic changes which are caused by the plant material used in the agriculture, medicine, paint and cosmetics industry of our work, finalizing the laboratory analyzes will be able to enter the plant; potential oxidative stress traces, plant stress response, lipid peroxidation and plant growth. The findings obtained at the end of the study will be a general evaluation, as well as the results of the analyses will provide insight into the advantages and disadvantages in the areas where coumarin is used to producers and consumers.

MATERIALS AND METHODS

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 hours of treatment (Finley and Tattersfield, 1952). The root tips that were 0.5 mm or higher were established 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.

Measurement of lipid peroxidation in the samples was determined by measuring malondialdehyde (MDA) content according to the method of Ohkawa *et al.* (1979). The absorbance of the supernatant at 410 nm was measured and the H_2O_2 concentration was calculated from the prepared standard curve.

CAT activity determination in plant tissue homogenates was determined according to Aebi (1984). An enzyme unit was calculated as the amount of enzyme destroying 1 µmol H_2O_2 per minute. The SOD activity in the samples was photochemically determined according to the method of Beauchamp and Fridovich (1971).

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RNA isolation from plant samples was performed according to the QIAGEN RNeasy plant mini kit protocol. The amount of RNA was determined on a thermo scientific NanoDrop ™ 1000 Spectrophotometer with spectrophotometric measurements. Real-time PCR cycles were performed using the Bio-RAD CFX Connect™ real-time PCR detection system thermal cycler.

In the roots obtained from the experimental groups, the expression of the genes in CAT, Mn SOD and Cu / Zn SOD genes was analyzed by real-time PCR (Table 1). The sequences of the genes were taken from the thesis study named effect of drought and salt stresses on the gene expression levels of antioxidant enzymes in lentil (*Lens culinaris* M.) (Aksoy, 2008). The gene expression levels were determined using the obtained data using the relative expression software tool (REST ©). The percentage of GC content of DNA has a significant effect on its Tm. Because the higher the percentage of GC content, the higher its Tm Jain *et al.* (2017).

The protocol that was optimized and used in real-time PCR is as follows; 1000 ng of cDNA, 5 µl of forward primer, 5 µl of reverse primer, 25 µl of GoTaq® qPCR master mix, 1X, final volume of 50 µl. The program in which the real-time PCR reaction was performed as pre-denaturation 95°C 2 minutes 1 cycle, 40 cycles of 95°C 15 seconds melting curve analysis 60- 95°C continuous reading 1 cycle. Statistically significant differences between the groups were compared using both one-way analysis of variance (ANOVA) and probit analysis. The data is displayed by mean ± standard deviation (SD) and p-values less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

EC_{50} values were found at 278 ppm for coumarin (Table 2). This was later adapted to a 300 ppm coumarin concentration. Therefore, all experimental procedures were carried out using coumarin concentrations of 300 ppm (EC_{50}) and 600 ppm (2x EC_{50}).When the amounts of MDA formed after 72 hours in the control group and coumarin applied L. *culinaris* roots were evaluated, the amount of 2,04 nmol/g MDA in the control group was determined. Increasing values were determined relative to the control at a concentration of (3,74 nmol/g) and 600 ppm of coumarin (4,30 nmol/g) at a concentration of 300 ppm of coumarin (Fig 1).

Table 1: Real-time PCR Primer sequences.

Primer	Sequence (5′→3′)	Primer Length(bp)	Tm (°C)	GC Content (%)
CAT F	CCTGTCATTGTGCGTTTCTC	20	47	50
CAT R	CTCCCACCTTAATGGCCTCT	20	49	55
Mn-SOD F	GGCGGAGGTCATATTAACCA	20		
Mn-SOD R	AAGCCACACCCATCCAGAC	19	48	58
Cu/Zn-SOD F	GCTTCCATATCCATGCCTTG	20	47	50
Cu/Zn-SOD R	AGCTACTCTGCCACCAGCAT	20	49	55
Lens- GAPDH F	TGTTGAGGGTCTCATGACCA	20	47	68
Lens- GAPDH R	CGCTGGGAATGATGTTAAATG	21	45	43

(F: Forward, R: Reverse)

Table 2: EC_{50} 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: Purity and quantitation of isolated RNAs.

Treatment	Concentration(ppm)	A_{260}	A_{280}	$A_{260/A280}$	$A_{260/A230}$	RNA ng/ml
Hoagland	0	25,587	12,099	2,11	2,26	1023,5
Coumarin	300	23,105	11,042	2,09	2,26	924,2
Coumarin	600	17,61	8,367	2,1	1,99	704,4

When the amounts of H_2O_2 formed after 72 hours in the control group and coumarin-treated *L. culinaris* roots were evaluated, a decrease in the concentration of 300 ppm coumarin(14,48 µM/g) was observed while 22.94 µM/g $H_2O_2$was observed in the control group (Fig 2). Moreover, increasing values compared to control in 600 ppm coumarin concentration (40, 15 µM/g) were determined.

At the end of 72 hours, when the CAT activity was different between the samples, it was observed that 600 ppm of coumarin application (4878, 28 U/ml) increased when 300 ppm of coumarin application (2998, 15 U/ml) decreased compared to control group (5163, 21 U/ml) (Fig 3).

At the end of 72 hours, when SOD activity was different between the samples, in 300 ppm (0, 40 U/mg) and 600 ppm of coumarin (0, 13 U/mg) it was both decreased compared to the control group (0, 51 U/mg) (Fig 4).

RNAs from the control and coumarin stressed samples were used for cDNA synthesis. Table 3 gives the quantities of the best occurring and the samples used in cDNA synthesis.

The level of expression of the CAT gene obtained by the REST program is given in Table 4. According to control the expression level of the CAT gene was reduced by in 300 ppm and in 600 ppm coumarin administration. According to this data, the difference between 300 ppm and 600 ppm coumarin applications.

The level of expression of the Cu/Zn-SOD gene obtained by the REST program is given in Table 5. According to the control, the expression level of Cu/Zn-SOD gene decreased in 300 ppm and 600 ppm coumarin administration.

The level of expression of the Mn-SOD gene obtained by the REST program is given in Table 6. The expression level of the Mn-SOD gene was increased in300 ppm coumarin and decreased in 600 ppm coumarin administration.

Table 4: CAT gene expression level obtained by REST program.

Time (Hours)	Applied dose	CAT gene expression level
72	300 ppm coumarin	-37,635
	600 ppm coumarin	-17,509

Table 5: Expression level of Cu/Zn-SOD gene obtained by REST program.

Time (hours)	Applied Dose	Cu/Zn-SOD Gene Expression Level
72	300 ppm coumarin	-7,042
	600 ppm coumarin	-3,211

Table 6: Expression level of Mn-SOD gene obtained by REST program.

Time (Hours)	Applied dose	Mn-SOD gene expression Level
72	300 ppm coumarin	1,928
	600 ppm coumarin	-3,673

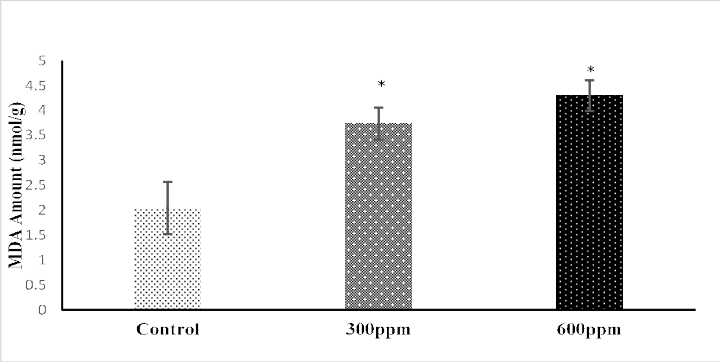


Fig 1: Amounts of MDA formed after 72 hours in the control group and *L. culinaris* roots of coumarin applied. * When compared to the control group, it is significantly different at p <0.05 level.

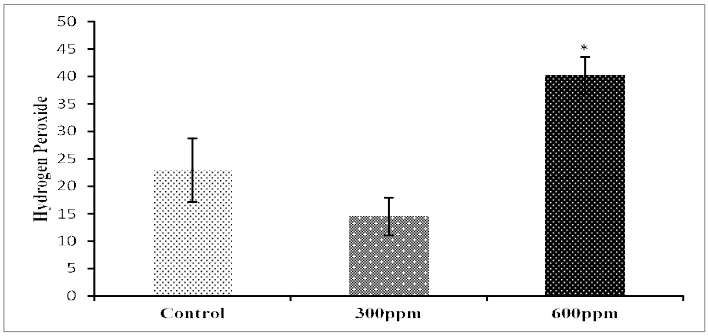


Fig 2: Quantities of H_2O_2 formed after 72 hours in the control group and coumarin applied *L. culinaris* roots were significantly different at the p <0.05 level compared to the control group.* When compared to the control group, it is significantly different at p <0.05 level.

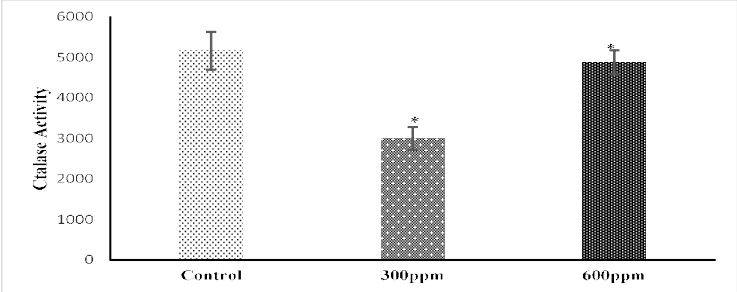


Fig 3: Values of CAT activity after 72 hours in the control group and coumarin applied *L. culinaris* roots were significantly different at the level of p <0.05 when compared with the control group.* When compared to the control group, it is significantly different at p <0.05 level.

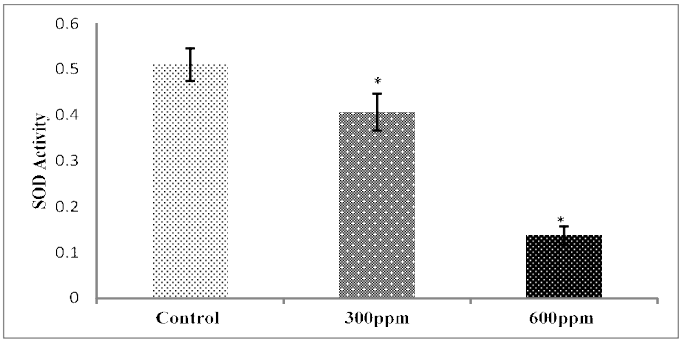


Fig 4: Values of SOD activity after 72 hours in the control group and *L. culinaris* roots of coumarin applied are significantly different at p <0.05 level compared to the control group.* When compared to the control group, it is significantly different at p <0.05 level.

Reactive oxygen species oxidize the lipids in the cell membranes by oxidizing the lipids, thereby reducing the selective permeability properties of the membranes. Intermediate malondialdehyde (MDA), which occurs in the oxidation reactions of lipids in cell membranes as a result of plant stress factors, is one of the decomposition products of unsaturated fatty acids in the bio membrane (Wise and Naylor, 1987). The resulting MDA affects the ion exchange in the cell membranes, causing cross-linking of the compounds in the membrane. This causes negative consequences such as alteration of ion permeability, increase or decrease of enzyme activities. Because of this property, MDA can react with the nitrogen-bearing groups of DNA; therefore it can be said that they are mutagenic, genotoxic and carcinogenic in nature. When the amount of MDA formed after 72 hours in the control group and in the coumarin-treated *L. culinaris* roots was evaluated, it was determined that the amount of MDA increased in the coumarin concentrations, which were increased compared to the control group. Lipid peroxidation causes cell membrane integrity to be destroyed and membrane permeability to be deteriorated. Influence of the intracellular and extracellular transit mechanisms of Ca and Na ions may cause changes in ATP consumption and affect the cell energy mechanism Kalender *et al.* (2002). The increase in intracellular calcium ions leads to the activation of enzymes such as proteases and phospholipases that can cause more damage to proteins and lipids. This can lead to enzyme inactivation or activation, which can lead to DNA conformation deterioration and cell death. The damage to the cell membrane of these chain reactions is irreversible. Ahrabi *et al.* (2011) investigated the biochemical and physiological responses of coumarin on canola. Despite the increase in antioxidant capacity of canola felids, lipid peroxidation in coumarin presence has increased. In our study, the amount of hydrogen peroxide and lipid peroxidation were significantly increased in plants under 600 ppm of coumarin stress.

H_2O_2 acts as a regulator that plays a key role in different physiological processes Dar *et al.* (2007) including senescence , respiration and photosynthesis (Ren *et al.*, 2014), stoma movement Hua *et al.* (2012), cell cycle Livanos *et al.* (2012). In our study, when coumarin stress was applied to the lentil plant, it was observed that the content of H_2O_2 in the root tissue increased significantly, especially at a concentration of 600 ppm coumarin. The reduction in the amount of hydrogen peroxide at 300 ppm may be due to the introduction of antioxidant systems. However, as this amount increases, the plant may be unable to cope with stress. In addition, the linear increase in the amount of MDA and H_2O_2 at a concentration of 600 ppm of coumarin supports the literature. (Saleh and Kebeish, 2017).

CAT is formed by β-oxidation of fatty acids and is responsible for removing H_2O_2 produced by oxidase enzymes

involved in photorespiration and purine catabolism (Gill and Tuteja, 2010). Formation of a stress-ending CAT inhibitor, the incorporation of enzyme synthesis, and the structural deformation of enzyme subunits, which are involved in the disruptions leading to enzyme inhibition (MacRae and Ferguson, 1985). Abenavoli *et al.* (2006) found that coumarin reduced the activities of enzymes such as APX, CAT, and suppressed the transcription of molecular chaperones located in the secretory pathways. Saleh and Kebeish (2017) have shown that coumarin inhibits SOD, CAT and ascorbate peroxidase activities. In this study, CAT activity decreased in the application of 300 ppm coumarin and 600 ppm in the coumarin application. It is stated that the CAT enzyme has a significant effect when the formation of H_2O_2 is increased. Also related to the increase in hydrogen peroxide production. They use hydrogen peroxide as substrate in catalysis catalyzed reactions. The amount of hydrogen peroxide is also in agreement with the increase in CAT activities. The increase in CAT activity may be due to the activity of protein synthesis, and it can be said that the result of the stress applied is the result of the increased amount of hydrogen peroxide.

SOD is the first defense mechanism against the harmful effects of reactive oxygen species (Gill and Tuteja, 2010). The highly reactive superoxide anion causes lipid oxidation as well as other biochemical components. Some investigators have reported that superoxide anion causes membrane damage, cellular toxicity and single chain fractures in DNA, lipid peroxidation Montero-Tavera *et al.* (2017) and Asada, (2006). Coumarin and its derivatives inhibit SOD, CAT and ascorbate peroxidase activities in wheat seeds Abenovoli *et al*. (2006). When the difference of SOD activity between the samples was evaluated at the end of 72 hours in our study, it was determined that SOD activity decreased in 300 ppm coumarin administration and 600 ppm coumarin administration according to the control group. Decreased SOD activity may be evidence that this enzyme is not rigid enough against coumarin or coumarin substance may have caused damage to the enzyme synthesis pathways.

Real-time PCR results showed that the expression level of CAT gene obtained by REST program decreased both in the application of 300 ppm coumarin and in the application of 600 ppm coumarin compared to the control. Similarly, the expression level of the Cu / Zn-SOD gene decreased in both 300 ppm coumarin and 600 ppm coumarin administration compared to the control. Expression level of Mn-SOD gene was found to be upregulated at 300 ppm coumarin application and downregulated at 600 ppm coumarin application to the control. Real-time PCR monitoring of the behavior of the genes of interest under stress conditions allows the determination of the functions of genes and the identification of stress-related genes Wu *et al.* (2004).Studies have shown that increases in the gene expressions of SOD enzymes have important roles in counter acting biotic and abiotic reactive oxygen species, in coping with stress and in contributing to maintaining vitality under stress conditions of plants. Many plant species have been reported to exhibit increases in SOD enzyme activity under various stress conditions Gapinska *et al.* (2008). The changes in the activities of the enzymes involved in the expression of the genes studied in our study and related genetic products have produced parallel results. Literature studies did not reveal any molecular studies performed by real-time PCR, which showed the effect of coumarin on *L. culinaris* or another plant. In this respect, our study shows that it is the first study to be performed with real-time PCR technique on *L. culinaris* roots by exposing coumarin stress.

CONCLUSION

This study will be an important source of data for future studies since it will allow the determination of the cytotoxic, genotoxic and molecular effects of the secondary compounds, coumarin, on the *L. culinaris.* The determination of the effects of allelopathic chemicals will also contribute to the determination of the toxic effects of the coumarin compound on eukaryotic organisms and to the safety and efficacy of both healthy and safe substances for consumers and the environment.

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