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CHANGES OF PHENOLIC COMPOUNDS IN TOMATO ASSOCIATED WITH THE HEAVY METAL STRESS

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Abstract

Heavy metals have restricted the plant regular life cycles affecting the plant primer and seconder metabolites by biochemical and physiological pathways. Phenolic compounds considered as products of metabolic alterations have been synthesized in various numbers and typical characteristic of plants. In this study, we aimed to investigate the variations of phenolic compounds with HPLC in leaves of tomato exposed to heavy metals. The applications of Cu, Cd and Pb significantly reduced the total phenolic content, levels of caffeic, chlorogenic and vanillic acids in all treated groups except for 50 and 20 ppm of Pb for total phenolics and vanillic acid, respectively. The level of benzoic acid is generally decreased by the application of heavy metals except for Cu at 50 ppm doses. Rutin is the most abundant phenolic compound in term of quantity among to analyzed phenolics and its content decreased depending on the heavy metal doses except for 10 ppm doses of Cd. The responses of tomato under heavy metals stress resulted in lower amount of phenolic compounds. In the present study, it was showed that total phenolic content has positive correlations with caffeic acid in all treatment of heavy metals.
Keywords: Heavy metal, phenolic compound, tomato

1. Introduction

There are a broad stress factors affected the plant life cycles and plants exposed to these environmental conditions. Plant growth, development and yield have flourished when stress situations are close to optimum. The main factors that are limited plant development are abiotic stresses including salinity, drought and metal ions. These abiotic factors have changed the plant phenolic compounds (Król et al., 2014). Heavy metals accumulate in agricultural areas with various ways such as fertilizations, irrigation water, herbicides, and pesticides. The excess of heavy metals affects the major mechanisms such as nutrient distribution, photosynthesis, water usage, defense system, and seconder metabolites. Cadmium and lead are unnecessary and non-useful elements, but copper is crucial micro nutrients for plant life cycles (Rascio & Navari-Izzo, 2011). Plant growth medium comprises various elements and compounds, and the high levels of these metals change the phenolic acids of plants. The plant responses have varied depending on the kind of elements and the presence of heavy metal mixtures (Elguera et al., 2013). Heavy metals change the responses of plant by molecular, biochemical and physiological pathways. The adaptability of plants to unfavorable conditions changes depending on the respond of plant defense systems. The primary responses of plants under the stress conditions are the formation of reactive oxygen species, and heavy metals trigger the oxidative stress, and plant defense systems have accompanied to overcome the free radicals. The basic antioxidant system employed by plants is enzymatic defense mechanisms such as ascorbate peroxidase, catalase, glutathione reductase, glutathione S-transferase, guaiacol peroxidase, and superoxide dismutase. Also, plants have synthesized various seconder metabolites including phenolic acids, flavonoids, coumarins and lignins (Gratão et al., 2005; Sannchez-Rodriguez et al., 2011).

Seconder metabolites have various functions and involved in response of higher plants to different environmental factors such as pathogen attack, extreme temperature, nutrient imbalance, drought, salinity, heavy metal. A typical characteristic of plants is to synthesize a wide variety of the seconder metabolites composed of

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various numbers of compounds such as phenylpropanoids, flavonoids, tannins and lignins. Phenolic compounds are considered as products of metabolic alterations and one of the main groups of secondary metabolites (Mustafa & Verpoorte, 2007; Król et al., 2014). Environmental factors can define the synthesis, profile, chemical composition and level of phenolic compounds through increasing or decreasing in plants. Also, phenolic contents of plant depended on plant age, genotype, tissues, season, and exposure time to stress (Sartor et al., 2013; Waśkiewicz et al., 2013). These phenolic performs a great number of biological functions such as being a structural component of cell walls and regulation of auxin transport. Phenolic compounds have one or more aromatic ring directly bonded hydroxyl group. Plant phenolics have been formed from phenylpropanoids or acetate/malonate pathways. Plants have the ability to synthesize various phenolic compounds that are unnecessary in the initial processes of life cycle but they have vital importance because of their interaction with the environmental situations. Abiotic stresses such as heavy metals and excessive nutrients affect accumulation of phenolic metabolites in plant tissues. The content of phenolic compounds is involved to various enzymes such as chalcone synthase, phenylalanine ammonia-lyase and phosphoenolpyruvate (PEP)-carboxylase which are responsible for the synthesis of phenolics (Bhattacharya et al., 2010; Cheynier, 2012; Bautista et al., 2016).

Phenolic compounds have different chemical structures characterized by hydroxylated aromatic rings. Benzoic acid (BA) is aromatic carboxylic acid that serves as a precursor for a range of essential phenolic compounds and natural product having a crucial role in plants. It provides carbon skeletons for a great number of specialized metabolites and biosynthesis begins from the shikimate pathway (Widhalm & Dudareva, 2015). Caffeic acid (CA) is cinnamic acid derivatives ubiquitously distributed in several plant varieties. This phenolic compound is substrate of polyphenol oxidases and may undergo oxidation in plants under the environmental conditions. CA has been accumulated in plant cell wall constitutes of the stress conditions in plants (Gülçin, 2006; Batish et al., 2008). Chlorogenic acid (CGA) is phenolic generated by esterification of cinnamic acid such as caffeic and ferulic acid, and produced through the phenylpropanoid pathways. CGA are present in all nearly all plants and have been related with responses against to environmental stressors (Farah et al., 2008; Ncube et al., 2014). Vanillic acid (VA) is the oxidized form of vanillin and one of the phenolic acids identified in many plants. It may be used as a natural antioxidant, and decrease peroxidation of lipids and significantly restored enzymatic antioxidants (Gitzinger et al., 2012; Calixto-Campos et al., 2015). Rutin is one of the bioactive flavonoids with physiological properties in plants. Rutin content in various herbs has been differed depending on environmental factors and growth stages of plants (Zhao et al., 2015).

Phenolic profiles of cereals have been intensively investigated in cereal grains, medicinal and aromatic plants due to their antioxidant activity and potential benefits, in recent years. Also, antioxidant enzymes of several plants subjected to biotic and abiotic stresses have been studied by plant physiologist (Gill & Tuteja, 2010; Farooq et al., 2013; Grace et al., 2014; Bajoub et al., 2015). Moreover, total and individual phenolics of plants which they have not exposed any special stresses have been working by chemists (Bajpai et al., 2005; Djeridane et al., 2006; Manguro & Lemmen, 2007; Dudonne et al., 2009; Zhang et al., 2011). In recent years, although the relationships between the abiotic stress and phenolic compounds have been newly investigated, there are no enough studies carried out individual phenolic compounds in plants exposed to environmental stress (Kováčik et al., 2009c; Kisa et al., 2016). In the present study, it is evaluated the effect of cadmium, copper and lead applied in different doses on total and individual phenolic compounds of tomato. Also, we want to indicate the interaction between total phenolic levels and individual phenolics in terms of studied parameters.

2. Materials and methods

2.1. Plant growth conditions

Tomato (*Lycopersicon esculentum*) seedlings were cultivated in pots having a 12 kg equal mixture of garden soil and peat in the greenhouse conditions with a randomized plot design. The experiment was carried with three replications with 16:8 photoperiods, at 25±2˚C, and B, Ca, Fe, Mg and Zn were treated in 20 in ppm for plant development. When plants reached the sufficient size about three weeks later, they were exposed to 10, 20 and 50 ppm of Cd, Cu and Pb from $CdCI_2$, $CuSO_4$ and $Pb(NO_3)_2$ as heavy metal sources, respectively. The treatments were performed triplicate with an interval of two days, one time a day in the mornings. The tomato leaves were harvested two weeks after the heavy metal exposures, and samples were kept at -80˚C for chemical analysis.

2.2. Plant phenolic compounds extraction

The tomato leaves (2g) were freshly crushed in liquid nitrogen with mortar and pestle, phenolic compounds were extracted from fine powder with chloroform-methanol (1:4), and sonicated with ultrasonic bath for 20 min at

room temperature. The suspension was centrifuged at 5000xg for 10 min, the supernatant was used for analysis of total and individual phenolic compounds.

2.3. Total phenolic content determination

The total phenolic contents of tomato leaves were determined by using phenol reagent method described previously (Singleton et al., 1965). The extract (100 µL) was diluted with distilled water (4.5 mL) and one milliliter of Folin-Ciocalteu was added to the mixture. After the incubation for 3 min at room temperature, the mixture was added to 300 µL of 2 % Na_2CO_3, and it was kept at 25˚C for two hours. The absorbance was read at 760 nm with UV-Vis spectrophotometer (Varian, Carry-50). The results were given as milligrams of gallic acid equivalents using a standart curve prepared from GA.

2.4. HPLC analysis of major phenolic compounds

The identification of major individual phenolic compounds in leaves extracts was achieved by using high performance liquid chromatography equipment (HPLC). The extracted methanol-chloroform samples were filtered through a syringe filter (0.22 µm) and the filtrate was injected into HPLC system for analysis. Identification of phenolic compounds in samples was characterized by matching peak retention and UV spectra of chromatogram with those of known standard phenolic compounds. HPLC (Shimadzu) equipped with LC 20AT pump and DAD-M20A detector. Phenolic compounds separation was done by a reversed phase C18-EPS column (150 x 4.6 mm). The mobile phase comprised of solvent A (deionized water) and B (acetonitrile: water, 5:90) and the following gradient program was briefly performed: at 0-8 min, the A:B proportion was 10:90; at 8-29 min, 15:8; at 29-40 min, 30:70; at 40-50 min, 55:45; at 60 min, 0:0 washing and equilibration of column. The mobile phase was delivered at a rate of 1 mL min^{-1}, column oven temperature was set a 40˚C, and the absorbance read at 280 nm. Identification of phenolic compounds in samples was characterized by matching peak retention and UV spectra of chromatogram with those of known standard phenolic compounds. The original phenolics were purchased from Merck and Sigma-Aldrich, and the results were given as mg kg^{-1} in fresh weight.

2.5. Statistical analysis

The work results were performed by one-way analysis of variance (ANOVA), followed by Duncan's multiple tests using the SPPSS (SPSS 20.0 software). Significant differences in relation to the control groups were accepted if $p< 0.05$ and data were expressed mean ± SD.

3. Results

3.1. Total phenolic contents of tomato leaves

The treatment of heavy metals to the plant growth medium affected the total phenolic contents in leaves of tomato under the different concentration of Cd, Cu and Pb. The addition of Cd and Cu in plant cultivated soils significantly decreased the phenolic contents in all treated groups. The exposures of Pb reduced the phenolic contents in 10 and 20 ppm doses, but there are no significant changes at the high doses (50 ppm) compared to control plants. The decreases of total phenolic contents have showed similar reduction in leaves of tomato exposed to Cd and Cu and all results are shown in Fig. 1.

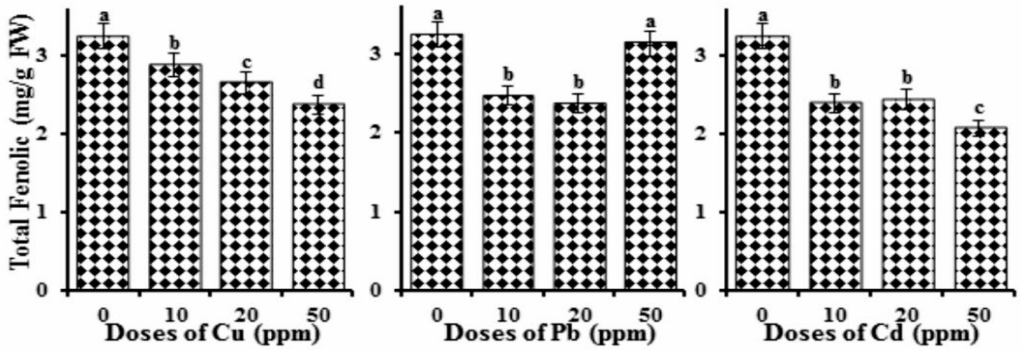

Fig 1. The effect of heavy metals on total phenolic compounds of tomato leaves. Different letter on bar were considered as a significantly different at $p \leq 0.05$. Data expressed as milligrams of gallic acids equivalents per gram fresh weight.

3.2. The effect of heavy metals on the content of major individual phenolic compounds

The effect of heavy metal on the content of benzoic acid, caffeic acid, chlorogenic acid, rutin and vanillic acid was investigated in tomato leaves under the heavy metals. The responses of the major phenolic contents of the leaves are usually decreased by the treatment of Cu, Cd and Pb. The contents of caffeic acid and chlorogenic acid significantly reduced in all application of heavy metals, but a wide difference has been occurred the applied doses of heavy metals with regard to their contents. The level of vanillic acid is significantly reduced by the all applications of Cu and Cd. However, the treatment of Pb significantly decreased level of vanillic acid at 10 and 50 ppm doses, but it remained unchanged at 20 ppm compared to controls. The content of rutin has been changed the applied doses and heavy metal types. The application of Cu (10, 20 ppm), Pb (20 ppm) and Cd (20, 50 ppm) significantly decreased the content of rutin and other doses of heavy metals slightly reduced the rutin level except 10 ppm doses of Cd. The low doses of Cd (10 ppm) slightly increased the content of rutin. Also, rutin is the most abundant phenolic compound in terms of quantity among to investigated phenolics in tomato leaves. The quantity of benzoic acid in leaves of tomato growth in heavy metal containing soil is usually decreased by the application of heavy metal except for Cu at 50 ppm doses. The variations of individual phenolic compounds of tomato discussed above are shown in Fig. 2 and 3.

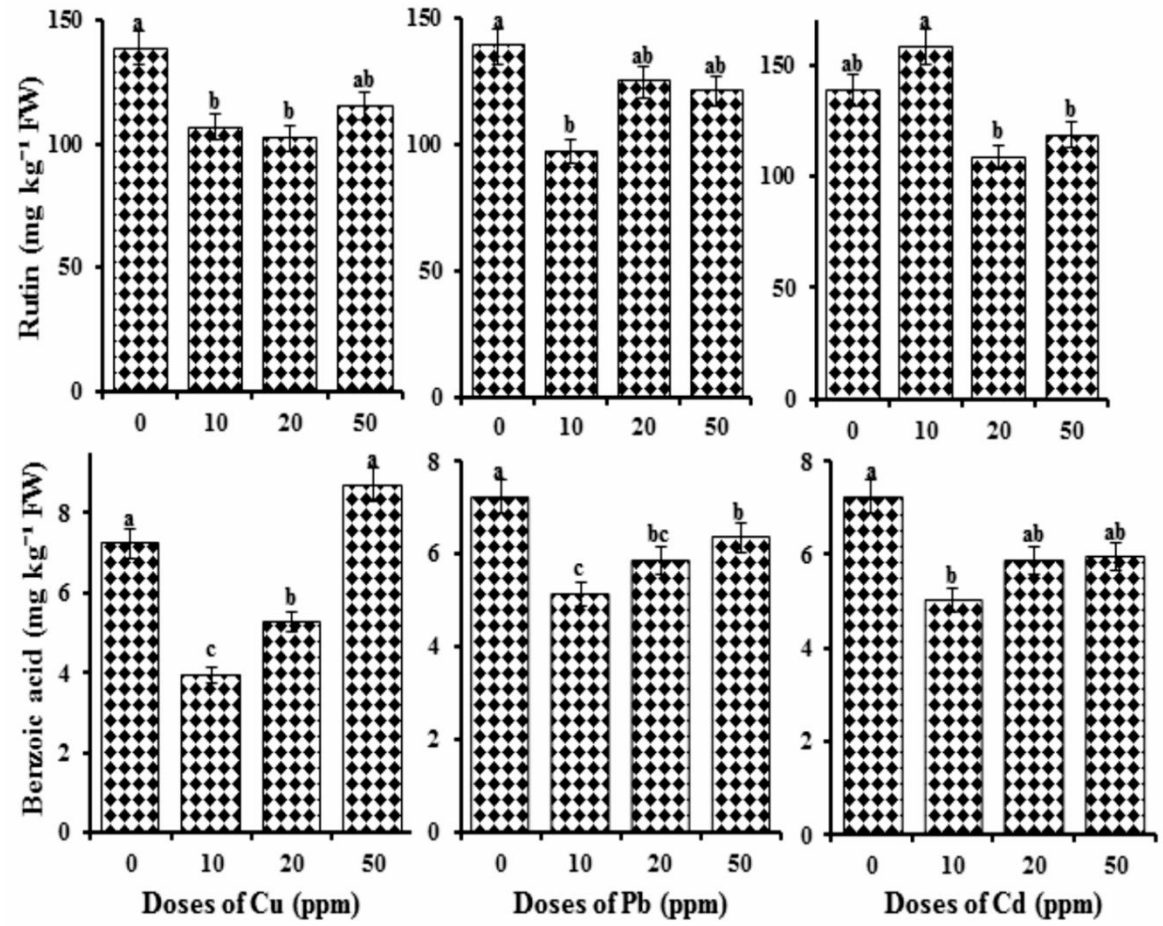

Fig 2. The level of caffeic acid, vanillic acid and chlorogenic acid of tomato leaves exposed 0, 10, 20, 50 ppm applications of Cu, Pb and Cd. Different letter on bar were considered as a significantly different at $p \leq 0.05$. Data expressed as mg kg^{-1} FW.

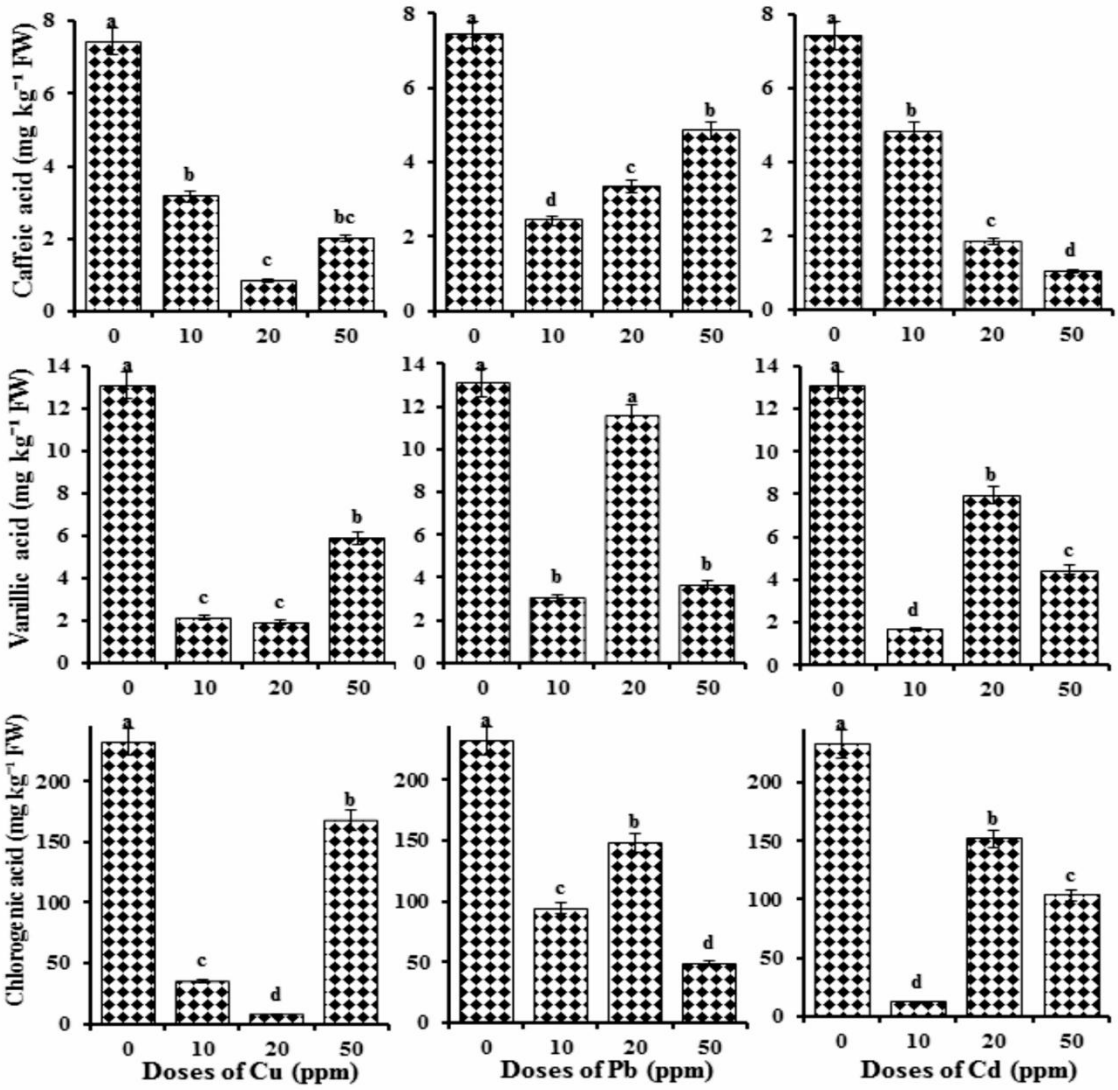

Fig 3. The level of benzoic acid and rutin of the leaves of tomato exposed 0, 10, 20, 50 ppm applications of Cu, Pb and Cd. Different letter on bar were considered as a significantly different at $p \leq 0.05$. Data expressed as mg kg^{-1} FW.

3.3. The correlation analysis for phenolic compounds of tomato

The relations between the phenolic compounds of tomato planted in Cd, Cu and Pb-containing medium was analyzed with pearson's correlation. It is indicated that total phenolics have positive correlations with caffeic acid, vanillic acid and rutin under the Cu treatment; caffeic acid, rutin and benzoic acid under the Pb application, and caffeic acid, chlorogenic acid, vanillic acid, and benzoic acid under the Cd exposures. Also, there are some positive correlation among the individual phenolic compounds under the different concentration of heavy metals and their doses, and the correlations of all results are given in Table 1.

	T. Phenolics	Caffeic acid	Vanillic acid	Rutin	Chlorogenic acid	Benzoic acid
T. Phenolics	1					
Caffeic acid	0,851**	1			**Cu application**	
Vanillic acid	0,587*	0,899**	1			
Rutin	0,643*	0,926**	0,990**	1		
Chlorogenic acid	0,314	0,752**	0,937**	0,923**	1	
Benzoic acid	-0,279	0,200	0,595*	0,538	0,785**	1
	T. Phenolics	**Caffeic acid**	**Vanillic acid**	**Rutin**	**Chlorogenic acid**	**Benzoic acid**
T. Phenolics	1					
Caffeic acid	0,886**	1			**Pb application**	
Vanillic acid	0,140	0,571	1			
Rutin	0,609*	0,854**	0,820**	1		
Chlorogenic acid	0,220	0,628*	0,903**	0,673*	1	
Benzoic acid	0,848**	0,970**	0,619*	0,912**	0,598*	1
	T. Phenolics	**Caffeic acid**	**Vanillic acid**	**Rutin**	**Chlorogenic acid**	**Benzoic acid**
T. Phenolics	1					
Caffeic acid	0,876**	1			**Cd application**	
Vanillic acid	0,849**	0,503	1			
Rutin	0,269	698*	-0,264	1		
Chlorogenic acid	0,738**	0,346	0,982**	-0,416	1	
Benzoic acid	0,779**	0,477	0,849**	-0,217	0,947**	1

Table 1. The correlations among the phenolic compounds of tomato leaves exposed to 0, 10, 20, 50 ppm applications of Cu, Pb and Cd. Correlation coefficient is significant at the 0.01 (**) and 0.05 (*) level (2-tailed).

4. Discussion

Plants have ability to synthesize very different phenolic compounds and the responses of the plants to various environmental conditions are very complex. The content of phenolics show adherence to plant species, variety, tissue, soil characteristic, climate, duration of stress, and biotic and abiotic factors. Plant phenolic compounds are regarded as secondary metabolites known to have several functions and plant responses have varied depending on the kind of element and potential presences of heavy metals in the plant growth medium (Weidner et al., 2009). Environmental stress including drought, salinity and heavy metals have been considered to main causes of secondary stress such as oxidative and osmotic stress and the generated stress have negatively affected normal growth, development and metabolism of plants. Abiotic stress can cause a decline or an increase in phenolic compounds in plants (Król et al., 2014).

In the current study, addition of Cu, Cd, and Pb to the plant cultivated medium has generally decreased the total phenolic compounds compared to control leaves of tomato plants. It was previously reported that total phenolics in leaves of *Erica andevalensis* generally didn't show a significant change by the treatment of $CdSO_4$ (Márquez-García et al., 2012). Total phenolic content of sprouts were significantly decreased at 10 and 50 mM NaCI applications compared to controls in radish (Yuan et al., 2010). Different studies have carried in order to evaluate total phenolic content showed that cold stress significantly decreased the total phenolic in the leaves of grape (Amarowicz et al. 2010; Król et al. 2015). It was previously observed that total phenolic tended to decrease with increasing concentration of Cd in leaves of *Lepidium sativum* (Elguera et al., 2013). Phenolic

content of Greek olive cultivars (*Olea europea* L.) depended on cultivated months and cultivars, and total phenolic of gaidourelia, kalamon and megaritiki didn't show significant changes by the treatment of water deficit stress during May, but there was a slightly increase in June at the leaves of gaidourelia, kalamon, koroneiki and megaritiki (Petridis et al., 2012)). However, many authors demonstrated that content of total phenolic increased in various plants under the abiotic stress (Kováčik et al., 2009c; Lim et al., 2012; Sytar et al., 2014; Manquian-Cerda et al., 2016). These results indicate that the total phenolic compounds of tomato decreased compared to control and these decreases have changed to treatment doses. The decrease in the total phenolic content can be related to the decreases of individual phenolic compounds in the leaves of tomato exposed to heavy metals.
Seconder metabolites of plants changes depending on the cultivated soils and environmental conditions, responses of plant to stress factors are different and the synthesis of phenolics is a carefully controlled process in plants (Sannchez-Rodriguez et al., 2011). In the present study, we analyzed the major phenolic compounds of tomato leaves, and it can be observed that individual phenolic compounds were globally higher in leaves of control groups than in tomato leaves planted under heavy metals. The content of caffeic acid and chlorogenic acid significantly decreased in the leaves of tomato under the each heavy metal treatments compared to control plants. The treatment of Cd and Cu in the plant growth medium resulted in lower amount of the vanillic acid, while the level of rutin changed depending on types and doses of heavy metals in tomato leaves. Also, benzoic acid has been usually reduced by the addition of heavy metals on the tomato growth medium except for 50 ppm of Cu. Similar results have been obtained with different researcher as followed studies. The content of caffeic acid was lower under the cold stress than in the control groups of *Vitis vinifera* (Król et al., 2015). It was declared that the contents of benzoic acid, caffeic acid, and chlorogenic acid decreased while the accumulation of vanillic acid increased, and the amount of total phenolic remained unchanged in leaves of *Matricaria chamomilla* under the salt stress (Kovacık et al., 2009b). Application of $CdCI_2$ decreased the free phenolics in leaves of *Lepidium sativus* such as benzoic acid, caffeic acid and chlorogenic acid (Elguera et al., 2013). It was previously reported that biotic (nematode) and abiotic (water stress) decreased the content of rutin, while they increased the level of chlorogenic acid in the tomato (Atkinson et al., 2011). The content of vanillic acid didn't show significant changes by the treatment of copper salts, but the level of chlorogenic acid showed slightly increase in chamomile leaves (Kovacık et al., 2008). Also, application of $NiCI_2$ raised the content of caffeic acid and chlorogenic acid and changed the content of vanillic acid depending on treated doses in chamomile leaves (Kováčik et al., 2009a). Also, It was revealed that the level of chlorogenic acid and rutin have been raised, but contents of caffeic acid and ferulic acid reduced in leaves of corn growth medium containing heavy metals (Kisa et al., 2016).

In the present study, it was demonstrated that treatment of heavy metals globally decreased total phenolic content and major individual phenolics of the tomato leaves with some exceptions. The contents of phenolic compounds are vigorously dependent on cultivating conditions and the appearance of stressors in fields. Secondary metabolism in plants has been widely intensified in response to cold, temperature and osmotic stress (Akula & Ravishankar, 2016). The decreases in the phenolics should be results of the decline in the activity of crucial enzymes involved in the biosynthesis of phenolic compounds under the heavy metal stress. Polyphenols are generated in various plants through the phenylpropanoid pathways and the involved reactions have comprised several enzymes such as chalcone synthase, cinnamate 4-hydroxylase, phenylalanine ammonia-lyase, p-coumarate 3-hydroxylase, and etc. (André et al., 2009). In this study, it is indicated some phenolic compounds of tomato exposed to heavy metals, but supplementary investigations can be carried to obtain new perspectives which further phenolic compounds and related studies such as expressions and activities of involved enzymes.

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