

Antioxidants as a defensive shield in thyme (*Thymus vulgaris* L.) grown on the soil contaminated with heavy metals

Kamila Kulbat,^{*} Joanna Leszczyńska

Institute of General Food Chemistry, Lodz University of Technology
Wolczanska 171/173, 90-924 Lodz, Poland

*kamila.kulbat@wp.pl

Abstract: *The objective of this study was to investigate the effect of excessive concentration of selected heavy metals - nickel, copper and zinc on aromatic plants *Thymus vulgaris*. The present work examines the concentration of phenolic compounds, total antioxidant capacity and flavonoids content in leaves obtained from plants *Thymus vulgaris* grown on the soil contaminated with different concentration of these heavy metals. It was assumed, that selected metals, playing the role of micronutrients, cause toxic effect in their excessive concentrations, inhibit the growth and development of plants. Adverse impact on the plant is most likely due to the oxidative stress at a cellular level. It was demonstrated, that the lowest applied concentrations of heavy metals lead to the increased antioxidant content, which then decreases with increasing metal concentrations.*

Keywords: *heavy metals, antioxidants, phenolic compounds, flavonoids, *Thymus vulgaris* L.*

Introduction

The rapid development of technology largely affect the environment. Industrial factories are one of the main sources of the biosphere pollution, producing various types of toxic substances. Anthropogenic activities contribute to the increased concentration of heavy metals in soil and groundwater. Plants growing on contaminated areas show a reduction in growth and yield. Heavy metals, such as copper, nickel and zinc, play the role of micronutrients and are essential for proper growth and development of plants, being an integral part of many enzymatic proteins. However, high concentrations of these elements lead to increased accumulation in tissues disrupting basic cell metabolism [1]. Excessive concentrations of metal ions generate the production of reactive oxygen species by auto-oxidation and the Fenton reaction, which is typical for transition metals such as iron, copper, nickel and zinc [2].

Heavy metals have also ability to block the essential functional groups of biomolecules (by binding to thiol groups – SH in enzymes active centers) or substitute the required metal ions in the protein molecules, resulting in losing

their functionality [3]. From the point of presented paper, the most important fact is that transition metals such as nickel, copper and zinc, in excessive concentration can cause severe oxidative stress in plant tissues, leading to cell structures damage [4, 5]. There is no conclusive evidence that stress can be mitigated by the increased activity of antioxidant systems. The reason may be that the transition metals initiate the formation of very reactive hydroxyl radical that initiates free radical chain reactions leading to the destruction of many intracellular structures. The best studied is membrane lipid peroxidation [6]. According to most of the publications, metal accumulation in plant tissues increases with increasing metal concentration in soil, but the degree and rate of accumulation of the different metals varies between plant species [7]. In higher plants, both biotic and abiotic stress (particularly due to the presence of heavy metals) often induces the synthesis and accumulation of the same defensive secondary metabolites [7].

The toxic effects of heavy metals, is not limited to the oxidative damage of cell components such as lipids, proteins and nucleic acids, but can also cause inhibition of the synthesis of chlorophyll, which is reflected in the form of leaf chlorosis. The decrease in the chlorophyll content and consequently, inhibition of photosynthesis, reduces plant growth and development. Research conducted by Shakya et al. (2008) on bryophytes treated with three heavy metals Cu, Zn and Pb showed a significant decrease of chlorophyll a, chlorophyll b, and total chlorophyll content due to accumulation of copper ions. Zinc and lead slightly decreased chlorophyll content and the degree of inhibition depended on the concentration of metal. This study indicated a more destructive effect of copper on the chlorophyll content in plant tissues. High concentrations of Cu are known to produce oxidative damage to cellular structures, primarily changes in the properties of biological membranes by lipid peroxidation [8]. A similar pattern is observed in higher plants. Research on seedlings of bean (*Phaseolus vulgaris* L.) treated with different concentration of heavy metals: Pb, Cu, Cd and Hg showed a significant decrease in total chlorophyll content under the influence of increasing metals concentrations, while increasing the content of proline and non-enzymatic antioxidants: retinol, α -tocopherol and ascorbic acid [9]. Similar results were obtained by Zengin (2006) where contamination of soil with Zn and Co decreased the total chlorophyll content and total protein, while increase accumulation of proline [10].

Experimental

The aim of this study was to determine the total phenols content and antioxidant activity as well as total flavonoid content in leaves obtained from aromatic plants *Thymus vulgaris* grown on the soil contaminated with different concentration of heavy metals – nickel, copper and zinc.

It was our hypothesis that an increase concentration of selected heavy metals causes oxidative stress at the cellular level and stimulate biosynthesis of secondary metabolites with antioxidant properties. The study also assumed

that the excessive concentrations of these metals may inhibit germination and negatively affect plant growth.

Materials

The present study focused on the aromatic plant *Thymus vulgaris* L. from the *Lamiaceae* family. The plants were cultivated on a universal soil with pH 6.45±1, KCl 1.0 g dm⁻³, CaCl₂ 150 mg dm⁻³, P₂O₅ 170 mg dm⁻³ in a photoperiodic system 16/8 hours and the temperature oscillating around 24/18±2°C day/night. The relative humidity of the soil was around 65-70%. The seeds were subjected to 4-days incubation at 4°C to initiate germination (25-30 seeds per pot). A suitable weight of soil was emptied onto a large dish and combined with a solution of heavy metal salts to obtain the appropriate concentrations. All salts were used in the form of acetates. Analyzed the degree of soil contamination has been selected on the basis of the Regulation of the Polish Minister of the Environment on 9 September 2002 Poland (standards for soil quality and land quality). Soil quality standards were established for three categories of land. The lowest concentrations of metals responds to maximum permissible concentration for a group of land B, classified as agricultural land except land under water in ponds and ditches, forest land, as well as urban areas with the exception of industrial land, fossils areas and land used for transportation. The highest concentrations correspond to a maximum permissible concentrations for a group of land C classified as industrial, mining and transportation areas. Control plants are defined as plants cultivated on a universal soil without any contamination.

The concentrations of heavy metal ions (nickel, copper and zinc) applied to the soil are shown in the Table 1.

Table 1. Concentrations of heavy metal ions applied to the soil [mg kg⁻¹]

Heavy metals	[mg kg ⁻¹]		
Nickel	100	210	500
Copper	200	500	1000
Zinc	720	1500	3000

Methods

After 45 days of cultivation all the leaves of the plants (excluding cotyledons) were collected. The samples of fresh tissue of thyme were extracted with 80% methanol. For this purpose, 0.25 g of fresh plant tissue was homogenized in 5 ml of 80% methanol and incubated on a laboratory shaker (TTS 2, Yellow Line, IKA – Werke GmbH & Co. KG, Staufen, Germany) for 10 min. Subsequently the samples were centrifuged (896 RCF, 10 min) (Sigma 2-16P, Polygen, Wrocław, PL) and the supernatants were transferred into new tubes and stored until analysis at -20°C.

Experimental results are means ± SD of three independent replications. Statistical analyses were done using STATISTICA, Version 10: New Features and Enhancements. Differences between means were analyzed using ANOVA

analyses of variance followed by the Duncan multiple range post hoc test. P values < 0.05 were regarded significant and marked using different letters.

Antioxidant activity - Ferric reducing power assay (FRAP)

Total antioxidant activity was evaluated according to Ferric Reducing Antioxidant Power (FRAP) method with modifications [11, 12]. FRAP working solution containing 0.3 M CH₃COONa • 3H₂O (pH = 3.6), 40 mM HCl, 10 mM 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ) dissolved in 40 mM HCl and 20 mM FeCl₃ • 10H₂O was freshly prepared and kept away from light. 15 µl of extract solution was added to 1.98 ml of FRAP working solution. The mixture was incubated for 4 min at 37°C in a water bath. Absorbance was measured at 593 nm using the spectrophotometer (UV-Vis 8453, Hewlett Packard). FRAP working solution with distilled water instead of a sample was used as a blank. Total antioxidant capacity was calculated using the equation obtained from ascorbic acid calibration curve and expressed as µmol ascorbic acid per gram of fresh weight (F.W.). All the tests were performed in triplicate.

Total phenolic compounds content

The content of total phenolic compounds was determined by using the Folin–Ciocalteu (FC) reagent according to the method by Singleton and Rossi in 1965 [13]. The reaction mixture containing 50 µl of extract solution, 3.85 ml of distilled water and 100 µl of FC reagent was incubated in the dark at room temperature for 3 minutes. After this time, 1 ml of Na₂CO₃ was added and the mixture was incubated in the dark at room temperature for 60 min. The absorbance was measured at 725 nm using a spectrophotometer. The concentration of total phenolic compounds was calculated using a calibration curve for gallic acid and expressed as mg of gallic acid equivalent per gram of fresh weight (F.W.). All determinations were carried out in triplicate.

Total flavonoid content

The total flavonoid content was determined by the aluminium chloride colorimetric method [14]. 500 µl of extract solution was added to the mixture containing 1.5 ml of 80% methanol, 0.1 ml of 10% AlCl₃ • 6 H₂O and 0.1 ml of 1M CH₃COONa. The mixture was incubated in the dark at room temperature for 30 min. The absorbance was measured at 415 nm using a spectrophotometer. The total flavonoid content was calculated from the calibration curve for quercetin, and the result was expressed as mg of quercetin equivalent per gram of fresh weight (F.W.). All measurements were carried out in three replicates.

Results and Discussion

The results obtained for total antioxidant capacity, phenolic compounds content and flavonoid content in leaves are presented on Figures 1-3. The data are means ± standard deviation (±SD) of three replicates.

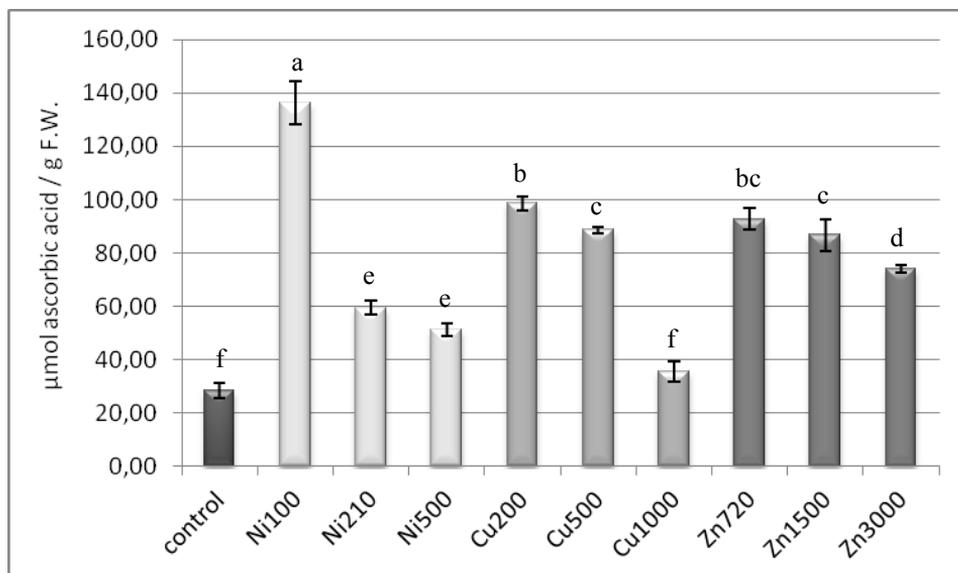


Figure 1. Total antioxidant capacity of the leaves of *Thymus vulgaris* L. The values are means \pm SD of three replicates. Bars with different letters are significantly different at $P < 0.05$

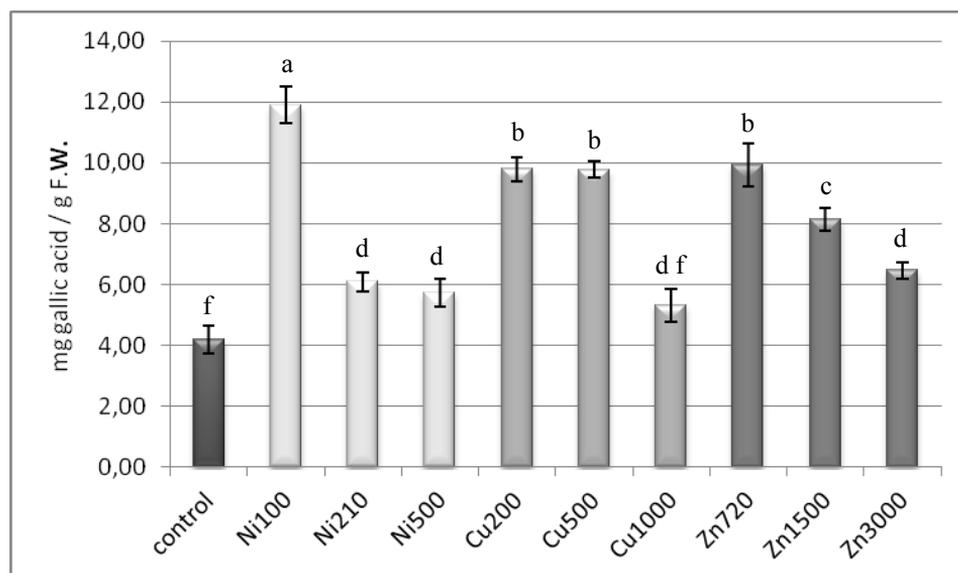


Figure 2. Total phenolic compounds content of the leaves of *Thymus vulgaris* L. The values are means \pm SD of three replicates. Bars with different letters are significantly different at $P < 0.05$

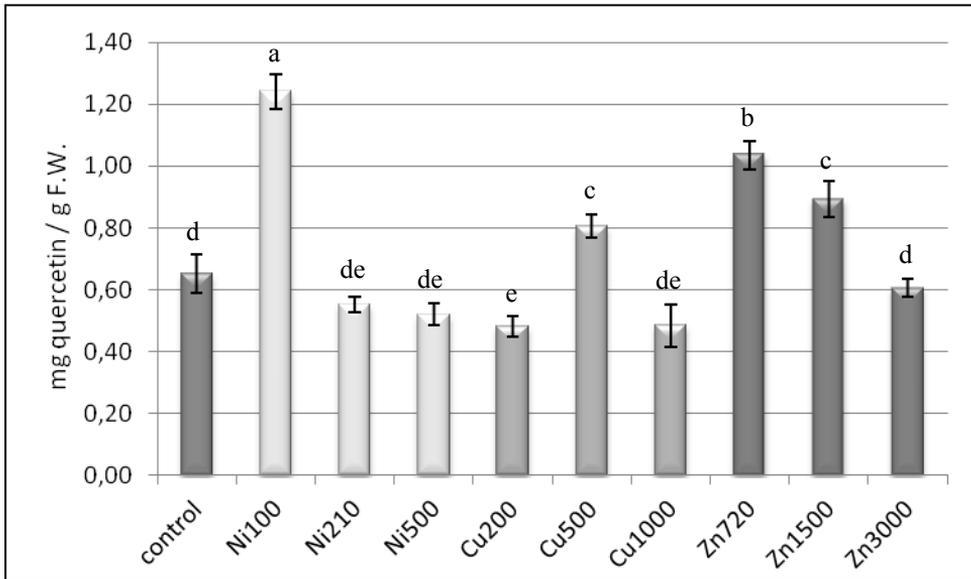


Figure 3. Total flavonoid content of the leaves of *Thymus vulgaris* L. The values are means \pm SD of three replicates. Bars with different letters are significantly different at $P < 0.05$

Little is known about the effect of excessive concentration of microelements - nickel, copper and zinc on the phenolic metabolism of *Lamiaceae* family.

The aim of this study was to determine the total phenols content and antioxidant activity, as well as, total flavonoids content in leaves obtained from aromatic plants *Thymus vulgaris* L. grown on the soil contaminated with different concentrations of these heavy metal ions.

The germination process runs concurrently for control plants and plants grown on soil contaminated with nickel and copper. For plants grown on soil contaminated with zinc, the growth rate and the number of germinated seeds were strongly limited and dependent on zinc concentration. Thyme plants grown on soil contaminated with nickel ions characterized by normal growth, slightly lower or equal to that of the control plants. Plants grown on soil contaminated with copper also characterized by normal growth, similar to the control. Only plants grown on soil contaminated with zinc expressed negative morphological changes. The lowest zinc concentration (720 mg kg^{-1}) resulted in inhibited growth and development when compared to the control. Higher concentration of zinc (1500 mg kg^{-1}) strongly inhibited growth of plants and caused yellowing of their leaves. The highest zinc concentration tested (3000 mg kg^{-1}) resulted in yellowing and then necrosis of the leaves. At the end of cultivation the highest zinc concentration appeared to be toxic enough to cause death of almost all the plants.

Simultaneous absorption of heavy metal ions into plant cells contributed to the oxidative stress and may lead to the damage of cellular organelles.

The highest antioxidant capacity among all the tested plants was detected in the ones germinating under nickel stress at the lowest concentration (Ni 100 mg kg⁻¹). Antioxidant capacity was significantly higher than the control in all stressed plants with the exception of plants grown on the soil contaminated with copper at the highest concentration (Cu 1000 mg kg⁻¹). Total antioxidant capacity decreased with the increasing concentration of heavy metal ions in the soil (Figure 1).

Similar results were obtained for phenolic compounds. The greatest increase in total phenolic content was observed for plants grown on the soil contaminated with nickel at its lowest concentration (Ni 100 mg kg⁻¹). As in the case of total antioxidant capacity, phenolic content was higher in all stressed plants except for copper (Cu 1000 mg kg⁻¹). It is worth to note that the highest concentration of total antioxidants as well as phenolic compounds was observed in plants cultivated on the soil contaminated with the lowest concentration of all tested heavy metal ions. Higher concentration of compounds with antioxidant properties may suggest that heavy metal ions initiate oxidative stress which triggers defensive response at a cellular level. Antioxidants, especially phenolic compounds are involved in defense reaction against oxidative stress mediated by metal ions. Accumulation of antioxidants helps to protect living cells from oxidative damage.

The total phenolic contents for plants grown under nickel stress were 11.90 ± 0.61 mg g⁻¹ (Ni 100 mg kg⁻¹), 6.09 ± 0.32 mg g⁻¹ (Ni 210 mg kg⁻¹) and 5.73 ± 0.46 mg g⁻¹ (Ni 500 mg kg⁻¹) in comparison to the control, where the concentration was 4.20 ± 0.45 mg g⁻¹. The concentration of phenolic compounds in plant tissues grown on the soil contaminated with copper were 9.79 ± 0.41 mg g⁻¹ (Cu 100 mg kg⁻¹), 9.77 ± 0.27 mg g⁻¹ (Cu 500 mg kg⁻¹) and 5.32 ± 0.55 mg g⁻¹ (Cu 1000 mg kg⁻¹) whereas for zinc were 9.93 ± 0.71 mg g⁻¹ (Zn 720 mg kg⁻¹), 8.14 ± 0.39 mg g⁻¹ (Zn 1500 mg kg⁻¹) and 6.47 ± 0.27 mg g⁻¹ (Zn 3000 mg kg⁻¹) (Figure 2).

Plants grown on the soil contaminated with the lowest concentration of nickel were also characterized by higher level of flavonoids content. The total flavonoid content for contaminated samples were 1.24 ± 0.056 mg g⁻¹ (Ni 100 mg kg⁻¹), 0.55 ± 0.023 mg g⁻¹ (Ni 210 mg kg⁻¹) and 0.52 ± 0.034 mg g⁻¹ (Ni 500 mg kg⁻¹). For plants grown on the soil contaminated with copper were 0.48 ± 0.033 mg g⁻¹ (Cu 200 mg kg⁻¹), 0.81 ± 0.037 mg g⁻¹ (Cu 500 mg kg⁻¹) and 0.48 ± 0.069 mg g⁻¹ (Cu 1000 mg kg⁻¹) whereas for zinc 1.04 ± 0.046 mg g⁻¹ (Zn 720 mg kg⁻¹), 0.89 ± 0.058 mg g⁻¹ (Zn 1500 mg kg⁻¹) and 0.61 ± 0.028 mg g⁻¹ (Zn 3000 mg kg⁻¹) in comparison to control sample 0.65 ± 0.062 mg g⁻¹ (Figure 3).

The total content of phenolic compounds as well as antioxidant capacity gradually decreased in all stressed plants with the concentration of heavy metal ions in the soil. The relatively high concentration of all antioxidants was observed in plants grown on the soil contaminated with nickel at the lowest

concentration. It is worth to note that phenolic compounds are strong antioxidant components and radical scavengers [3]. A positive correlation between total antioxidant capacity and total phenolic content suggested that phenolic compounds are the dominant antioxidants in tested plants. It is possible that in plants grown on the soil contaminated with the highest concentration of heavy metal ions, phenolic compounds have been oxidized and the level of oxidative damage was too high to be inhibited by cellular antioxidant system. The middle and the highest concentration of zinc seems to be the most toxic for plants of *Thymus vulgaris*, strongly inhibited growth and development.

Similar results were obtained by Márquez-García et al. (2012) where contamination of soil with cadmium increased the total flavonoids and phenolic content, as well as, the total antioxidant capacity of *Erica andevalensis*, an endemic species of South Western Iberian Peninsula growing on post-mining soils [15]. The increased biosynthesis of phenolic compounds was also observed in buckwheat (*Fagopyrum esculentum*) sprayed with nickel-containing aerosol. The authors observed Ni accumulation in plant tissues and increased concentration of malondialdehyde (MDA) after foliar treatment. Total phenolic content significantly increased in time and dose-dependent manner [16].

Phenolic compounds are also known for their ability to chelate heavy metal ions, most likely because of the presence of suitable substituents - hydroxyl and carboxyl groups. Already in 1997, Moran et al. proved that phenolic compounds from soybean have ability to bind iron [17].

The binding of heavy metals Hg, Pb, Cr by methanol extract rich in polyphenols was proven in the study with *Nympheae* [18].

According to Trebichalský et al. (2015) total content of polyphenols was correlated with Cd and Pb contents in selected cultivars of strawberries [19]. Similar results have been provided by Wojcieszek et al. (2016) who demonstrated that polyphenols are able to bind copper ions in berries – Brazilian açai (*Euterpe oleracea* Mart.) and Polish bilberry (*Vaccinium myrtillus* L.) Probably, the same mechanism works for other metals [20].

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