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Title: Growth, photosynthetic pigments, phenolic content and biological activities of Foeniculum vulgare Mill., Anethum graveolens L. and Pimpinella anisum L. (Apiaceae) in response to zinc

Article Type: Research Paper

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Keywords: Anethum graveolens L., Foeniculum vulgare Mill., Pimpenilla anisum L., Zinc, antioxidant activity, enzyme inhibition

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Abstract: The effect of zinc (Zn) on phenols, antioxidant activities (free radicals' scavenging activities, inhibition of lipid peroxidation, chelating activity and reducing power), and enzyme inhibition activities of acetylcholinesterase, lipoxygenase and tyrosinase of anise, dill and two cultivars of fennel [Latina" (FL) and "Doux de Florence" (FDF)] aqueous extracts was evaluated for the first time. At the same time, growth and photosynthetic pigment contents were also estimated.
A significant decrease in all growth parameters, such as plant height, fresh and dry weights of aerial parts and roots was statistically proved in the presence of 2 mM Zn. An enhancement of Zn accumulation was observed, particularly in aerial part. Exposure to 2 mM Zn induced quantitative changes in the phenols of all tested extracts. In fennel extracts, a decrease of phenol content was observed when treated with Zn, whereas an increase was found in dill and anise extracts submitted to Zn treatment. All samples, either in the presence or absence of Zn, showed antioxidant activity, independent on the tested assay, with the exception of the cultivar FDF (treated and non-treated), which was unable to scavenge NO radicals. Zn-treated dill and anise presented usually higher antioxidant activity. Concerning inhibitory activities against acetylcholinesterase, lipoxygenase and tyrosinase enzymes, both treated and non-treated FDF did not inhibit acetylcholinesterase activity. Zinc-treated FDF was also unable to inhibit lipoxygenase activity. The remaining species presented capacity for inhibiting those enzymes and tyrosinase as well, but their abilities varied in response to Zn excess.

Date: 04/08/2017

Subject: Manuscript Ref. No.: INDCRO-D-17-01815R2

Title: Growth, photosynthetic pigments, phenols and in vitro biological activities of aqueous anise, dill and fennel extracts in response to 2 mM Zinc

Dear Prof. Maria Pascual-Villalobos,

We thank you for your e-mail of 24 July 2017 with the comments on our manuscript (MS) INDCRO-D-17-01815R2 on “Growth, photosynthetic pigments, phenols and in vitro biological activities of aqueous anise, dill and fennel extracts in response to 2 mM Zinc”.

We have read it carefully and we do understand most of the referee’s comments. Please find bellow our reply and comments following each point raised by the reviewers. All corrections and additions performed in the MS text have been marked in blue.

Reviewers' comments:

Reviewer #1: The revised version of the manuscript INDCRO-D-17-01815R2 meets all the criteria of scientific work.

Paper may be published after several technical corrections:

- Suggestion for the title (please accept the proposal, the current version is incorrect):

Growth, photosynthetic pigments, phenolic content and biological activities of Foeniculum vulgare Mill., Anethum graveolens L. and Pimpinella anisum L. (Apiaceae) in response to zinc

RESPONSE:This proposal made by reviewer 1 was taken into account.

- Technical errors in paper need to be corrected, for example line 76 and 78 chronology, 110 (ZnSO4x7H2O is ok, not with point ZnSO4·7H2O), 184 (50% is ok, not with space 50 %) 190, ...803 (pat or part)....etc..

RESPONSE: The group of references cited in the text was listed alphabetically, according to the guide for authors. ZnSO4·7H2O was used, and not ZnSO4x7H2O, according to the generally found in chemical manuscripts.

All cases in which % was separated of the number was corrected.

pat was corrected and replaced by part.

- The Latin names of the species should be in italics as well as in vitro, in vivo etc. (lines 577, 597, 634, 650, 658, 666, 675, 689, 700, 713, 716, 739, 765, 784, 792, 798 etc...)

RESPONSE: All cases were corrected.

- Grammatical errors were noticed in the text of paper and not respected the rules in writing the English language.

RESPONSE: Tentatively done. The revision of the text by an English native was not carried out.

- There are errors in the text when typing, spacing, double spacing, etc.. all the errors should be corrected.
RESPONSE: All cases were corrected.

- References should be technical checked in accordance with the instructions for authors. Also, a number of typographical errors have been noted in the references.
RESPONSE: All cases were corrected.

- Conclusion must be accurately, clearly and precisely written, based on the results.
RESPONSE: Conclusion was re-written.

Reviewer #2: The present manuscript studied the effect of Zn in the antioxidant activity of fennel, dill and anise. Zn is a heavy metal and is also the responsible for the weakness of several soils that cannot be used for agriculture so that is very important to control the amount of this metal.
The introduction is very complete as also the other sections, especially the results and discussion that are clear and compared with the available literature, and the authors concluded that the Zn concentration directly affects the antioxidant activity and also the amount of phenolic compounds. This is very interesting given the importance of the safety in the agriculture practices.
This manuscript was already revised and after revision the authors already made the requested corrections according to the journals rules.
The manuscript can be accepted in the present form.

Reviewer #3: I have evaluated the manuscript (INDCRO-D-17-01815R2) titled "Growth, photosynthetic pigments, phenols and in vitro biological activities of aqueous anise, dill and fennel extracts in response to 2 mM Zinc" submitted to Industrial Crops and Products. The study is original and content of the study was studied properly. My comments on the paper were given below.
1- The title of the manuscript is not appropriate.
RESPONSE: The title was re-written according to the proposal of Reviewer 1.

2- Give more details about the statistical analysis. Please specify the type of statistical analysis for each experiment. Also, indicate if data showed normality and homocedasticity, or if you assumed them directly before analysis.
RESPONSE: A chapter of statistical treatment was introduced in the present form. By lapse it was not introduced in the previous version of the manuscript.

3- The results of the work do not show much novelty. The effects of heavy metals (including Zn excess) have already been studied on many plants, although it does not seem that they have been tested on fennel, dill and anise.
RESPONSE: I agree with the reviewer, nevertheless this subject is new for these species.

4- Deep explanation of the mechanisms of action of the aqueous extracts is required in "Results and discussion" section. Authors must establish comparisons between them and possible conclusions of the work itself.
RESPONSE: The discussion was tentatively ameliorated, at least in some cases.

5- It would be useful to determine the chemical composition of the aqueous extracts of anise, dill and fennel.
RESPONSE: The chemical composition was not introduced, because it is not done.

6- All information necessary to understand the table should be included in the title or subtitle.
RESPONSE: Information introduced.

7- I suggest to delete Table 8. It is too long and not necessary. The explain of the major points in the table is enough.

RESPONSE: Table 8 was deleted from the main document, but at this moment it is a supplementary information. We consider that the information compiled in previous Table 8, now Table S1, is important and must remain.

We hope that we have addressed correctly all the reviewers and Editor remarks and questions.

Hoping that everything is in the correct form and looking forward to hearing from you.

Yours sincerely,

Graça Miguel

Highlights

Zn excess reduced growth and photosynthetic pigments level in fennel, anise and dill

Zn excess affected phenol amounts and antioxidant potency of extracts

Zn treatment affected the enzymatic inhibitory ability of extracts

Growth, photosynthetic pigments, phenolic content and biological activities of *Foeniculum vulgare* Mill., *Anethum graveolens* L. and *Pimpinella anisum* L. (Apiaceae) in response to zinc

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Abstract

The effect of zinc (Zn) on phenols, antioxidant activities (free radicals' scavenging activities, inhibition of lipid peroxidation, chelating activity and reducing power), and enzyme inhibition activities of acetylcholinesterase, lipoxygenase and tyrosinase of anise, dill and two cultivars of fennel [Latina" (F_L) and "Doux de Florence" (F_{DF})] aqueous extracts was evaluated for the first time. At the same time, growth and photosynthetic pigment contents were also estimated. A significant decrease in all growth parameters, such as plant height, fresh and dry weights of aerial parts and roots was statistically proved in the presence of 2 mM Zn. An enhancement of Zn accumulation was observed, particularly in aerial part. Exposure to 2 mM Zn induced quantitative changes in the phenols of all tested extracts. In fennel extracts, a decrease of phenol content was observed when treated with Zn, whereas an increase was found in dill and anise extracts submitted to Zn treatment. All samples, either in the presence or absence of Zn, showed antioxidant activity, independent on the tested assay, with the exception of the cultivar F_{DF} (treated and non-treated), which was unable to scavenge NO radicals. Zn-treated dill and anise presented usually higher antioxidant activity. Concerning inhibitory activities against acetylcholinesterase, lipoxygenase and tyrosinase enzymes, both treated and non-treated F_{DF} did not inhibit acetylcholinesterase activity. Zinc-treated F_{DF} was also unable to inhibit lipoxygenase activity. The remaining species presented capacity for inhibiting those enzymes and tyrosinase as well, but their abilities varied in response to Zn excess.

Keywords: *Anethum graveolens* L., *Foeniculum vulgare* Mill., *Pimpenilla anisum* L., Zinc, antioxidant activity, enzyme inhibition

1. Introduction

Zinc (Zn), presented in available form, is an essential micronutrient for plants, animals, and microorganisms (Dhankhar et al., 2012). It plays crucial roles as a functional, structural, and regulatory cofactor in many enzymes and regulatory proteins (Fujiwara et al., 2015). Regarding plant physiology and metabolism, Zn is known to be required at optimal concentration for growth and is involved in the protective effect of plasma membrane integrity and permeability (Paradisone et al., 2015). However, various soils are detrimental for plant growth owing to high concentration of metal ion, either due to natural processes or man's activities (Levitt, 1980). As a heavy metal, Zn toxicity is one of the major problems of worldwide agricultural production causing a potential health risks (Marichali et al., 2014). In Tunisia, soils around open-cast mining exhibit a very high content of Pb, Zn, and Cd (Sebei et al., 2005). Owing to this fact, plants on these mining areas may accumulate some metals such as Pb, Zn, and Cd with an average of 0.7%; 0.9% and 0.003% respectively (Sebei et al., 2006). High amounts of zinc accumulated in plants leads to the induction of oxidative stress and, consequently, to the alteration of their physiological processes, according to the investigation made in model plants. Nevertheless this sort of research, using medicinal and aromatic plants is scarce (Marichali et al., 2016).

Foeniculum vulgare Mill. (fennel) is a perennial umbelliferous (Apiaceae) herb, used by humans since antiquity, due to its characteristic aniseed flavor. Fennel is native from the Circum-Mediterranean area but is cultivated throughout the world (Muckensturm et al., 1997). This aromatic plant has been traditionally used for the preparation of herbal teas and even for daily consumption, in the raw form as salads, stewed, boiled, grilled, or baked in many dishes (Barros et al., 2010). Several studies have demonstrated the effective *in vitro* activity of fennel as antimicrobial, antiviral, antiprotozoal (Dua et al., 2013), antioxidant, antitumor, anti-

inflammatory, cytoprotective, hepatoprotective, hypoglycemic, and oestrogenic (Badgujar et al., 2014).

Anethum graveolens L. (dill) is an annual and sometimes biennial umbelliferous (Apiaceae) herb, native to south-west Asia or south-east Europe, in the Mediterranean region (Zehtab-Salmasi et al., 2006; Oshaghi et al., 2017). This species has been used in traditional medicine for digestive disorders as carminative, stimulating lactation, and antispasmodic (Zehtab-Salmasi et al., 2006; Oshaghi et al., 2017). Other properties have also been attributed to dill: lowering lipids, anticancer, antimicrobial, antidiabetic, antigastric irritation, antioxidant, and anti-inflammatory (Oshaghi et al., 2017). In addition, dill has also been used as a vegetable and as an inhibitor of sprouting in stored potatoes (Zehtab-Salmasi et al., 2006).

Pimpinella anisum L. (anise), a plant belonging to the Umbelliferae family (Apiaceae), which can be found in the Eastern Mediterranean region, is known for its medicinal and culinary properties. This genus exhibits biological and pharmacological properties, such as antimicrobial, galactogogue, antioxidant, antiseptic, digestive, and anti-inflammatory activities (Martins et al., 2016).

There are growing interests for *F. vulgare*, *A. graveolens* and *P. anisum* therapeutic and medicinal activities but little attention has been paid to the zinc monitoring (toxic heavy metal) in these medicinal plants. To the best of our knowledge, there are no reports in the literature investigating the biological activities, particularly *in vitro* antioxidant, acetylcholinesterase, lipoxygenase and tyrosinase inhibitory activities of *F. vulgare*, *A. graveolens* and *P. anisum* in response to Zn excess. Thus, this study highlights the capacity of accumulation of Zn by fennel, dill and anise as well as its effect on some morphophysiological parameters, along with the effect on some biological properties in terms of antioxidant, anti-inflammatory activities, anti-acetylcholinesterase and anti-tyrosinase activities effectiveness.

2. Material and Methods

2.1. Plant cultivation and Zn treatment

Two cultivars of sweet fennel plants: "Latina" (F_L) and "Doux de Florence" (F_{DF}), as well as dill (D) and anise (A) plants, were used in the present study. The plants were cultivated according to the recommended technology reported by Senatori et al. (2013). Surface-sterilized seeds were germinated and grown in the spring of 2014 at the experimental greenhouse, in a plane area of North of Tunisia (EL Alia, Bizerte, Latitude: 37°10′ 08″ N; Longitude: 10°02′00″ E; Elevation above sea level: 102 m), filled with a loamy sand soil, naturally lit with sunlight, with a temperature range of 20 - 30 °C and a relative humidity range of 50−80%, and supplied with distilled water every 3 days. The 30-d-old seedlings were transferred to individual pots (26 cm upper diameter, 18 cm lower diameter, 25 cm in height) of 10 L volume, filled with the same soil. Throughout the 60 days of the experiment, healthy and uniform seedlings were submitted to Zn treatment using 2 mM $ZnSO_4 \cdot 7H_2O$, that is renewed every 5 days. A control treatment (0 mM Zn) was made in the same conditions but without adding Zn. The experimental design was a randomized block with two treatments (control and treated with Zn) of each variety arranged in individual pots, with one plant each, and nine replicates. Samples, obtained by mixing the aerial part collected at the end of the experiment from the nine replications of each treatment, were submitted for analysis.

2.2. Determination of growth parameters

For each treatment, the fresh and dry weights, and high of roots and aerial part were determined.

2.3. Determination of chlorophyll content

The pigments *Chla*, *Chlb*, total chlorophyll and carotenoids were extracted from fresh material in 80% acetone at room temperature. The chlorophyll contents were determined spectrophotometrically as reported by Marichali et al. (2014) and using the followed equations:

$Chla = 10.05\ OD_{663} - 1.97\ OD_{645}$

$Chlb = 16.36\ OD_{645} - 2.43\ OD_{663}$

$Chla + Chlbb = 7.62\ OD_{663} + 14.39\ OD_{645}$

Where *Chla* = Chlorophyll a; *Chlb* = Chlorophyll b; *Chla* + *Chlb* = total chlorophyll; OD = optical density (nm).

Carotenoid amounts were evaluated in the same extracts and were calculated according to Lichtenthaler (1987) using the formula:

$C_{carotenoïds} = (1000\ OD_{470} - 1.82\ Chl_a - 85.02\ Chl_b) / 198$

Where *Chla* = Chlorophyll a; *Chlb* = Chlorophyll b; OD = optical density (nm).

2.*4. Determination of zinc (Zn) content*

Fennel, dill and anise treated and non-treated samples (roots and aerial part) were digested by applying the optimized procedure using nitric and perchloric acids. In brief, a 0.05 g of well-powdered samples were added to 5 mL of HNO_3 and $HClO_4$ (3:1) (v/v) and the mixtures were digested firstly in ambient temperature for 24 h and then with an increased temperature starting from 60°C to 90°C and finally to 105°C until total dissolution. After cooling of the digest, an equal volume of Milli Q water was added to dilute left over acid. Blank solution was prepared by following the same procedure but without samples, only with reagents. The

amount of Zinc in the sample solutions was determined by MP-AES (Microwave Plasma Atomic Emission Spectrometry) (Agilent 4200 MP-AES, Santa Clara, CA).

2.5. Preparation of the extracts

The air dried material of fennel, dill and anise species were powdered by using electric mixer grinder and were weighed (5 g for each) and then macerated with distilled water (100 mL) without agitation, at room temperature, for 24 h, filtered, and finally, the filtrate was freeze dried.

2.6. Determination of total phenols

Phenolic compounds were determined in accordance with Folin–Ciocalteau's method (El-Guendouz et al., 2016). In brief, a serial of dilutions of aqueous extracts (50 µL) were prepared and mixed with 750 µL of Folin–Ciocalteau's reagent and 600 µL of sodium carbonate (75 mg/mL). The reaction mixtures were further incubated at room temperature for two hours. Afterward, absorption was measured at 760 nm versus a blank prepared without extract. Tests were carried out in triplicate. A number of dilutions of gallic acid (standard) were obtained to prepare a calibration curve. Total phenol content of the extracts was expressed as gallic acid equivalent/mL extract (mg GAE/mL extract).

2.7. Determination of total flavonols and flavones

Flavonols and flavones content of the extracts were evaluated by aluminum chloride colorimetric method (El-Guendouz et al., 2016). The extracts (150 µL) were mixed with aluminum chloride (2%) (75 µL) and sodium acetate (1%) (75 µL). After reaction for 5 min, 150 µL of sodium hydroxide (40 mg/mL) was added and left at room temperature for 30 min. The absorbance of the reaction mixture was measured at 510 nm. The flavonols and flavones content of the extracts were expressed as quercetin equivalents (mg QE/mL extract)

2.8. Flavanone and dihydroflavonol content

The total quantification of flavanone and dihydroflavonol compounds was determined according to El-Guendouz et al. (2016). Briefly, 75 µL of sample or standard (naringin) and 2 mL DNP (2,4-dinitrophenylhydrazine) (1 g DNP in 2 mL 96% sulphuric acid diluted to 100 mL with methanol) were heated at 50 ºC, for 50 min. After cooling at room temperature, 175 µL of KOH 10% were finally added to the mixture. The resulting solution was diluted to 3 mL with methanol. The absorbance was measured at 486 nm. All determinations were performed in triplicate. The values are expressed as naringin equivalents (mg NE/mL extract).

2.9. Antioxidant activity

2.9.1. Inhibition of lipid peroxidation by Thiobarbituric Acid Reactive Species (TBARS) method

This assay was performed using two distinct lipid substrates: egg yolk and liposomes.

The TBARS assay is the most commonly used method for measuring lipid peroxidation. The assay was carried out as described by Boulanouar et al. (2013). Briefly, egg yolk (100 mg/mL) (250 µL) homogenates were used as a lipid-rich medium and mixed with acetic acid (750 µL) and the solution of TBA (8 mg/mL) (750 µL) dissolved in sodium dodecyl sulfate (SDS) (11 mg/mL). The method involved heating the reaction mixture for one hour in water bath at 95 °C. After cooling at room temperature, the final volume was adjusted to 2 mL by adding butanol followed by vigorous vortexing and centrifugation for 10 min at 3,000 *g*. The absorbance of the upper layer was measured at 532 nm against a blank that contained all reagents minus the sample; the percentage of inhibition was calculated as follows:

$[(A_0 - A_1 / A_0) \times 100]$, in which A_0 is the absorbance of the control reaction (without extract), and A_1 is the absorbance of the extracts. Analyses were run in triplicate. The

inhibition percentage was plotted against extract concentration (w/v) and IC_{50} values were determined (concentration of extract able to prevent 50% of lipid peroxidation).

The liposome assay started with the preparation of liposome solution: 0.4 g lecithin in 80 mL chloroform (Boulanouar et al., 2013). This solution was dried and submitted to nitrogen flux for 30 s and then to vacuum for at least two hours until complete dryness. In brief, the reaction was initiated by mixing 50 µL of different extract dilution with 100 µL of liposome suspension, Fe Cl_3 (100 µL, 4 mM) and 50 µL of ascorbic acid (0.18 mg/mL diluted to 1/10). After incubation at 37 °C for one hour, 2 mL of TBA solution (0.6%) was added and the resulting mixture was further heated for 10 min at 95 °C. Afterwards, 2 mL butanol was subsequently added and final solution was centrifuged for 5 min at 3,000 *g*. The absorbance of the supernatant was determined at 532 nm. Tests were carried out in triplicate. The assay was performed as reported above for thiobarbituric acid reactive species (TBARS) method.

2.9.2. Ability for scavenging $ABTS^{+\bullet}$ free radicals

The scavenging activity against ABTS cation [2,2-azinobis(3-ethylbenzothiazoline)-6-sulfonic acid)] radical assay was carried out as described by Boulanouar et al. (2013). The $ABTS^{+\bullet}$ solution was diluted with ethanol to obtain an absorbance ranging between 0.700 and 0.800, at 734 nm. Then, 25 µL of samples were mixed to 275 µL $ABTS^{+\bullet}$ solution and the absorbance was read at 734 nm and recorded for 6 min. Three independent experiments were performed. The ABTS radical cation scavenging activity was expressed as:

$[(A_0 - A_1 / A_0) \times 100]$, in which A_0 is the absorbance of the control reaction (without extract), and A_1 is the absorbance of the extract. The results were expressed as IC_{50} values.

2.9.3. Ability for scavenging DPPH free radicals

The method of DPPH (2,2-diphenyl-1-picrylhydrazyl) radical-scavenging activity previously reported by Boulanouar et al. (2013) was followed by mixing a methanolic solution of DPPH 25 µM (275 µL) with extract (25 µL) and then maintained in the dark at room temperature for 1 h. The absorbance of the reaction mixture was measured at 517 nm. The percentage inhibition of the DPPH radical of samples was calculated by the formula:

$[(A_0 - A_1 / A_0) \times 100]$ in which A_1 and A_0 are absorbances of solvent with and without sample, respectively. The results were expressed as IC_{50} values.

2.9.4. Total antioxidant activity (Phosphomolybdenum assay)

The total antioxidant activity of the samples was evaluated by phosphomolybdenum method as reported by Zengin et al. (2015) with slight modifications. Fifty microliters of sample were added to 1 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The mixture was incubated for 90 min at 95 ºC and the absorbance was measured at 695 nm. The total antioxidant capacity was expressed as Ascorbic Acid equivalent (mg AA/mL extract)

2.9.5. Ferric reducing power activity

The reducing power of each extract was measured as previously reported by Boulanouar et al. (2013) with slight modifications. Fifty microliters of each sample was mixed to 0.5 mL of potassium phosphate buffer (200 mM, pH 6) and 0.5 mL of potassium ferricyanide (1%). The reaction mixtures were incubated at 50 °C in a water bath, for 20 min .Subsequently, 0.5 mL of trichloroacetic acid (10%, w/v) was mixed and the solution was centrifuged at 3,000 *g*, for 10 min. Finally, distilled water (0.5 mL) and 0.1 mL $FeCl_3$ (0.1%, w/v) were added to the supernatant and the absorbance was measured at 700 nm. The ferric reducing power capacities of the extracts were expressed graphically by plotting absorbance against concentration.

2.9.6. Ferrous chelating metal ions assay

Ferrozine can chelate with Fe^{2+} and form a complex with a red color which can be quantified. The ferrous ion-chelating effect of all extracts was estimated according to Aazza et al. (2013). Briefly, the reaction was initiated by mixing samples with 25 µL of $FeSO_4 \cdot 7H_2O$ (0.52 mg/mL), 150 µL of distilled water and 25 µL of ferrozine (2.5 mg/mL). The absorbance of the reaction mixture was measured at 562 nm. The ratio of inhibition of ferrozine–Fe^{2+} complex formation was calculated as follows: $[(A_0 - A_1 / A_0) \times 100]$ in which A_0 is the absorbance of the control and A_1 is the absorbance of the test sample. The results were expressed as IC_{50} values.

2.9.7. Nitric oxide scavenging capacity

The nitric oxide (NO) scavenging activity of samples was determinate in accordance with the method previously reported by Aazza et al. (2013) with slight modifications. In brief, 75 µL of extracts were added to 75 µL of 10 mM sodium nitroprusside into a 96-well plate and left standing at ambient temperature for 35 min. The reaction was initiated by adding Griess reagent [40 µL of sulfanilamide solution and 40 µL of *N*-1-napthylethylenediamine dihydrochloride (NED) solution] to the mixture and the absorbance was measured at 532 nm without previous incubation. The percentage of inhibition was calculated from the formula: $[(A_0 - A_1 / A_0) \times 100]$ where A_0 is the absorbance of the control reaction (without extract), and A_1 is the absorbance of the extracts. The results were expressed as IC_{50} values.

2.9.8. Superoxide anion scavenging activity (non-enzymatic method)

The superoxide anion scavenging activity of samples was determinate according to the method previously described by Albano and Miguel (2010). The method involved mixing the extract (25 µL) with 25 µL nitroblue tetrazolium (0.42 mg/mL) and 25 µL NADH (1.32 mg/mL) and ethanol (125 µL) followed by 25 µL phenazine methosulfate (PMS)

(0.25 mg/mL). The absorbance was measured at 560 nm, after 10 minutes of reaction. The decrease of absorbance at 560 nm indicates the consumption of superoxide anion in the reaction mixture. Tests were carried out in triplicate. The percentage inhibition was calculated by the formula given bellow: $[(A_0-A_1/A_0) \times 100]$ in which A_1 and A_0 are absorbances of solvent with and without sample, respectively. The results were expressed as IC_{50} values.

2.10. Inhibitory activity of enzymes

2.10.1. Lipoxygenase (LOX) inhibitory activity

The inhibition of lipoxygenase enzyme was performed by following the previous method described by El-Guendouz et al. (2016) with some modifications. The reaction consisted on the mixture of 10 µL of each sample and 5 µL of enzyme solution (0.054 g/mL) and 50 µL of linoleic acid (0.001 M) and borate buffer 937 µL (0.1M, pH 9). The measurement of the absorbance was recorded at 234 nm. The analyses were carried out in triplicate. The percentage of inhibition was calculated from the formula: $[(A_0-A_1/A_0) \times 100]$, where A_0 is the absorbance of the control reaction (without extract), and A_1 is the absorbance of the sample solution. The results were expressed as IC_{50} values.

2.10.2. Acetylcholinesterase (AChE) inhibitory activity

Acetylcholinesterase inhibitory activity was carried out according to the method reported by El-Guendouz et al. (2016) with minor modification. Briefly, an aliquot of sample (60 µL), 425 µL Tris-HCl buffer (0.1 M, pH 8) and 25 µL enzyme (0.28 U/mL) were mixed and incubated for 15 min at room temperature. After pre-incubation, 75 µL of substrate (0.005 g of iodine acetylcholine in 10 mL of buffer) and 475 µL of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) (0.059 g in 50 mL of buffer) were added to terminate the reaction. The absorbance of the reaction mixture was read after 30 min of incubation. The control was prepared in the same way, except that the sample solution was replaced by Tris-HCl buffer. Each experiment

was conducted in triplicate, and enzyme inhibitory rates of samples were calculated as follows: $[(A_0-A_1/A_0) \times 100]$. The results were expressed as IC_{50} values.

2.10.3. Tyrosinase inhibitory activity

Tyrosinase inhibitory activity was measured according to the method reported by El-Guendouz et al. (2016). An aliquot of each sample (40 µL) was mixed with (10 µL) enzyme solution (100 U/mL) and 140 µL phosphate buffer (50 mM, pH 6.8) in a 96-well microplate. The mixture reaction was incubated for 40 min at room temperature, then 60 µL of L-3,4-dihydroxyphenylalanine (L-DOPA) (5 mM) was finally added as substrate. The absorbance was measured at 492 nm. The percentage of inhibition of tyrosinase activity was calculated as follows: $[(A_0-A_1/A_0) \times 100]$. The results were expressed as IC_{50} values.

2.11. Statistical analysis

Statistical analysis was performed with the Statistical Package for the Social Sciences (SPSS) 23.0 software (SPSS Inc., Chicago, IL, USA). Statistical comparisons were made with one-way analysis of variance followed by Tukey's multiple comparisons. The level of significance was set at $p < 0.05$. Correlations were achieved by Spearman's correlation coefficient (r) at a significance level of 95% or 99%.

3. Results and discussion

3.1. Zinc content

Table 1 depicts the Zn amounts in roots and aerial parts of fennel, anise and dill. Zn accumulation varied according to the organ. Zn levels recorded in non-treated and treated roots ranged from 21.89 to 89.02 µg/g DW (dry weight) and from 61.02 to 1312.77 µg/g DW, respectively. Concerning the aerial part, anise and dill exposed to Zn excess exhibited a

remarkable increase in their Zn content reaching a maximum values of 16940.27 µg/g, DW, and 23665.70 µg/g, DW, respectively.

Zinc toxicity involves perturbation of metabolic activity in terms of competition for uptake of other elements and inhibition of enzymatic action (Marichalli et al., 2016). It has been reported by Cayton et al. (1985) that the absorption and translocation of plant nutrients such as Fe, Mg, K, P and Ca were related to Zn concentration in soil. Metal may enter inside plant cells by simple diffusion, passive transport, through channel proteins and finally by active transport through carrier proteins (the most important). Zinc, through this process, enters in the cytosol via members of the ZIP transporter family (ZRT-IRT like protein: **Z**inc-regulated transporter, **I**ron-regulated transporter **P**rotein). After entering, there are mechanisms that establish plant Zn homeostasis. Such may be achieved through Zn efflux transporters which are involved in Zn and other metals redistribution, translocation, and detoxification (Lin and Aarts, 2012). Plants that are unable to prevent entry or improve efflux of Zn or other metals, for surviving, they have other mechanisms that are based on sequestration of such metals in order to store them in storage tissues or specific organelles (e.g. vacuole). For example, the metal tolerance protein 1 (MTP1), is probably the most important Zn vacuolar sequestration transporter in plants (Lin and Aarts, 2012), or the heavy metal ATPase 3 (HMA3) (Chilian et al., 2015).

Our findings show that the accumulation of Zn in the aerial part of fennel (independent on the variety) is mediated by different mechanisms when compared to those of dill, in which the amounts of Zn in the aerial parts are much higher than in roots. According to Chilian et al. (2015) such could be attributed to higher HMA4 activity than that of HMA3, because HMA3 protein mediates Zn accumulation in root vacuoles, whereas HMA4 is involved in the metal translocation from root towards the shoot (Lin and Aarts, 2012). Following this reasoning, we could hypothesize that in dill, HMA4 could act more intensely than in fennel or anise, due to

the higher amounts of Zn in the aerial part. However, these hypotheses need further confirmation since it was not the aim of the present work.

3.2. Effects of Zn on plant growth and its pigment content

Zinc (Zn), manganese (Mn), iron (Fe), copper (Cu) and nickel (Ni) are essential micronutrients necessary for normal plant growth (Bashir et al., 2016; Sarwar et al., 2017). Several cellular organelles need these microelements for plant growth and development. Fe, Cu and Mn regulate chlorophyll synthesis and photosynthesis; Fe and Cu regulate respiration; and zinc is a co-factor required for the structure and function of diverse proteins (more than 300 enzymes and 200 transcription factors essential for maintaining membrane integrity, reproduction and auxin metabolism) (Bashir et al., 2016; Grotz and Guerinot, 2006; Singh et al., 2016). Deficiencies or high amounts of such elements may lead to dysfunctional chloroplasts and mitochondria (Bashir et al., 2016). The toxic effects due to higher amounts of essential elements, such as Zn, include inhibition of growth and photosynthesis, altered water balance and nutrient assimilation, senescence and plant death (Singh et al., 2016). Some of these effects were observed in the species and varieties studied in the present work, nevertheless more noticeable in dill and anise plants (Table 2), which can suggest that these species are more sensitive to high concentration of Zn than fennel.

Results depicted in Table 2 show that all tested plants remained alive and have grown until the end of treatment, nevertheless a depressing effect caused by Zn exposure in all growth parameters (plant height, root length, fresh and dry weight of aerial parts and roots) was observed. Indeed a strong negative correlation ($p < 0.01$) between Zn amount and growth parameters was estimated, which confirmed the toxic effect of Zn treatment on samples (Table 3). The action of Zn on growth plant was more evident in anise, independent on the plant part.

For all samples, the results presented in Table 1 indicate that the chlorophyll *a* content (*Chla*) is lower in treated plants comparing with non-treated samples. The same trend was noted for the chlorophyll *b* content (*Chlb*) after the treatment with zinc. The Zn exposure caused a significant decrease of carotenoid contents in all studied samples. The results confirmed that these cultivars of fennel, dill and anise are sensitive to Zn excess.

According to the results depicted in Table 4, the measured parameters such as high length, dry weight and fresh weight of roots and aerial part are positively correlated with total chlorophyll and carotenoid contents.

Our results also showed a dramatically reduction in carotenoid contents in response to Zn excess as compared to other photosynthetic pigments. In addition, the significant decrease in chlorophyll contents (total chlorophyll (*Chla* + *Chlb*), *Chla*, and *Chlb*) seems to be more pronounced in *Chlb* than *Chla*, which is similar to the results of Marichali et al. (2016) who reported that the content of photosynthetic pigments of *Nigella sativum* was significantly reduced as well as *Chlb* content, being this pigment more sensitive to Zn excess than *Chla*. The photosynthetic pigments decline, induced by Zn, might be resulted from several causes such an iron deficiency; an inhibition of enzyme activities involved in the chlorophyll biosynthesis; inhibition of other enzymes (δ-aminolevulinic acid dehydratase and protochlorophyllide reductases); a Mg removal from chlorophyll; advanced peroxidation of chloroplast membrane lipids by reactive oxygen species (ROS) which would inhibit the reductive steps in the biosynthesis pathway of chlorophylls; degradation of thylakoids; and reduction in the rate of RubisCO synthesis and/or modification in its activities (Di Baccio et al., 2009; Fernàndez-Martínez et al., 2014; Islam et al., 2014; Marichali et al., 2016; Parlak and Yilmaz, 2012; Subba et al., 2014).

3.3. Zn effects on total phenols and flavonoid contents

As shown in Table 5, the accumulation of phenols and flavonoids depends on the variety. Under stressful conditions, the two varieties of fennel plants showed a decrease in phenol and flavonoid contents, whereas in the other species higher accumulation of phenols was detected in the Zn treated plants (dill followed by anise) than in non-treated samples. Data from Table 5 show that treatment had a significant increment ($p < 0.05$) in the amount of dihydroflvonols in the F_L as well as in anise, whereas in dill their accumulation in treated plants decreased by two-fold, when compared to the control.

The results of the present work show a variability of total phenol contents for the different tested samples. Treated anise and dill samples showed a significant increase of their total phenol concentrations while a reduction in phenolic production was observed for both F_{DF} and F_L cultivars exposed to elevated Zn concentration. Thus, it can be argued that the response in terms of phenolic accumulation due to Zn excess is species dependent. Such higher amounts of total phenols in dill and anise samples were coincident with the highest levels of Zn detected in the same samples. This phenol accumulation may be a response to the oxidative damage induced by Zn in order to permit plant survival, such as previously reported for different plant species by several authors (Marichali et al., 2014; Morina et al., 2010). In fact, some studies have revealed that metal application, including Zn, are associated with the increased activities of enzymes of secondary pathway namely shikimate dehydrogenase, phenylalanine ammonialyase (PAL), and polyphenols oxidase (PPO) (Ali et al., 2006; Castáneda and Pérez, 1996; Van de Mortel, 2006; Wang et al., 2011). Nevertheless, Basak and co-workers (2001) have been reported that *Camellia sinensis*, submitted to Ni treatment, resulted in reduction of their phenolic amounts despite the stimulation of PAL activity as observed for our samples of fennel submitted to Zn. These dissimilar results are in accordance with those obtained in the present work, whereby the effect of metals on phenol accumulation in plants is dependent on the species. Therefore, no significant correlation between aerial part

Zn accumulation and phenol content was observed (Table 6). Hence, these results may support the observation of Kováčik et al. (2009) suggesting the use of modulators of phenolic metabolism in response to Ni treatment. In addition, they reported that phenolic accumulation and type of phenol are also dependent on the type of metals and on the plant organ.

Most of reports have studied the effect of Zn excess on the oxidative machinery but little information is available about the relationship between Zn excess and the non-enzymatic antioxidant activity.

3.4. Zn effects on antioxidant activities of samples

Generally, the extracts of anise had higher antioxidant activity than fennel (Table 7). On the other hand, the zinc exposure enhanced the activity in dill and anise extracts, in contrast to the fennel extracts. In this case and in both varieties, zinc had a negative effect on the antioxidant activity (Table 7). Accordingly, our results revealed that treated samples, rich in polyphenols, when compared to the non-treated ones, responded by an enhancement of their capacity for scavenging DPPH, ABTS, nitric oxide and superoxide radicals, as well as through the total antioxidant measured by phosphomolybdenum method. The antioxidant activity correlated well with the phenols' amounts. FRAP assay showed that dill extracts (treated and non-treated), and Zn-treated anise extract possessed the greatest reducing power followed by anise, and fennel (F_L and F_L Zn). However, treated and non-treated F_{DF} was not able to reduce the iron metal ion. Moreover, the activity was dose-dependent (Figure 1).

In order to discuss our results, we present a reference Table gathering the previous investigations about fennel, anise and dill (Table S1) which is organized as follows: the type of assay studied in the work followed by the plant part used, extraction type as well the solvent used and, finally, the chemical composition for finding a possible relationship between activity and chemical composition.

The results found for fennel samples are within the range reported by other authors for extracts obtained by diverse methods, but particularly for DPPH method, and solvents (Table S1). This Table only compiles antioxidant activities, measured through diverse methods, of the aerial parts of fennel, anise and dill as a whole or as parts (leaves, stems, shoots, inflorescences and flowers). The term "Plant material" was also considered. Fruits and seeds were not taken into account, because we considered that we should compare results obtained from the same plant part. In the evaluation of the antioxidant activity, the authors almost always use, at least two assays, although the capacity for scavenging DPPH radicals predominates in practically all works. Beyond the diversity of unities used by the authors for presenting the results, Table S1 permit to show great differences among the results of antioxidant activity, whereby our ones are within the large range found by other authors. Type of extraction and solvent, part of plant used and plant itself can be altogether factors that determine this variability.

In the majority of cases, the chemical composition of the extracts was not performed, nevertheless in those in which such was done (nine), practically did not contribute to correlate the activity with the components identified in the extracts. Only in three cases, the authors described a correlation between the antioxidant activity of extracts with some components detected in the same extracts (Faudale et al., 2008; Hossain et al., 2011; Parejo et al., 2004). In these cases, the authors considered that chlorogenic acid isomers, rosmarinic acid, gallic acid and luteolin-7-*O*-glucoside were determinant in the antioxidant activities found in extracts.

Independent on the assay tested, the authors of the works (Table S1) found a correlation between the activities and the concentration of total phenols, such was observed in our investigation (Table 8). In the present work, a correlation was also found between the amounts of flavones/flavonols and antioxidant activity, but not detected between activity and

446 flavanones and di-hydroflavonols (Table 8). However, the capacity for preventing lipid
447 peroxidation, either using egg yolk or liposomes as lipid substrates, as well as the capacity for
448 scavenging NO free radicals did not correlate with total phenol content. Only the
449 concentrations flavone/flavonols correlate with the capacity for preventing lipid oxidation,
450 when egg yolk was used as lipid substrate, although no correlation had been found when
451 liposomes constitute the lipid substrate of the reaction (Table 8).

452 The antioxidant activity of the aerial parts of anise and dill (Table S1) was also reviewed
453 and much lower references were found when compared to fennel. When the activity unities
454 were presented as IC_{50}, it was possible to compare the results of the present work with them.
455 They were within the same range. Correlations between antioxidant activities of extracts and
456 the phenols identified in the same extracts were only reported by Zielinski et al. (2015) and
457 Stankevičius et al. (2011) for anise and dill samples, respectively (Table S1). The former
458 attributed the capacity for scavenging DPPH free radicals to gallic acid, catechin, *epi*catechin
459 and quercetin. Although the importance of the identification of the phenol compounds of the
460 extracts by the authors, they did not give additional information about their role as
461 antioxidants. Nevertheless they also had detected correlation between the concentration of
462 total phenols and antioxidant activity as observed in the present work in the majority of tests.

463 Despite numerous papers describing different antioxidant assays testing the ability of
464 fennel, dill and anise extracts, little investigations has been performed for evaluating the
465 potentiality of these plants under Zn excess conditions, whereby our work permit finding the
466 effect of this metal not only in the production of phenols but also in the antioxidant activity.
467 Such results allowed detecting that fennel, independent on the variety, presented a negative
468 effect by decreasing the activity whereas in anise and dill such was not evident.

The accumulation of low molecular phenols by plants in stress or pollution conditions has been reported by diverse authors. According to Mongkhonsin et al. (2016), caffeic acid and rutin were at higher amounts in the leaves of *Gynura pseudochina* treated with zinc and/or cadmium. However, there was a threshold above which a decrease in the contents of those compounds was observed. In the same work, the authors also reported that higher amounts of rutin and caffeic acid correspond stronger capacity for scavenging DPPH free radicals. *Echium vulgare* plants exposed to Zn or lead (Pb) stress increased accumulation of chlorogenic acid and rosmarinic acid (Dresler et al. (2017). The young leaves of *Betula pubescens* trees, near to copper-nickel smelter (Finland), had higher amounts of 1-*O*-galloyl-*β*-D-(2-*O*-acetyl)-glucopyranose, neochlorogenic acid (*trans*-5-caffeoylquinic acid), *trans*-5-*p*-coumaroylquinic acid and quercetin-3-*O*-*β*-D-galactopyranoside. Mature leaves had higher concentrations of 1-*O*-galloyl-*β*-D-(2-*O*-acetyl)-glucopyranose and neochlorogenic acid (Loponen et al., 1997). Some of these compounds were reported by some authors (Faudale et al., 2008; Hossain et al., 2011; Parejo et al., 2004; Stankevičius et al., 2011; Zielinsli et al., 2015) (Table 8) as being constituents of fennel, anise and dill that correlated well with the capacity for scavenging free radicals.

Recently, the findings of Marichali et al. (2016) highlighted the importance of studying the induced-Zn toxicity in Tunisia toward antioxidant response of *Nigella sativa*. The results revealed that treatment of *N. sativa* with Zn excess was accompanied by an enhanced antioxidant activity of this plant. On the other hand, in comparison with previously tested metals, such increase was observed in FRAP values assay which indicates that excess of boron application stimulates the non-enzymatic antioxidant mechanism in the apple rootstock EM 9 explants (Molassiotis et al., 2006). These results supported the conclusion of Elzaawely et al. (2007) suggesting that improvement of antioxidant activity of plants may be caused by their exposure to abiotic stress. Copper-treated plants, with higher amount of phenols, were found

494 to increase their antioxidant activity determined by DPPH radical scavenging and β-carotene
495 bleaching methods, which could be suggested as a protective response of these plants from
496 copper induced damages (Elzaawely et al., 2007).

497 Anise plants under Zn toxicity, with higher amounts of phenolics, revealed a capacity for
498 metal chelating four-fold more efficient than non-treated plants (Table 7). All treated samples,
499 without exception, increase their ability to inhibit lipid peroxidation, particularly when
500 liposomes were used as lipid substrate, in response to Zn stress. These results explain the
501 strong correlation between the effective ability of samples against lipid peroxidation and their
502 Zn accumulation (Table 6). In contrast, the higher accumulation of Zn in all treated samples
503 did not correspond to stronger capacity for scavenging free radicals (DPPH, ABTS,
504 superoxide, NO) in all samples, which may explain the absence of correlation between zinc
505 accumulation and capacity for scavenging some types of free radicals. Zinc is a mineral
506 antioxidant, such as selenium, that does not act on free radicals, but act preventing lipid
507 peroxidation (Prasad et al., 2004).

508 *3.5. Zn effects on inhibitory activity of enzymes*

509 Inhibitory activity of extracts on lipoxygenase (LOX), acetylcholinesterase (AChE) and
510 tyrosinase were performed.

511 Anise extracts exhibited strong LOX inhibitory activity with IC_{50} values of 0.015 and
512 0.035 mg/mL for treated and non-treated samples, respectively. F_L treated samples (F_LZn)
513 showed the lowest ability to inhibit LOX activity (IC_{50} = 0.062 mg/mL), followed by non-
514 treated ones (IC_{50} = 0.052 mg/mL). The extract from non-treated F_{DF} plants revealed an IC_{50}
515 value of 0.049 mg/mL, whereas Zn treated plants (F_{DF}Zn) did not present the ability for
516 inhibiting 5-LOX enzyme. The exposure to Zn excess showed insignificant changes in the

potency of dill extract as a LOX inhibitor (Table 9). These results implying that Zn application influence the LOX inhibitory capacity of samples but depending on variety and species.

The LOX inhibition assay can be considered not only as an indicator of antioxidant but also an indicator of possible anti-inflammatory activity (Albano and Miguel, 2010; Kontogiorgis et al., 2016). These authors studied the antioxidant activity and LOX inhibitor activity of *F. vulgare* extracts. However, there is no study, at least to our knowledge, focused on fennel and anise Zn treated extracts as enzymatic inhibitors. It has been previously ascribed by Molassiotis et al. (2006) that treatment with boron was associated with an enhancement in LOX activity in leaves and stems of explants of the apple rootstock EM 9 (*Malus domestica* Borkh). According to these authors, LOX activity may be considered as indicator of oxidative stress. Indeed, it has been suggested that propagation of lipid peroxidation in plants under stress condition induced by higher lipolytic activity on the membrane was associated with stimulation of LOX activity (Lacan and Baccou, 1998).

As shown in Table 10, all samples had a significantly moderate ability for inhibiting AchE except F_{DF} samples (treated and non-treated), which were not able to inhibit AchE. Moreover, different trends were observed in the studied varieties after treatment with Zn. Extracts from Zn stressed F_L and anise plants revealed higher capacity of AchE inhibition than extracts from the respective non-treated plants, while the opposite occurred with the extracts form dill plants treated and non-treated with Zn.

Up to date, searching for new AchE inhibitors derived from natural sources with few side effects is required (Hasbal et al., 2014) but no relevant studies have been reported about anticholinesterase activity of fennel, dill and anise exposed to Zn excess which prompted us to concentrate on the efficiency of our samples for AchE inhibition. Among several investigations, Gomathi and Manian (2015) suggested the effectively AchE inhibitory effect

stem bark and leaves of *Stevia crenata* might be attributed to their increased amounts of non-enzymatic antioxidants.

In this work, although anise and some fennel plants were shown to possess an inhibitory effect against AchE, it was not revealed a significant correlation between AchE inhibitory activity and phenolic compounds including dihydroflavonols, suggesting the contribution of other bioactive constituents against AchE (Table 6). It is noteworthy to refer that there is not a clear trend on the AchE inhibitory activity of tested samples under Zn excess. In fact, no correlation between zinc content and the inhibitory effect of samples against AchE was observed in the present data (Table 6), and to best of our knowledge, scientific information remains absent for better understanding the zinc contributing function in the inhibitory AchE activity.

In the present research, all tested extracts exhibited tyrosinase inhibitory activities; however these were higher for dill and anise plants than for both F_{DF} and F_L cultivars, which were in the lower range (Table 9). In addition, the inhibitory activity values of dill and anise Zn treated extracts were 1.3 fold higher than those measured for non-treated extracts, suggesting that Zn treatment increased the ability of samples to inhibit tyrosinase enzyme. The extract form dill plants treated with Zn was found as a potent tyrosinase inhibitor. The inhibitory activity of tyrosinase of dill extracts was better than those reported by Orhan et al. (2013). The tyrosinase inhibitory activity, expressed as IC_{50}, was negatively correlated with the amounts of total phenols and flavonoids (Table 8), which can be explained by the essential role of hydroxyl group of phenolic compound for forming hydrogen bond with a site of tyrosinase enzyme.

There was no correlation between zinc accumulation and antityrosinase activity. Tyrosinase inhibitory activity of kojic acid and its synthetic derivatives is related to the ability to coordinate metals (Lachowicz et al. 2015). Synthetic kojic acid derivatives obtained by

these authors had affinity for Fe^{3+}, Al^{3+}, Zn^{2+}, and Cu^{2+}. However, the chelating capacity for Zn^{2+}, and Cu^{2+} was weaker. The absence of correlation between zinc accumulation and antityrosinase activity of extracts may be supported by the finding of Lachowicz et al. (2015). The chelating ability of the compounds present in the extracts with antityrosinase activity for zinc is weak and, consequently, without any supplemental inhibitory effect on tyrosinase activity.

4. Conclusions

The results of the present work showed that growth parameters and photosynthetic pigment contents were negatively affected by Zn treatment but depending on the plant species and even variety. An accumulation of zinc was observed in both roots and aerial parts of all species studied, though more noticeable in the aerial parts of anise and dill. The plants responded differently to Zn excess exposure, especially in their accumulation of phenols and, therefore, in their abilities for scavenging free radicals and inhibit tyrosinase activity. The accumulation of zinc in plants was only negatively correlated with the IC_{50} values of TBARS assay, independent on the lipid substrate used (egg yolk and liposomes), which may indicate the positive role of zinc on the prevention of lipid peroxidation.

The extracts of zinc-treated anise and dill plants had higher antioxidant activity and the extracts of anise-treated plants also had higher ability for inhibiting the activity of acetylcholinesterase, lipoxygenase and tyrosinase. This study gives new insights about the effect of Zn excess exposure on the ability of tested extracts against some enzymes but it is important to pay attention about the high accumulation of Zn in the aerial parts of these plants which make their consumption dangerous for human health, due to the toxicity of this metal. On the other hand, the ability of dill and anise plants to accumulate high amounts of Zn raises the hypothesis of their use for bioremediation of soils contaminated with this metal.

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773

Table 1

Zn content in roots and aerial parts of non-treated and Zn-treated *F. vulgare*, *A. graveolens* and *P. anisum* plants

Samples	Zn (µg/g DW)	
	Roots	**Aerial Part**
F_{DF}	56.84±7.50 ^{cd}	52.38±0.84^{c}
F_{DF} Zn	87.93±11.21^{c}	71.03±0.08^{c}
F_{L}	21.89±0.54^{d}	54.12±0.40^{c}
F_{L} Zn	61.02±12.3 9^{cd}	82.51±4.11^{c}
D	89.02±5.23^{c}	66.62±4. 99^{c}
D Zn	186.27±9.42^{b}	23665.70±3466.64^{a}
A	59.75±6.26^{cd}	30.45±4.73^{c}
A Zn	1312.77±25.64^{a}	16940.27±2466.04^{b}

DW: Dry Weight.

Each value represents the mean of three replicates ± S.E (standard error). Values with different letters in the same column are significantly different at $p < 0.05$.

Table 2

Growth parameters of non-treated and Zn treated *F. vulgare*, *A. graveolens* and *P. anisum* plants and their photosynthetic pigment amounts

	Growth (cm)		**Fresh weight (FW) (g)**		**Dry weight (DW) (g)**		**Pigment contents (mg/g FW)**			
Sample	Aerial part	Roots	Aerial part	Roots	Aerial part	Roots	*Chla*	*Chlb*	*Chla+Chlb*	*Carotenoids*
F_{DF}	90.50±3.85^{b}	17.33±1.46^{b}	399.55±63.33^{a}	24.84±2.00^{a}	296.22±41.78^{b}	18.06±1.24^{a}	0.98±0.01^{d}	0.68±0.01^{b}	1.67±0.02^{c}	0.48±0.01^{a}
F_{DF} Zn	73.08±3.88 ^{d}	12.13±1.68^{d}	273.08±3.88^{bc}	17.26±2.94^{c}	195.86±41.71^{cd}	14.93±1.09^{b}	0.66±0.00^{f}	0.40±0.04^{d}	1.06±0.05^{e}	0.08±0.01^{cde}
F_{L}	79.27±2.81^{cd}	16.40±1.20^{b}	262.94±34.37^{c}	23.48±3.65^{ab}	356.72±44.30^{a}	17.04±1.43^{a}	1.12±0.05^{bc}	0.84±0.08^{a}	1.97±0.03^{a}	0.49±0.06^{a}
F_{L} Zn	62.17±2.74 ^{e}	11.55±1.56^{d}	214.26±41.34^{d}	13.32±1.10^{d}	156.54±20.34^{ef}	11.37±1.29^{c}	0.80±0.01^{e}	0.59±0.01^{bc}	1.39±0.00^{d}	0.01±0.00^{e}
D	106.63±4.46^{a}	24.30±1.73^{a}	304.02±2.42^{bc}	21.23±1.11^{b}	203.74±2.53^{cd}	14.61±0.95^{b}	1.32±0.06^{a}	0.56±0.04^{c}	1.88±0.02^{b}	0.55±0.01^{a}
D Zn	80.88±6.13^{c}	15.1±1.49^{bc}	166.62±30.66^{e}	11.12±1.60^{d}	130.15±5.42^{f}	9.21±1.39 ^{d}	0.46±0.04^{i}	0.25±0.03^{ef}	0.72±0.00^{f}	0.12±0.01^{cd}
A	59.73±2.85 ^{e}	22.28±3.02^{a}	273.65±34.06^{bc}	16.11±0.87^{c}	156.98±15.32^{ef}	12.58±1.37^{c}	1.03±0.01^{cd}	0.91±0.02^{a}	1.94±0.01^{ab}	0.31±0.02^{b}
A Zn	12.90±1.05 ^{f}	12.90±1.05^{cd}	114.90±2.72^{f}	7.28±1.39^{e}	70.40±4.55^{i}	4.70±1.13^{e}	0.32±0.01^{i}	0.16±0.03^{f}	0.48±0.00^{i}	0.06±0.02^{de}

Each value represents the mean of three replicates ± S.E. Values with different letters in the same column are significantly different at $p < 0.05$.

Table 3

Spearman correlation coefficients between Zn content and growth parameters

	Growth		Fresh weight		Dry weight	
	Roots	Aerial part	Roots	Aerial part	Roots	Aerial part
Zn content	-0.265*	-0.470**	-0.720**	-0.284**	-0.744**	-0.390**

* Significant at $p < 0.05$; ** Significant at $p < 0.01$.

Table 4

Spearman correlation coefficients between chlorophyll / carotenoid content and growth / fresh weight

	Chlorophyll content (mg/g FW)	Carotenoid content(mg/g FW)
Growth(cm)	0.511**	0.717**
Fresh weight(g)	0.654**	0.766**

FW: Fresh Weight

* Significant at $p < 0.05$; ** Significant at $p < 0.01$.

Table 5

Phenolic contents of aqueous extracts of non-treated and Zn treated *F. vulgare*, *A. graveolens* and *P. anisum* plants

Samples	Phenols (mg GAE/mL Extract)	Flavonols and flavones (mg QE/mL Extract)	Dihydroflavonols and flavanones (mg NE/mL Extract)
F_{DF}	24.42±0.06^{f}	13.86±0.13^{d}	9.51±0.31^{bcd}
F_{DF} Zn	20.61±0.16^{i}	10.48±0.08^{e}	9.80±0.07^{bc}
F_{L}	35.76±0.08^{d}	14.46±0.60^{d}	2.60±0.13^{f}
F_{L} Zn	20.85±0.01^{i}	10.65±0.31^{e}	10.22±0.07^{b}
D	67.22±0.64^{b}	27.80±0.88^{b}	10.44±0.52^{b}
D Zn	74.07±0.3^{a}	35.40±0.50^{a}	5.06±0.53^{e}
A	29.08±0.08^{e}	15.01±1.14^{d}	8.43±0.04^{d}
A Zn	38.25±0.03^{c}	22.63±1.21^{c}	14.49±0.01^{a}

GAE: Gallic Acid Equivalent; QE: Quercetin Equivalent; NE: Naringin Equivalent.

Each value represents the mean of three replicates ± S.E. Values with different letters in the same column are significantly different at $p < 0.05$.

Table 6

Spearman correlation coefficients between Zn amount ,antioxidant and enzyme inhibitory activities

	Zinc
Total phenol	0.331
Flavones + flavonols	0.294
Dihydroflavonols + flavanones	0.310
DPPH	-0.539
ABTS	-0.166
Phosphomolybdenum	0.307
Ferric chelating	-0.331
TBARS	-0.644**
Liposome	-0.709**
Superoxide	-0.257
NO	0.298
Lipoxygenase	-0.166
Acetylcholinesterase	-0.038
Tyrosinase	-0.271

** Significant at $p < 0.01$.

Table 7

Antioxidant activity (IC_{50}, mg/mL) of aqueous extracts of non-treated and Zn-treated F. *vulgare*, *A. graveolens* and *P. anisum* plants, measured by using different assays

Samples	Phospho-molybdenum (mg AAE/mL)	DPPH	ABTS	Ferric chelating	Liposome	NO	TBARS	Superoxide
F_{DF}	31.692±0.793^{l}	0.379±0.005^{a}	0.020±0.000^{bc}	0.116±0.004^{b}	0.067±0.000^{a}	-	0.021±0.000^{d}	0.021±0.002^{c}
F_{DF} Zn	27.121±0.465^{j}	0.354±0.001^{b}	0.025±0.002^{a}	0.145±0.001^{a}	0.010±0.000^{i}	-	0.048±0.001^{b}	0.028±0.002^{b}
F_{L}	42.700±0.462^{de}	0.073±0.002^{d}	0.007±0.000^{fi}	0.056±0.001^{c}	0.042±0.000^{c}	1.735±0.023^{c}	0.051±0.002^{a}	0.005±0.000^{e}
F_{L} Zn	24.498±0.744^{k}	0.310±0.007^{c}	0.012±0.000^{de}	0.143±0.001^{a}	0.017±0.000^{e}	3.798±0.117^{a}	0.004±0.000^{f}	0.040±0.001^{a}
D	82.717±1.223^{b}	0.032±0.000^{e}	0.010±0.001^{ef}	0.023±0.000^{e}	0.062±0.001^{b}	2.007±0.027^{b}	0.037±0.000^{c}	0.010±0.000^{d}
D Zn	95.713±0.457^{a}	0.017±0.000^{f}	0.014±0.002^{d}	0.016±0.000^{f}	0.012±0.000^{f}	1.821±0.047^{b}	0.021±0.000^{d}	0.003±0.000^{ef}
A	38.008±0.515^{f}	0.306±0.010^{c}	0.018±0.000^{c}	0.040±0.000^{d}	0.023±0.000^{d}	0.009±0.000^{d}	0.050±0.001^{ab}	0.005±0.000^{e}
A Zn	46.716±1.298^{c}	0.071±0.000^{d}	0.010±0.000^{ef}	0.010±0.000^{i}	0.009±0.000^{i}	0.007±0.000^{d}	0.013±0.000^{e}	0.004±0.000^{ef}

-: Not detected; AAE: Antioxidant Activity Equivalent.

Each value represents the mean of three replicates ± S.E. Values with different letters in the same column are significantly different at $p < 0.05$.

Table 8

Spearman coefficients between phenolic compound amounts and antioxidant and enzyme inhibitory activities

	Total phenol	Flavones+flavonols	Dihydroflavonols + flavanones
Total phenol	1	0.953**	-0.118
Flavone+flavonol	0.953**	1	-0.110
dihydroflavonol	-0.118	-0.110	1
DPPH	-0.892**	-0.855**	0.04
ABTS	-0.563**	-0.437*	-0.067
Phosphomolybdenum	0.959**	0.953**	-0.173
Ferric chelating	-0.897**	-0.893**	-0.108
TBARS	-0.040	-0.006	-0.626**
Liposome	0.021	0.001	-0.277
NO	0.381	0.312	0.044
Superoxide	-0.801**	-0.825**	0.320
Lipoxygenase	-0.022	-0.131	-0.159
Acetylcholinesterase	0.382	0.370	-0.188
Tyrosinase	-0.949**	-0.957**	0.098

** Significant at $p < 0.01$

Table 9

Enzyme inhibitory activities (IC_{50} = mg/ mL) of aqueous extracts of non-treated and Zn-treated *F. vulgare, A. graveolens and P. anisum* plants

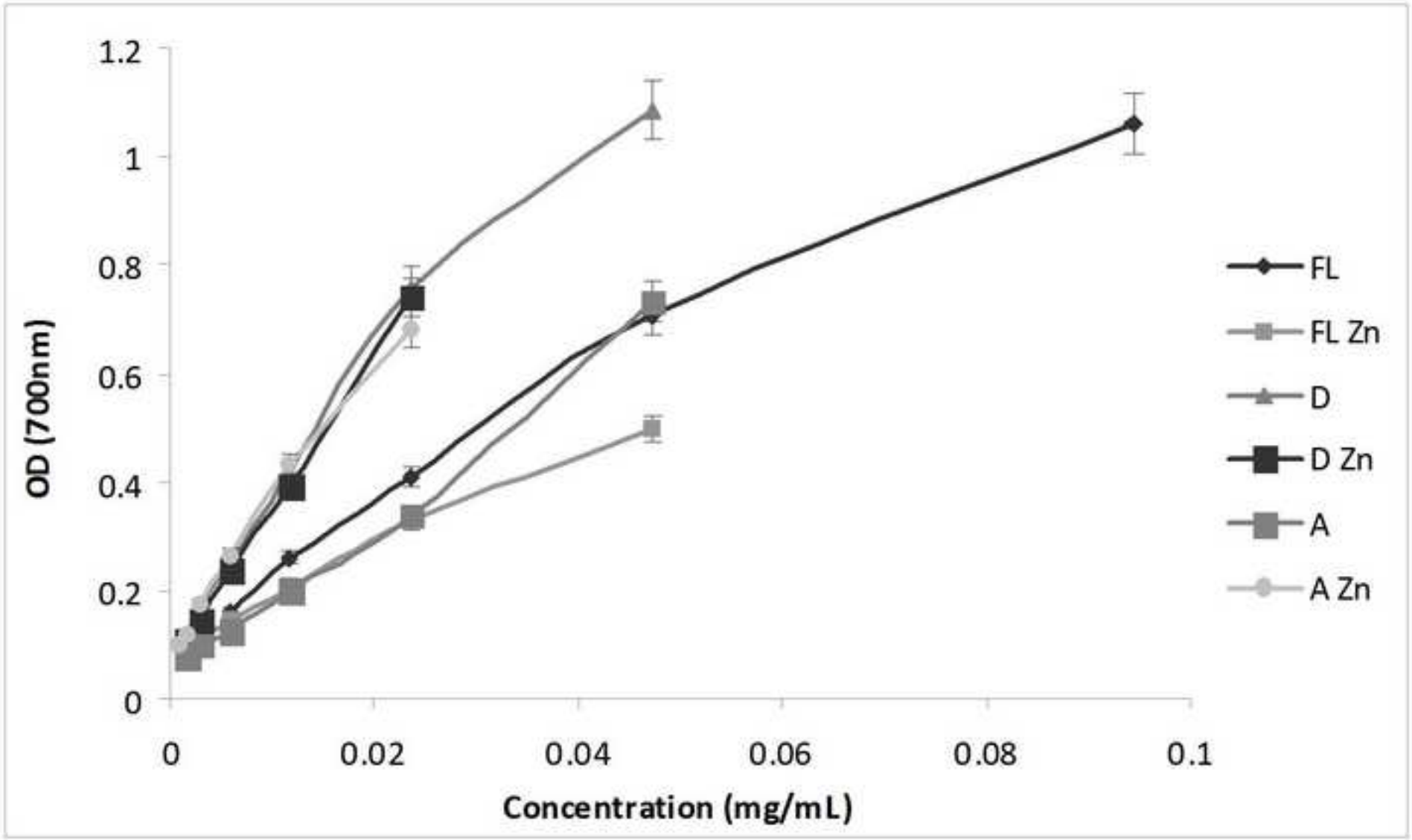
Samples	Lipoxygenase	Acetylcholinesterase	Tyrosinase
F_{DF}	0.049±0.000^{b}	-	0.826±0.015^{b}
F_{DF} Zn	-	-	0.941±0.010^{a}
F_{L}	0.052±0.001^{b}	0.331±0.015^{bc}	0.592±0.019^{c}
F_{L} Zn	0.062±0.000^{a}	0.209±0.010^{cd}	0.953±0.035^{a}
D	0.043±0.000^{c}	0.120±0.007^{de}	0.190±0.004^{f}
D Zn	0.041±0.000^{c}	0.227±0.007^{bcd}	0.157±0.001^{f}
A	0.035±0.000^{d}	0.809±0.128 ^{a}	0.403±0.008^{d}
A Zn	0.015±0.000^{e}	0.351±0.026^{b}	0.308±0.005^{e}

Each value represents the mean of three replicates ± S.E (standard error). Values with different letters in the same column are significantly different at $p < 0.05$.

-not detected

Figure. 1. Ferric reducing power of aqueous extracts of non-treated and Zn-treated *F.vulgare, A. graveolens and P. anisum* plants.

Figure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Supplementary Interactive Plot Data (CSV)

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