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Heavy Metals Chelating Ability and Antioxidant Activity of *Phragmites australis* Stems Extracts

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ABSTRACT

In this study, the ability of hexane (HSE), chloroform (CSE), ethyl acetate (EASE) and methanolic (MSE) stems extracts from *Phragmites australis* or EDTA (as standard) to chelate iron using ferrozine method or zinc and copper using the murexide method is carried out *in vitro*. When the increased volumes of the HSE studied were taken from a stock solution of a fixed concentration 1 mg/ml at 25–175 µl for the iron and zinc chelating assay, 1–7 mg/ml for the copper chelating assay gave a significant ($p \leq 0.01$) activity. The obtained results showed that the HSE have the highest capacity to chelate ferrous ions below the EDTA (standard chelator) with absorbance arrive to the lesser extent 0.24±0.005, 0.04±0.013 which expresses 86% and 97% (compared to the control) of inhibition, respectively. For the murexide chelation, the results obtained also showed that the HSE and EDTA have a good ($p \leq 0.01$) chelating dose dependent effect towards zinc and copper ions with increased absorbance 0.45±0.02 and 0.42±0.02 with 54% and 56% of inhibition, respectively, for the zinc chelation and 0.66±0.03, 0.13±0.005 represent 44% and 88% for copper chelation. In contrast to the antioxidant capacity, the extract of hexane, ethyl acetate, chloroform and methanol from leaves, stems and roots of the *Phragmites australis* plant have a very low scavenger effect to the radical DPPH where the maximum inhibition is approximately 13.79%, obtained with the maximum volume. Finally, the HPLC analysis of effective extract (HSE) confirmed the presence of oxalic, citric, malic, succinic, fumaric, formic, acetic, propionic and butyric or iso butyric acid.

Keywords: Chelation, *Phragmites australis*, Common reed, DPPH, Heavy metal.

INTRODUCTION

Phragmites australis (*P. australis*), a worldwide distributed wetland grass, is traditionally used in food preparation and as a spice in China. However, the pharmacological effect of this plant is poorly understood [Zhu et al. 2017].

Heavy metals are the chemical elements that currently correspond to the generic term of ETM, these elements actually designate a large number of metals with an interest more and more important in the life of modern societies and toxic nature to the environment and to humans [Niencheski and Baumgarten 2000].

Globally, a large number of plants possess very interesting biological properties which find their application in various fields, especially in environment. For instance, *Phragmites australis* is a macrophyte plant of ecological interest that is frequently found in wetlands and in temperate and tropical regions. This plant has a strong ability to chelate heavy metals such as Fe, Zn, and Cu [Jiang and Wang, 2007, Rocha et al. 2014].

The evaluation of phyto-therapeutic properties, such as chelation ability and antioxidant is considered very important and useful, especially for the plants which are widely used in folk medicine. These plants represent a large source of biologically active substances. Indeed, their active ingredients exhibit interesting biological activities that are the subject of numerous studies, both *in vivo* and *in vitro* [Sellal et al. 2016].

MATERIALS AND METHODS

Collection and Extraction preparation of plant materials

The young stems of *P. australis* were collected from the Elhamadia region situated south of Bordj Bou Arreridj (east of Algeria) in March 2013. The collected plant materials were rinsed with running tap water and dried in shade for four weeks. After drying, the samples were ground in moulinex mill until a fine powder of clear green color was obtained. The powder was prepared just prior to extraction.

The air-dried stem powders (25 g) were successively extracted through soxhlet extraction using solvents (190 ml) of increasing polarity of hexane, chloroform, ethyl acetate and methanol for six cycles for each one. The extracts were then concentrated to dryness at 45°C in a rotary vacuum evaporator (Buchi). Finely, the extracts were dried and stored at 4°C in a sterile container for further use [Sanjeevkumar et al. 2014].

Ferrous in chelating activity

The chelation of iron (II) ions by the *P. australis* extracts was determined as described by Gulzar et al. (2013) with some modifications. It involved 500 µl of hexane, chloroform, ethyl acetate and methanol extracts solutions (1 mg/ml in DMSO) or EDTA (1 mg/ml in distilled water) which was mixed with 50 µl $FeSO_4$ (2 mM) in distilled water and 450 µl methanol. Five minutes later, 50 µl of ferrozine (5mM in distilled water) was added to the mixture. The controls contained all the reaction reagents except the extract. After a 10 min equilibrium period, the absorbance of the solution was measured spectrophotometrically at 562 nm. The percentage of inhibition of ferrozine-Fe^{2+} complex formation was given by the formula:

$$\% \text{ inhibition} = [(A_C - A_S)/ A_C] \times 100 \quad (1)$$

where: A_C was the absorbance of the control and A_S was the absorbance in the presence of the sample of *P. australis* extracts and standard.

Zinc and copper chelating assay

The chelating activity of zinc ions for *P australis* stem extracts solution 1 mg/ml or 10 mg/ml in DMSO for Zn and Cu chelating activity, respectively, was measured using the method of murexide described by Watak and Patil, (2012) with a slight modification. 0.5 ml of DMSO containing different volumes (25, 50, 75, 100, 125 and 175 µl) of HSE, CSE, EASE, MSE or EDTA (1 mg/ml in deionized water as a standard) for Zn chelation assay or 0.5ml of different concentrations (1, 2, 3, 4, 5, 7 mg/ml of extracts) or (10, 20, 30, 40, 50, 70 µg/ml of EDTA) for copper chelation was mixed with 1.6 ml deionized water and 0.1 ml of $ZnCl_2$ or $CuSO_4$ 8 mM (in HCl buffer 0.1M prepared by addition of KCl 0.1M at pH 5). After 30 seconds, 0.1 ml of murexide (5 mM in the same buffer) was added. Murexide reacted with the divalent zinc or copper to form stable magenta complex species that where very soluble in water. The mixture was then kept at room temperature in the dark for 10 min, and the absorbance was measured at 462 nm. The control containing deionized water without sample. A lower absorbance of the reaction mixture indicated a higher Zn^{2+} or Cu^{2+} chelating activity. The test was carried out in triplicate. The percentage of inhibition of murexide-Zn^{2+} or Cu^{2+} complex formation was given by the formula:

$$\% \text{ inhibition} = [(A_C - A_S)/ A_C] \times 100 \quad (2)$$

where: A_C was the absorbance of the control and A_S was the absorbance in the presence of the sample of plant extracts and standard.

DPPH radical-scavenging assay

The free radical scavenging activity of the HSE, CSE, EASE and MSE of reed plant was measured by 1,1-diphenyl-2-picrylhydrazil (DPPH) using the method described by Que et al. (2006), Sellal et al. (2012) with slight modification. Practically, 0.1 mM solution of DPPH· in ethanol was prepared. 2 ml of this solution were mixed with 2 ml distilled water containing various volumes of different extracts solutions (10 mg/ml in DMSO) or ascorbic acid solution (0.5 mg/ml in distilled water) as a standard. Then, the mixture was kept at room temperature in the dark for 30 min, and the absorbance was measured at 517 nm. The control contained distilled water without sample.

A lower absorbance of the reaction mixture indicated a higher DPPH radical-scavenging activity. The test was carried out in triplicate.

HPLC

The analyses by HPLC were performed at 55°C under isocratic conditions. The mobile

phase consisted of H_2SO_4 0.005 mol/L solution. This solution was filtered through a 0.45 µm Millipore membrane (omnipore TM merck Millipore Ltd) and degassed by sonication for 10 min before use. Flow rate was 0.9 ml/min and the injection volume was 20 µl. Short chain aliphatic fatty acids (fumaric, formic, malic, acetic, propionic, isobutyric or butyric, acetic, oxalic, citric and succinic acids) were analyzed with UV-Vis detection at 206 nm. The analytical column used was HPX-87H (300 mm 7.8 mm and 9 µm) [Sa et al. 2011].

Statistical analysis

The results are expressed as mean ± SD of three parallel replicates. Student's *t* test was used to compare all products (extracts and standard) against the control using Microsoft Excel software. Parametric one-way analysis of variance (ANOVA) was followed by LSD comparisons to compare the mean values among the groups was performed using CoStat software. The *P* values < 0.01 were regarded as significant.

RESULTS

Ferrous ion chelating activity

The iron-chelating activity is based on the absorbance measurement of iron (II)–ferrozine complex. This complex produced a red chromophore with a maximum absorbance at 562 nm. The chelator agents are able to capture ferrous ion before ferrozine. Fig. 1 demonstrates that the addition of increased volumes of hexane stem extract and EDTA (as a standard) induces a significant ($p \leq 0.01$), dose-dependent decrease in the optical density at 562 nm compared to the control, suggesting that this extract possesses a high capacity to chelate iron, where the obtained absorbance decreased to a lesser extent 0.24±0.005, compared to the EDTA (standard chelator) 0.04±0.013. The addition of the same volumes of the other stem extracts (chloroform, ethyl acetate and methanol) showed a high stable absorbance which is still higher than 1. This proves that those extracts have a lesser capacity to chelate ferrous ions compared to the EDTA (standard chelator) and hexane stem extract.

The difference between hexane extract and the control was statistically significant with maximum inhibition that exceeds 86% from 100 µl. The EDTA (standard chelator) produced to a significant ($p \leq 0.01$) effect (97%) compared to the control. The comparison of variances between our products used (extracts and EDTA) showed a significant difference in the following order EDTA > HSE > MSE > CSE > EASE. This analysis also showed that EDTA and HSE regroup in the same range.

Zinc and copper chelating assay

For this assay, the chelator was able to capture the ions and inhibit the formation of Zn^{2+} or

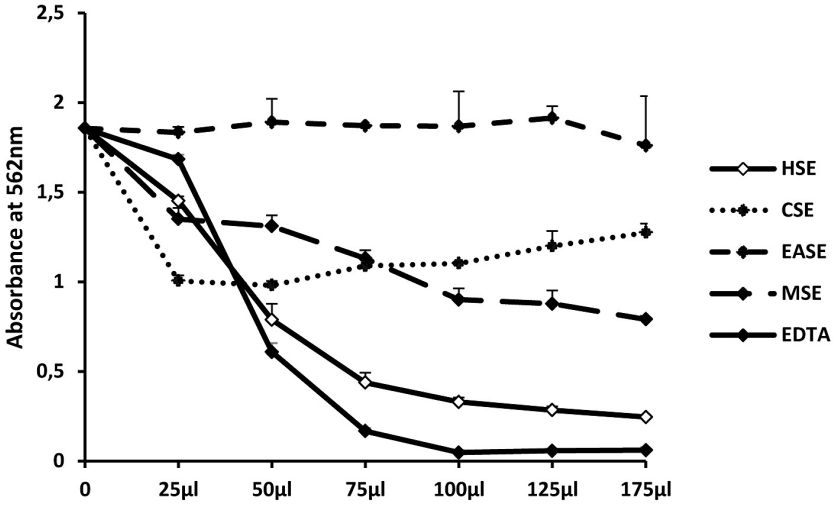

Figure 1. Iron chelation activity by increasing volumes (25 to 175 µl) of hexane stem extract (HSE), chloroform stem extract (CSE), ethyl acetate stem extract (EASE), methanol stem extract (MSE) of *P.australis* and EDTA (as a standard) at 562 nm. The results present mean ± SD (n = 3). The results were compared by the Student's t-test with a P < 0.01 taken as significant.

Cu^{2+}- murexide complex which has an absorption maximum at 462 nm, where the low absorbance indicates a high chelating activity. For zinc, the addition of different volumes (25, 50, 75, 100, 125 and 175µl) of HSE, CSE, EASE and MSE or EDTA (as standard chelator) showed that HSE and EDTA have the highest significant ($p \leq 0.01$) capacity dose dependent among them with increased absorbance 0.45±0.01 and 0.42±0.02 (fig. 2) and highest percentage of inhibition (54% and 56%) at 100 µl, respectively.

For the copper chelation, using different concentrations (1, 2, 3, 4, 5, 7 mg/ml) of the HSE or (10, 20, 30, 40, 50, 70 µg/ml) of the EDTA showed a significant ($p \leq 0.01$) dose dependent ability to chelate copper ions with 0.66±0.03 (fig. 3 A) at 7 mg/ml, compared to the control and the other extracts (CSE, EASE and MSE) which presented a high stable absorbance. However, this capacity was still low compared to the standard chelator which gave a very significant ($p \leq 0.001$) capacity with 0.13±0,005 at only 70 µg/ml (fig. 3 B).

DPPH assay

In the DPPH assay, the antioxidants were able to reduce the stable radical DPPH (purple) to the yellow colored diphenyl-picrylhidrazine. The method is based on the reduction of an alcoholic DPPH solution in the presence of hydrogen-donating antioxidant due to the formation of the non-radical form of DPPH-H [Bougatef et al. 2009].

Fig. 4 illustrate that the absorbance obtained with reed extracts remains high and stable, despite the increasing volumes compared to the ascorbic acid as a standard. The difference between the extracts and the control was not statistically significant, where the maximum inhibition is approximately 13.79% obtained with the maximum volume of the MSE. In contrast, ascorbic acid produced a highly significant ($p \leq 0.01$) effect (95%) compared to the control. These results reflect the absence of different scavenger compounds in the reed extracts.

HPLC

Quantitative and qualitative HPLC / UV-vis analyses were performed using stem hexane extract from the reed (*Phragmites australis*). The identification is based on the comparison between the retention times of the extract constituents with those of the different standards analyzed under the same conditions. The data on standard retention times and stem hexane extract are presented in Fig. 5 and 6, respectively; the quantitative data are presented in Table 1. The comparison of the retention times enabled to suspect the presence of seven acids (oxalic, citric, malic, fumaric, formic, acetic and propionic acid).

DISCUSSION

Iron, zinc and copper chelating activity

The total chelating potential of hexane stem extract is mainly caused to the presence of short

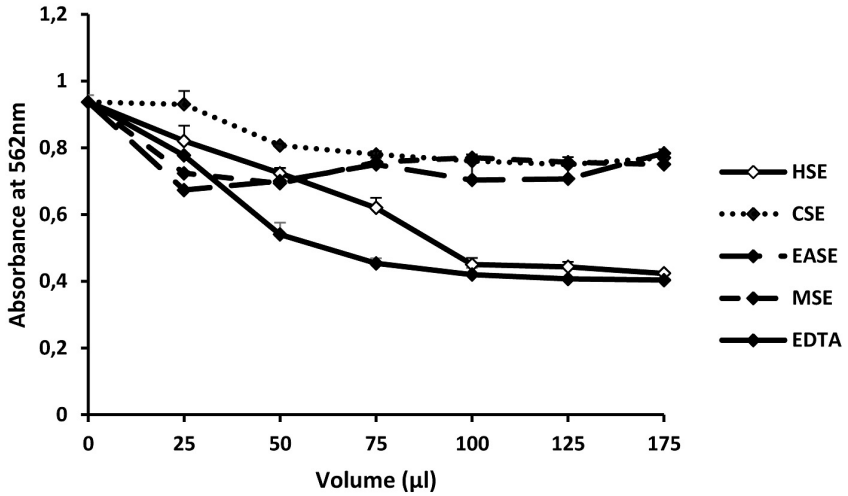

Figure 2. Zinc chelation activity by increasing volumes (25 to 175 µl) of hexane stem extract (HSE), chloroform stem extract (CSE), ethyl acetate stem extract (EASE), methanol stem extract (MSE) of *P.australis* and EDTA (as a standard) at 562 nm. The results present mean ± SD (n = 3). The results were compared by the Student's t-test with a $P < 0.01$ taken as significant.

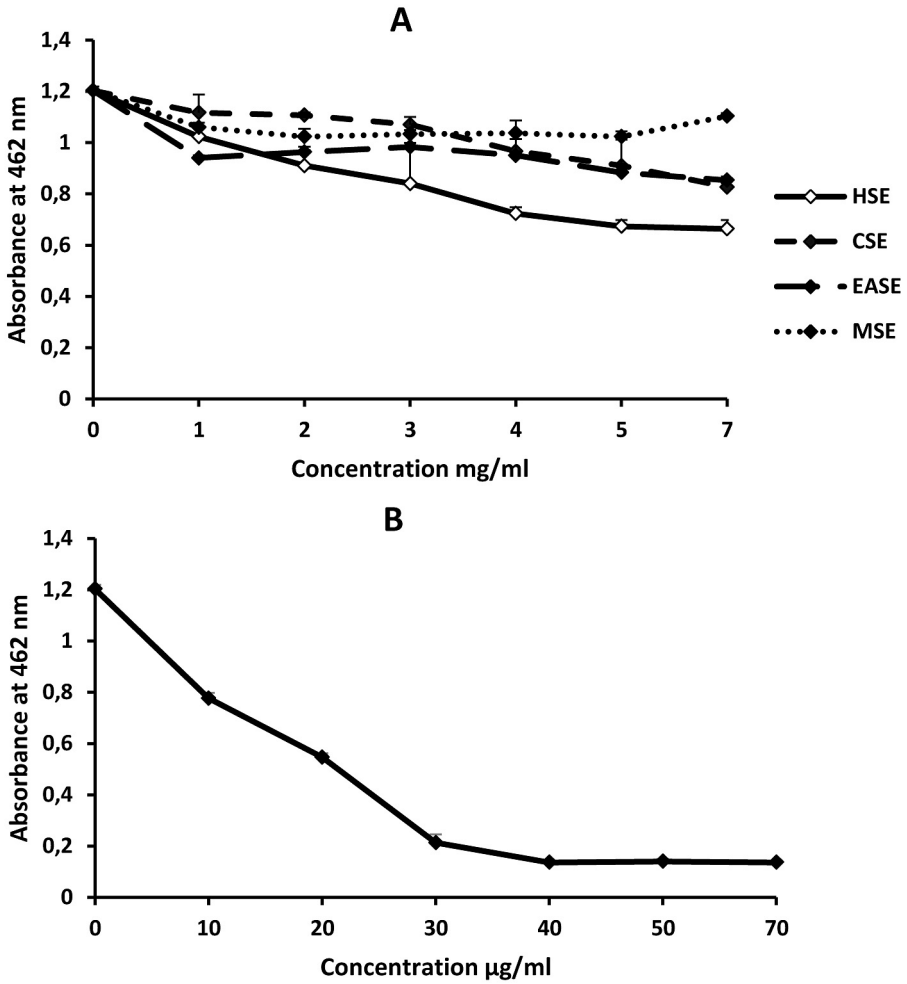

Figure 3. Copper chelation activity **A:** of hexane stem extract (HSE), chloroform stem extract (CSE), ethyl acetate stem extract (EASE), methanol stem extract (MSE) of *P.australis* **B:** EDTA (as a standard) at 462 nm. The results present mean ± SD (n = 3). The results were compared by the Student's t-test with a $P < 0.01$ taken as significant.

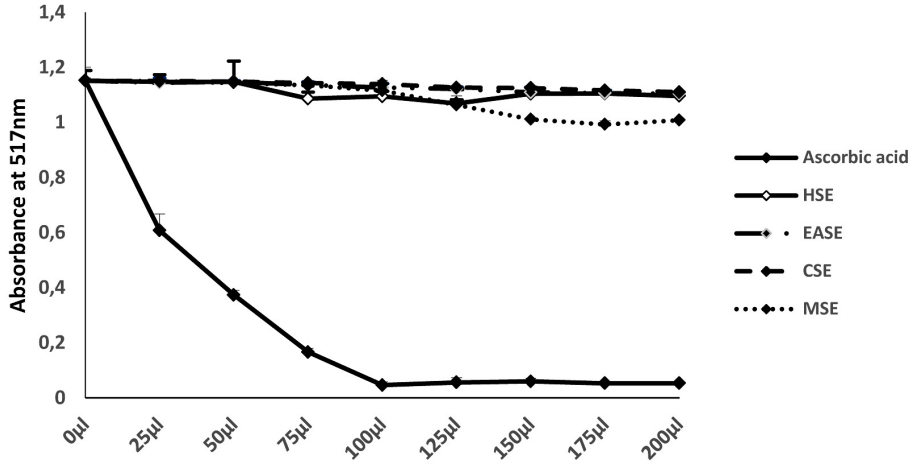

Figure 4. DPPH radical scavenging activity by increasing volumes (25 to 200 µl) of HSE, CSE, EASE and MSE extracts and ascorbic acid as a standard at 517 nm. The results present mean ± SD (n = 3). The results were compared to the control by the Student's t-test (compared to the positive control).

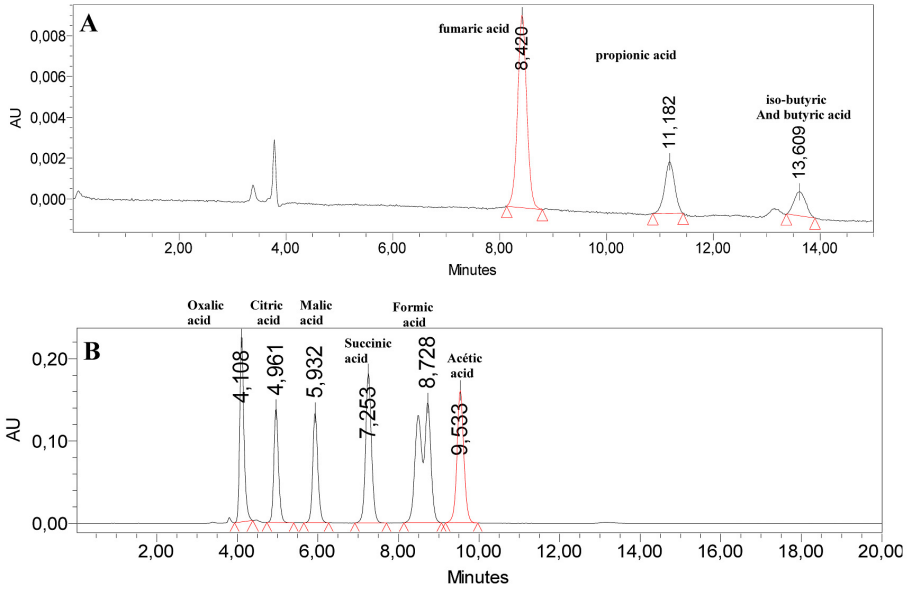

Figure 5. HPLC chromatograms of standard organic acids. **A:** fumaric acid, propionic acid and iso-butyric or butyric acid. **B:** oxalic, citric, malic, succinic, formic and acetic acids.

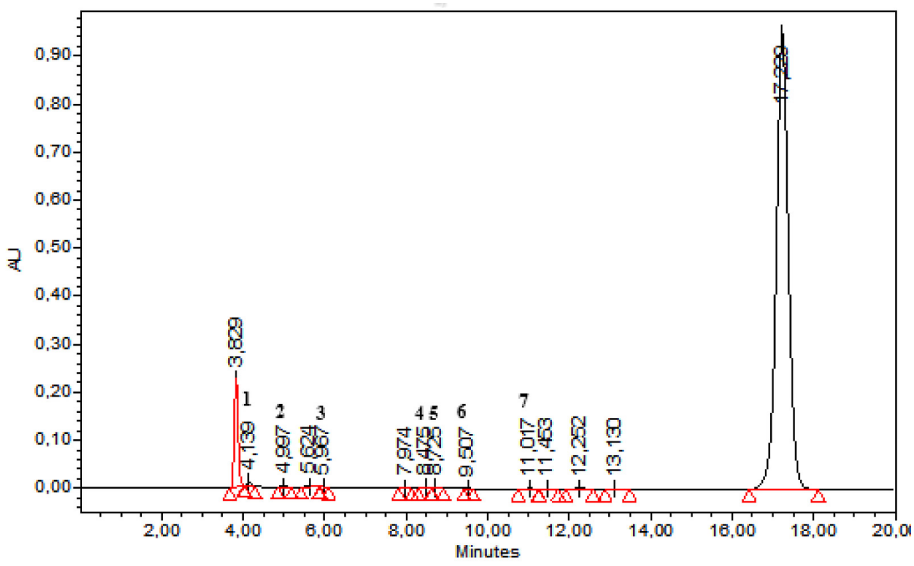

Figure 6. HPLC chromatogram of HSE.

Table 1. Organic acid profile of the reed (*Phragmites australis*)

Constituents	HSE mg /kg	Surface	RT
Oxalic acid	0.001	42350	4.139
Citric acid	0.001	19770	4.997
Malic acid	0.0064	2092	5.967
Fumaric acid	4.01	10878	8.475
Formic acid	0.001	9520	8.725
Acetic acid	0.0312	2582	9.507
Propionic acid	14.13	8820	11.017

fatty acids. There are many reports of the presence of active compounds in reed hexane extract including fumaric, propionic and iso-butyric acid, which have the ability to form complexes with bivalent ions of iron, zinc and copper [Shivhare 2011].

Many studies are in accordance with our results. Indeed, it was reported that macrophyte plants such as the reed are able to accumulate important concentrations of heavy metals such as zinc, iron and lead significantly and transfer them to the aerial parts at the cellular vacuoles in the form of complexes with organic acids such as the citrate, acetate, oxalate and fumarate [Ullah et al. 2015].

Several studies also showed that the organic acids present in plant extracts such as oxalate, acetate, malate and citrate have a high affinity for bivalent ions [Harmens et al. 1994].

Some studies also showed that *Phragmites australis* is capable of absorbing and accumulating metallic trace elements such as Zn, Cu, Fe, Cd and Pb. The accumulated metals, will be transported to the aerial parts in cationic forms in order to be finally stored in vacuoles as complexes with organic acids [Kleche et al. 2013].

DPPH assay

The lesser DPPH radical scavenging capability obtained reflect the absence of different antioxidants compounds in different stem extracts.

The results of [Balcerek et al. 2009] show that the aerial parts of *Phragmites australis* contains very low concentrations of total phenolic components, which do not exceed 0.81±0.03%. This is confirmed also by the bibliography; indeed, certain works found that the *P. australis* methanolic extract with 80% contains only 5.78±0.07 mg equivalent gallic acid per gram of dry matter [Kahkonen et al. 1999, Balcerek et al. 2009].

Other studies showed that the scavenger activity of the radical DPPH of the roots of *Phragmites communis* is very low with high concentrations that comes up 2500 mg/l and gave only a percentage of 7.8±0.32% [Boo et al. 2012].

CONCLUSION

The ability of HSE, CSE, EASE and MSE from *Phragmites australis* plant to chelate iron, zinc and copper was evaluated *in vitro*. The obtained results showed that the hexane extract has a very good chelation capacity of the metals, which is of the order $Fe^{a} > Zn^{b} > Cu^{b}$. The HPLC confirmed the presence of oxalic, citric, malic, fumaric, formic, acetic and propionic acid responsible for the metals sequestration. In contrast to the antioxidant ability, the results showed the absence of different scavenger compounds in all extracts used.

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