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Research Article

Study of the Chelating Ability of Hexane, Chloroform, Ethyl acetate and Methanolic Root Extracts from Algerian *Phragmites australis* Species

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ABSTRACT

Introduction: Heavy metals that enter organisms can cause illness and disease. Chelation therapy is a solution that uses chelating agents to protect these organisms by high accumulation. **Materials and methods:** The objective of this study is to evaluate the capability of different roots extracts from reed or EDTA (as standard) to complexate iron using, ferrozine-ferrous reducing assay or Zn and Cu using the murexide-Zn or Cu reducing assay *in vitro*. **Results:** the results proved that the organic phase of the first extract and EDTA (standard chelator), have the strongest chelation activities with respective rates of 70% and 97% for iron, 55% and 56% for zinc and 47% and 88% for copper compared to the control groups considered to be 100% of the complexation. Finally, the HPLC analysis showed the presence of six organic acids, which are probably responsible for this effect. **Conclusion:** The hexane extract and EDTA (standard), have the strongest chelation activities.

Keywords: *Phragmites australis*, Chelation, Heavy metals, Organic acids, Ferrozine, Murexide.

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INTRODUCTION

Pollution is an unfavorable change in the environment caused totally or partially by humans either directly or indirectly, as it can occur naturally under the influence of different physical, chemical and biological agents and therefore resulting in deterioration of the quality of water, air or soil. It affects the distribution of energy flows, radiation levels and the physico-chemical properties of the ecosystem. This change can affect humans directly or through agricultural, water or biological resources. The degradation of the quality of the environment as a result of pollution is due to the loss of vegetation, biological diversity, excessive amounts of harmful chemicals in the atmosphere and in cereals intended for food (Zhao *et al.* 2019)¹.

Heavy metals are considered a major source of environmental contamination. They are toxic, persistent and can negatively affect all life forms. In humans they are absorbed directly by ingestion or inhalation and concentrated in different parts of the human body resulting in chronic or acute effects on human health, including physiological disorders of the respiratory system, renal and gastrointestinal (Sellal *et al.* 2019a)².

Chelation is a treatment that uses chelating agents to treat disorders caused by an abnormally high accumulation of metal ions in organisms. Although heavy metals are toxic to humans and plants and can cause pollution of water, soil and air, they need to be removed before they are released to the environment. (Wu, 2016)³.

Chelating agents are organic or inorganic compounds that have the ability to bind to metal ions to result in the formation of a "chelate" which is a complex ring-like structure (Flora and Pachauri, 2010)⁴.

The short-chain aliphatic fatty acids of the plant *Phragmites australis* represent a group of monocarboxylic organic acids, which are also classified among the natural chelators of heavy metals (Cu, Zn and Fe) thanks to the presence of carboxylic group (Kumar *et al.* 2015)⁵.

The aim of this research is to study the chelating power of different extracts from the root part of the *Phragmites australis* plant and to identify the active substances in the effective extract.

MATERIALS AND METHODS

Extracts preparation

The roots of reed plant were collected in the southern of Bordj Bou Arreridj (eastern Algeria). The material was rinsed and dried. The material is then ground up to obtaining a fine powder. Then, the 25 g of the last preparation were extracted by 190 ml of different solvents with increased polarity hexane, chloroform, ethyl acetate and methanol successively using soxhlet, and the obtained were then evaporated at 45 °C in a rotary vacuum evaporator (Buchi), dried and stored at 4 °C (Sanjeevkumar *et al.* 2014)⁶.

Ferrozine-ferrous reducing assay

The ferrous chelating ability of roots *P. australis* extracts was evaluated by the method described by Le *et al.* (2007) with some modifications⁷. The five hundred µl of Ethylene Diamine Tetra acetic Acid (1 mg/ml as standard) or different roots extracts (1 mg/ml as sample) are added to fifty µl FeSO₄ (2 mM) and 450 µl methanol. 5 minutes later, the complexing reaction is stimulated by the addition of fifty µl ferrozine (5mM) and the absorbance of the solution was measured spectrophotometrically at 562 nm after 10 min. The control group contained all product except the extract.

Murexide-zinc or copper reducing assay

The zinc and copper chelating ability of roots *P. australis* extracts was determined by the method described by Watak and Patil, (2012)⁸. practically, the volume of 0.5 ml containing 25, 50, 75, 100, 125 and 175 µl for each extract taken from a stock solution (1 mg/ml) or Ethylene Diamine Tetra acetic Acid (1 mg/ml) was prepared for Zn chelation assay. For copper chelation the same volume contains a different concentrations (1, 2, 3, 4, 5, 7 mg/ml of extracts) or (10, 20, 30, 40, 50, 70 µg/ml of Ethylene Diamine Tetra acetic Acid) is also prepared. Preparations are then added to 1.6 ml deionized water and 0.1 ml of ZnCl₂ or CuSO₄ (8 mM). 30 seconds later, the complexing reaction is stimulated by the addition of 0.1 ml of murexide (5 mM) and the mixture is incubated in the dark for 10 min. After, the absorbance was measured spectrophotometrically at 562 nm against a blank, which contains all the solutions used except the extracts.

The percentage of inhibition of ferrozine-Fe²⁺ or murexide-Zn or murexide-Cu complexes formations was given by the formula:

$$\% \text{ inhibition} = [(Ac - As) / Ac] \times 100$$

Where: AC was the absorbance of the control and As was the absorbance in the presence of the sample of *P. australis* extracts and standard.

HPLC

The analysis and identification of organic acids (fumaric, formic, malic, propionic, isobutyric, acetic, oxalic, citric and succinic acid) of the root hexane extract are carried out by the chromatographic method UV-Vis at 206 nm according to the method described by Sa *et al.* (2011)⁹.

Standard solutions (1000 mmol/l) are prepared separately in ultra-pure distilled water (HPLC grade) and stored in the freezer until 'in use.

The chromatographic chain (HPLC) used for the analytical control is brand waters 2695 and includes the following elements: a degasser (waters), two pumps (waters) of an isocratic system, an auto sampler and a waters 2996 detector connected with computer software (Empower). The analytical column used is an ion exchange column of the brand Aminex HPX-87H used for the analysis of carbohydrates, volatile fatty acids and fatty acids with a short aliphatic chain (length 300 mm, diameter 7.8 mm and the size of the 9 µm particles).

The mobile phase is a sulfuric acid solution at 0.005 mol/l introduced at 55 °C with a flow rate of 0.9 ml/min and injection volume of 20µl in isocratic mode after filtration (on a membrane omnipore TM merck millipore Ltd with a porosity of 0.45 µm) and degassing by ultrasound.

Statistical analysis

The results are presented as mean ± SD of three replicates. All products (extracts and standard) are compared against the control using Student's t test. The ANOVA was used to compare the means between groups using CoStat software. P < 0.01 considered as significant.

RESULTS

Ferrozine-ferrous reducing assay

Ferrozine forms a red complex with ferrous ions. the chelation effect is therefore inversely proportional with the red color. In general, hexane extract and EDTA showed significantly ($p \leq 0.01$) higher ferrous ion-chelating capacity than the other extracts, where the obtained absorbance dose-dependent decreased to a lesser extent of 0.54 ± 0.01 and 0.046 ± 0.01 respectively (Figure 1). In contrary, the same volumes of the other roots extracts (chloroform, ethyl acetate and methanol) showed a high stable absorbance which is still higher than 1. The difference between hexane extract or EDTA and the witness was statistically significant with maximum inhibition of 70% and 97% respectively.

Figure 1: The Iron chelating ability of different root reed extracts and EDTA (as a standard) at 562 nm. The absorbance's presented as mean ± SD of three replicates and P < 0.01 taken as significant.

Murexide-zinc or copper reducing assay

For zinc chelation, the hexane extract of root part and EDTA at a volume of 175 μ l taken from a stock solution of 1 mg/ml gives a lower highly significant absorbance's ($p \leq 0.01$) of 0.41 ± 0.005 and 0.40 ± 0.005 respectively than that of the negative control 0.93 ± 0.02 considered as 100% of the complexation and to the other extracts which are given high

and stable absorbances despite the increase in concentration (Figure 2).

The hexane roots extract from the reed is generated therefore an effect very close to that of EDTA where the zinc ions are chelated with an approximate percentage of 55% and 56% respectively compared to control group.

Figure 2: The murexide-Zn reducing ability of different root reed extracts and EDTA (as a standard) at 562 nm. The absorbance's presented as mean $\pm$ SD of three replicates and $P < 0.01$ taken as significant.

For copper chelation, the addition of hexane extract to the mixture induces a marked reduction in absorbance compared to the control (1.20 ± 0.01 , $p \leq 0.01$). Indeed, the hexane extract of the leaf reveals an absorbance of 0.63 ± 0.01 with a concentration of 7 mg/ml (Figure 3 A). The copper complexation with murexide was thus reduced by approximately 47% compared to the negative control considered as 100% of the complexation. These results

remain lower than those obtained with EDTA, where the minimum absorbance obtained with only a volume of 70 μ l is 0.13 ± 0.005 (Figure 3 B) which corresponds to a maximum inhibition of 88% compared to the control. Finally, the ANOVA comparison between all products used (extracts and EDTA) for the assays showed a significant difference in the following order EDTA > HRE > MRE > CRE > EARE.

Figure 3: The murexide-copper reducing results A: different root reed extracts. B: A standard. The results expressed as mean $\pm$ SD of three replicates and compared by the Student's t-test where $P < 0.01$ taken as significant.

High performance liquid chromatography analysis of hexane root extract

The qualitative analysis based on the comparison of retention times between the sample (hexane root extract)

and the standards proved the presence of six organic acids (figure 4 and 5) and the quantities presented in table 1 also calculated using the chromatograms areas comparison between the sample and standards.

Figure 4: Chromatograms of standard mixture at 206 nm. The concentration of analytes added was 1000 mmol/L. Chromatographic conditions: column Aminex HPX-87H, injection volume of 20 μ L, flow rate of 0.9 mL/min and temperature at 55° C.

Figure 5: Chromatograms after screening of hexane root extract under the same conditions.

Table 1: Organic acid quantities in the hexane root extract.

Constituents	HRE (mg /kg)	Surface	RT
Oxalic acid	0.0001	15119	4.086
Citric acid	0.033	48644	4.988
Malic acid	0.023	19146	5.983
Fumaric acid	14.62	34760	8.486
Formic acid	0.003	14656	8.779
Propionic acid	19.37	12318	10.992

DISCUSSION

Chelation means the insertion of a mineral ion into a complex cyclic structure by an organic or inorganic ligand which represents the chelating agent. Generally, sulfur, nitrogen and / or oxygen are the electron donor atoms that are found in the chelating agent because they contain pairs of solitary electrons. The strength of the chemical bonds within the complexes formed between the chelators and the metal ions rests on the elements involved and their stereochemistry (Sears, 2013)¹⁰.

Chelation is considered a favorable solution to reduce the toxicity of metals. Complex structures are formed (chelates) after binding of chelating agents with toxic metal ions, these structures are easily removed from inside or outside of cells (Flora and Pachauri, 2010)⁴.

The chelating capacity of the hexane extract is mainly due to the presence of organic acids and fatty acids with a short aliphatic chain. Several studies confirm the presence of active compounds in the reed hexane extract, in particular oxalic, citric, fumaric, formic and propionic acid, which have the capacity to form complexes with bivalent ions of iron, zinc and copper (sellal *et al.* 2019a)².

Chelating agents have "ligand" binding atoms which form either covalent bonds (an electron of each atom to form a pair of electrons binding the two atoms), or covalent coordination bonds (a covalent bond between two atoms for which the two electrons shared in the bond come from the same atom) or else the two types of bonds, covalent bonds and covalent coordination bonds.

The ligand atoms are generally sulfur, nitrogen and oxygen (S, N, O), they are found in the form of chemical groups such as (-SH), (-SS), (-NH₂), (=NH), (-OH), (-OPO₃H) or (>C=O). The majority of donor atoms act as bidentate ligands. Cyclic structures containing the metal ion and the atoms with two ligands bonded to the metal are then formed in the case of bidentate and polydentate ligands (Figure 6). In the simplest case, a proton (H⁺) of an organic acid that can absorb the lone pair of electrons of ligand-binding atom(s) of the chelator may be involved in the coordination complex formation. However, the positive charge on proton remains

since there is no loss or gain of electrons in the process (Flora and Pachauri, 2010)⁴.

Figure 6: Formation of metal ligand complexes using mono, bi and polydentate ligands (Flora and Pachauri, 2010).

Organic acids are organic compounds with acid properties and therefore capable of releasing a (H) or (H₃O) cation in the medium.

They are classified into various molecules such as monocarboxylic acids (propionic and butyric acids), dicarboxylic acids (fumaric, malic and oxalic acids) or tricarboxylic acids (such as citric acid). Organic acids are also involved in the complexation of metal ions at the cellular level and represent another group of natural chelators alongside metallothionein and phytochelatin (Kumar *et al.* 2015)⁵.

Fatty acids represent a group of organic monocarboxylic acids which are also classified among the natural chelators of heavy metals (Cu, Zn and Fe) thanks to the presence of carboxylic group (R-COOH). The latter is a donor group capable of releasing a proton (H⁺) which is replaced by a metal ion (M) according to four modes of coordination: ionic, monodentate, bidentate and by bridging (Figure 7) (Martin, 2015)¹¹.

Figure 7: Types of Metal Carboxylate Coordination Modes.

The roots of reed plant can absorb and accumulate high quantities of toxic metals. this capacity is mainly due to the parenchyma cortex which has large intercellular air spaces. Then the toxic elements were transferred in vacuoles to the leaf parts (Sellal *et al.* 2016)¹².

Our results also, confirm that the hexane extracts from the leaves and stems of the *Phragmites australis* plant have the strongest chelation activities with respective rates of 89% and 86% for iron, 56% and 54% for zinc and finally 64% and 44% for copper (Sellal *et al.* 2016 ; Sellal *et al.* 2019b)^{12, 13}.

CONCLUSION

The chelating power of different extracts from the root part of the reed plant against Fe, Zn and Cu was evaluated *in vitro*. The obtained results, showed that the hexane extract and EDTA (standard), have the strongest chelation activities with respective rates of 70% and 97% for iron, 55% and 56% for zinc and 47% and 88% for copper compared to the control groups. These effects are probably due to the presence of oxalic, citric, malic, fumaric, formic and propionic acids confirmed by an HPLC analysis.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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