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Research Article

## Total Phenolic Contents and Antioxidant Properties of Algerian *Alkanna tinctoria* aerial part Extracts

Thoraya Guemmaz\*, Lekhmici Arrar and Abderrahmane Baghiani

Laboratory of Applied Biochemistry, University of Setif 19000, Setif-1, Algeria

### ABSTRACT

**Objective:** Evaluation of the polyphenols, flavonoids and tannins contents of different aqueous extracts of the aerial part of *Alkanna tinctoria* and their antioxidant activities.

**Methodology:** Three extracts; decoction, macerate and infusion of the aerial part of *Alkanna tinctoria* were prepared. The content of total polyphenols, flavonoids and tannins of the extracts was carried out and antioxidant activity was assessed using DPPH (2,2'-diphenyl-1-picrylhydrazyl),  $\beta$ -carotene bleaching and iron chelation tests.

**Results :** The results showed that Infusion (Inf) was the richest in total polyphenols ( $189.66 \pm 30.77$  mg EGA / g of dried extract) and flavonoids ( $26.80 \pm 8.37$  mg EQ / g of dried extract). While the content of tannins is almost the same for Dec and Inf ( $40.27 \pm 15.00$ ,  $40.25 \pm 18.27$  mg ETA / g of dried extract). Moreover, the two extracts Inf ( $IC_{50}$  of  $0.09 \pm 0.0015$  mg / ml) and Dec ( $IC_{50}$  of  $0.17 \pm 0.031$  mg / ml) showed a very strong anti-radical activity towards the radical DPPH. In the  $\beta$ -carotene bleaching test, Inf and Dec showed a significant inhibitory activity of peroxidation with values of 100% and 99.8% and are more effective compared to BHT (98.15%). Dec and Mac showed strong chelating activity with  $IC_{50}$ s of  $0.003 \pm 0.0002$  mg / ml and  $0.005 \pm 0.00008$  mg / ml respectively. These chelating activities remain higher than EDTA.

**Conclusion:** It may be concluded that the extracts of the aerial part of *Alkanna tinctoria* exhibit significant both content of polyphenols and antioxidant activity.

**Keywords:** *Alkanna tinctoria*, polyphenols, flavonoids, antioxidant activity.

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### \*Address for Correspondence:

Thoraya Guemmaz, Laboratory of Applied Biochemistry, University of Setif 19000, Setif-1, Algeria

### INTRODUCTION

Medicinal plants have a wide variety of biological and pharmacological activities. They are reservoirs for a wide variety of secondary metabolites, including alkaloids, flavonoids, tannins and terpenoids<sup>1</sup>. These compounds are secondary metabolites involved in different functions in plants. They have various biological activities, in particular the antioxidant potential, in particular against the oxidative process induced by reactive oxygen species which are the direct cause of various pathological states such as aging and cancer<sup>2</sup>.

Among these plants, *Alkanna tinctoria* (AT) known as Orchanet, in Arabic Aoud Hawaa<sup>3</sup>. *Alkanna* is a genus of herbaceous plants including about 50 species of the family *Boraginaceae*, originally from Europe, the Mediterranean and Western Asia<sup>4</sup>. *Alkanna tinctoria* found in Europe, W. Asia, and N. Africa, and known to the Greeks (described by Theophrastus and the Romans), has been used since antiquity<sup>5</sup>. This plant is widely used against jaundice, and

kidney stones, it has an antibacterial, astringent and vulnerary effect. It is used in the treatment of ulcers, inflammation and burns<sup>6,7</sup>. To our knowledge, research on aqueous extracts from the aerial part of *Alkanna tinctoria* is very limited. This work was undertaken to evaluate the antioxidant activities of extracts from the aerial part of *Alkanna tinctoria*, a plant whose use is very limited in traditional medicine in Algeria.

### MATERIAL AND METHODS

#### Plant material

The plant (*Alkanna tinctoria*) AT was harvested at the end of June 2017, in the region of Setif, Algeria. It was identified by Pr. Oudjih Bachir, Elhadj Lakhdar University, Batna. The aerial part of this plant was washed and cut, then dried in the shade and at room temperature, then crushed and stored until use.

## Preparation of extracts

### Decoction

A weighing of 20 g of fine powder of the plant is added to 200 ml of boiling distilled water. The mixture was kept boiling with stirring for 20 min. The decoction (Dec) is then centrifuged and then filtered through filter paper and dried in an oven at 40 °C until total evaporation of water <sup>8</sup>.

### Maceration

A macerate of the plant is obtained by adding 20 g of fine powder to 200 ml of distilled water. The mixture was stirred for 24 hours at room temperature. The macerate (Mac) is then centrifuged and then filtered through filter paper and dried in an oven at 40 °C until complete evaporation of water <sup>9</sup>.

### Infusion

A weighing of 20 g of fine powder of the plant is added to 200 ml of boiling distilled water. The mixture is stirred for 15 min at room temperature. The infusion (Inf) is then centrifuged and then filtered through filter paper and dried in an oven at 40 °C until complete evaporation of water <sup>10</sup>.

## Quantitative estimation of polyphenols, flavonoids and tannins

### Estimation of total phenolic content

The total phenol content of the aqueous extracts was determined according to the Folin-Ciocalteu method <sup>11</sup>. Different concentrations of each extract were mixed with 500 µl of Folin Ciocalteu reagent (diluted 10 times) and incubated at room temperature for 4 minutes. Then 400 µl of 7.5% sodium carbonate solution was added and incubated again for 90 minutes at room temperature. The absorbance of all samples was measured at 760 nm. The concentrations of the phenolic compounds are determined from the calibration curve. The results are expressed in milligram equivalent of gallic acid per gram of extract (mg EAG / g).

### Estimation of flavonoids content

The method of Djeridane *et al.* <sup>12</sup> was used for this test. To 500 µl of each extract (prepared with suitable dilutions in methanol) is added 500 µl of AlCl<sub>3</sub> (2% in methanol). After 10 minutes of incubation, the absorbance reading is taken at 430 nm. The flavonoids concentration is calculated using a calibration range established with quercetin (2.5 - 40 µg / ml). The concentration is expressed in milligrams of quercetin equivalent per gram of extract (mg EQ / g).

### Estimation of tanins content

The modified method of Gharzouli *et al.* <sup>13</sup> was applied for this study. Briefly, one volume of each plant extract was mixed with an equal volume of hemolyzed bovine blood (absorbance = 1.6). After 20 minutes, the mixture was centrifuged at 4000 rpm for 10 minutes and the absorbance of the supernatant was measured at 576 nm. Results were expressed as mg tannic acid per gram of extract (mg EAT / g).

## Evaluation of antioxidant power

### DPPH radical-scavenging activity

The DPPH (2,2'-diphenyl-1-picryl hydrazyl) radical scavenging activity was determined according to the method described by Mayouf *et al.* <sup>14</sup>. A volume of 50 µl of the extracts dilutions was added to 1250 µl of a methanolic solution containing 0.004% DPPH. The mixture was kept at room temperature for 30 minutes before measuring its

absorbance at 517 nm. BHT (2, 6 di-tert-butyl-4-methyl phenol) was used as the reference standard. The radical scavenging activity was calculated as a percentage (I%) as follows:

$$I\% = 100 (\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}) / \text{Abs}_{\text{control}}$$

Abs control is the absorbance of the DPPH solution, Abs sample is the absorbance in the presence of the extract.

The concentration of the extract providing 50% inhibition (IC<sub>50</sub>) was calculated from the percentage of the inhibition curve relative to the concentration of the extract.

### Linoleic acid / β-carotene assay

The antioxidant capacity was also determined by measuring the inhibition of the formation of conjugated diene hydroperoxides resulting from the oxidation of linoleic acid <sup>15</sup>. An emulsion (2.5 ml) containing β-carotene, linoleic acid and Tween-40 was added to 0.35 ml of the sample, then incubated for 48 h at room temperature. The same procedure was repeated with the BHT as the positive control, and H<sub>2</sub>O as the negative control. The absorbance of the mixture was measured at 490 nm after 0, 1, 2, 4, 6, 24 and 48 h. The antioxidant activity of the extracts was evaluated as a percentage of β-carotene bleaching:

$$I\% = \text{Abs}_{\text{sample}} / \text{Abs}_{\text{sample}} (t_0) \times 100.$$

### Chelating power of iron

The chelation of ferrous iron by the extracts is evaluated by the ferrozine method described by Belkheiri *et al.* <sup>16</sup>. Suitably diluted extracts (250 µl) were mixed with 50 µl of 0.6 mM FeCl<sub>2</sub> and 450 µl of methanol. The reaction was initiated after 5 minutes by the addition of 50 µl of ferrozine (5 mM) and incubated for 10 minutes at room temperature. The absorbance of the Fe<sup>2+</sup> - ferrozine complex was measured at 562 nm. The chelating effect was calculated as a percentage and the IC<sub>50</sub> was defined as the effective concentration of the extract which produces 50% of the maximum chelating effect. EDTA (Ethylene diamine tetraacetic) was used as a standard.

$$\text{Chelating power (\%)} = [(A_c - A_s) / A_c] \times 100$$

A<sub>c</sub>: absorbance in the absence of chelator (control).

A<sub>s</sub>: absorbance in presence of chelator (extracts or EDTA).

### Statistical analysis

The values were generally expressed as a mean ± SD. The results of the different tests are analyzed by the uni varied ANOVA test followed by the Tukey test for multiple comparisons and determination of significance rates. The comparison of the means and standard deviations is determined using the software "Graphpad Prism" version 5.0.

## RESULTS

### Yields of extraction

The extraction yields showed that the Inf (7.25 %) gave the highest yield followed by Dec (4.9 %) then Mac (4.75 %). The differences observed between the yields of the aqueous extracts have no relation to the extraction solvent which is the same. This may be due to the extraction temperature.

### Determination of total polyphenol, flavonoids and tannins contents

In order to characterize the extracts prepared, estimation of polyphenols, flavonoids and tannins was carried out. The results are shown in table 1.

**Table 1** : The contents of total polyphenols, flavonoids and tannins in extracts from the aerial part of AT.

Extracts	Polyphenols (mg EGA/g E °)	Flavonoids (mg EQ/g E) °°	Tannins (mg ETA/g E) °°°
Decoction	173.28 ± 43.1	14.62 ± 1.71	40.27 ± 15.00
Maceration	144.50 ± 20.66	1.98 ± 0.46	17.69 ± 9.63
Infusion	189.66 ± 30.77	26.80 ± 8.37	40.25 ± 18.27

° : equivalent of milligrams of gallic acid per gram of extract weight (mg EGA/g E).

°° : equivalent of milligrams of quercetin per gram of extract weight (mg EQ/ g E).

°°° : equivalent of milligrams of tannic acid per gram of extract weight (mg ETA/ g E).

Values were expressed as means ± SD (n = 3).

Results of the estimation of polyphenols show that Inf is the richest in phenolic compounds (189.66 ± 30.77 mg EGA / g of extract) followed by Dec (173.28 ± 43.1 mg EGA / g of extract), then Mac (144.50 ± 20.66 mg EGA / g of extract). The quantitative estimation of the flavonoids shows that Inf (26.80 ± 8.37 mg EQ / g of extract) is the richest in flavonoids, followed by Dec (14.62 ± 1.71 mg EQ / g of extract) then the Mac (1.98 ± 0.46 mg EQ / g of extract) respectively.

The results of the assay also show that the content of polyphenols is significantly higher than that of flavonoids, this suggests that the polyphenols present are not all flavonoids, there may be presence of other polyphenols such as tannins which have been highlighted in the present study.

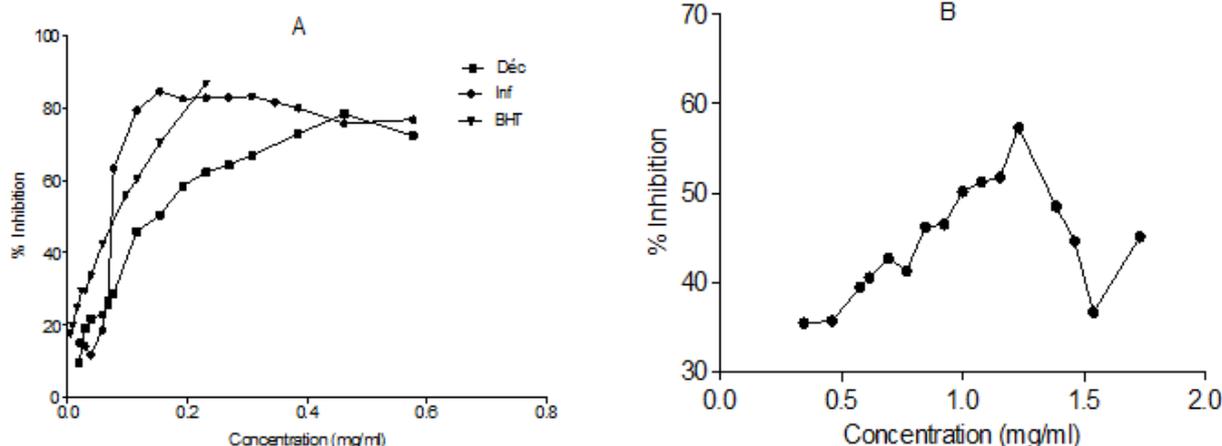
Estimation of total tannins reveal that Dec (40.27 ± 15.00 mg ETA / g of extract) and Inf (40.25 ± 18.27 mg ETA / g of

extract) are the richest in tannins with very similar values, followed by Mac (17.68 ± 9.63 mg ETA / g of extract).

#### Antioxidant activity of *Alkanna tinctoria* extracts

##### DPPH scavenging activity

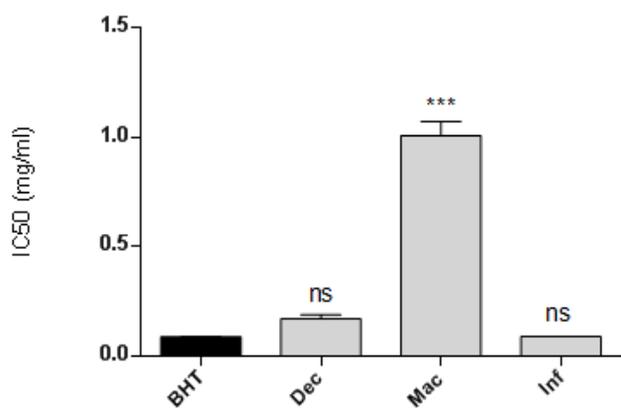
The anti-radical activity of the various extracts was evaluated by their inhibitory activity on a methanolic solution of DPPH, measured at 517 nm. The standard used was BHT. The anti-free radical activity of the various AT extracts, as well as the BHT, has been illustrated in figure 1. It is noted that the AT extracts have a dose-dependent anti-free radical activity, except the extract of the maceration, which has apparently a pro-oxidant effect. The IC<sub>50</sub> of the different extracts were determined. A lower value of the IC<sub>50</sub> (the concentration of the substrate that causes a 50% inhibition of DPPH activity) indicates higher antioxidant activity.



**Figure 1:** Scavenger effect of decoction (Dec), infusion (Inf) and BHT (A) and maceration (B) of *Alkanna tinctoria*, (The values are the average of three measurements).

The extract of the Inf presents an IC<sub>50</sub> of 0.09 ± 0.001 mg / ml followed by the extract of the Dec with an IC<sub>50</sub> of 0.17 ± 0.031 mg / ml and finally the Mac with an IC<sub>50</sub> of the order of 1.006 ± 0.111 mg / ml. The difference is not significant

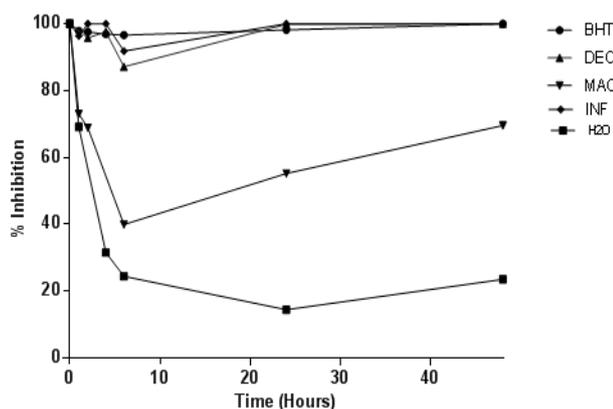
between the BHT (IC<sub>50</sub> of 0.087 ± 0.001 mg / ml) and the two extracts: Inf and Dec. On the other hand, the effectiveness of the macerate is very low compared to BHT (\*\*\*, p ≤ 0.001) (Figure 2).



**Figure 2:** IC<sub>50</sub> of the aqueous extracts of the aerial part of *Alkanna tinctoria* and of BHT on the DPPH radical, (The values of the IC<sub>50</sub> are the average of three measurements ± SD).

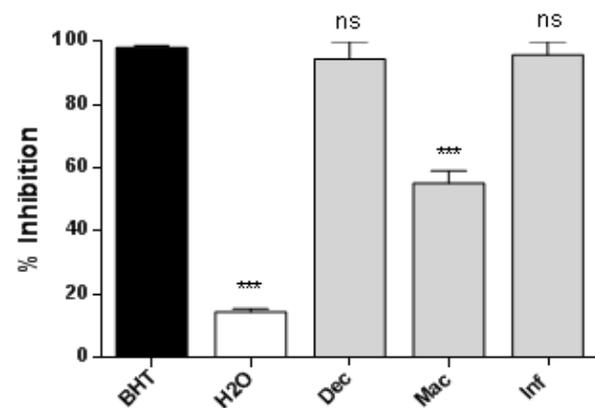
**Linoleic acid / β-carotene assay**

The bleaching of β-carotene was greatly slowed down in the presence of the two extracts Inf and Dec with antioxidant activities 100 ± 9.23 %, 99.82 ± 9.62 % respectively. While the Mac shows an activity of 55.22 ± 5.54 %. (Figure 3).



**Figure 3:** Bleaching kinetics of β-carotene at 490 nm in the presence of extracts of *Alkanna tinctoria* and BHT (the values are the average of three measurements).

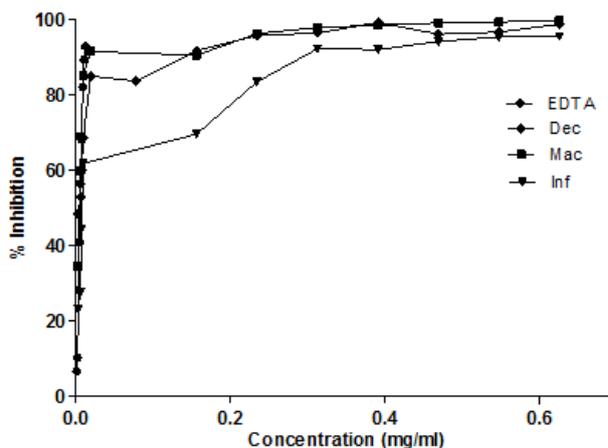
The difference is not significant between BHT and the two extracts Dec and Inf ( $p \leq 0.001$ ) (Figure 4). Moreover, the difference is significant between the BHT (98.15% ± 0.75) and the Mac (\*\*,  $p \leq 0.001$ ).



**Figure 4:** Antioxidant activity of extracts of the aerial part of *Alkanna tinctoria* and of BHT in the β-carotene / linoleic acid system, (the values are the mean of three measurements ± SD) ( $p \leq 0.001$ ).

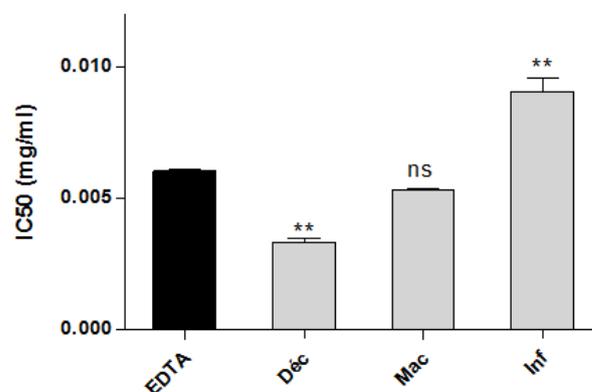
**Chelating power of iron**

The results obtained show that the aqueous extracts of the aerial part of AT have concentration-dependent chelating activity (Figure 5).



**Figure 5 :** Metal chelating activity at 562 nm in the presence of extracts of *Alkanna tinctoria* and EDTA, (Values are the average of three measurements).

The Dec extract has an IC<sub>50</sub> of 0.003 ± 0.0002 mg / ml followed by the Mac with an IC<sub>50</sub> of 0.005 ± 0.00008 mg / ml and finally the Inf with an IC<sub>50</sub> of around 0.009 ± 0.0008 mg / ml. So Dec has the strongest chelating activity among the three extracts. The difference is not significant between EDTA (IC<sub>50</sub> of 0.006 ± 0.00007 mg / ml) and MAC but significant between EDTA and the two aqueous extracts: Inf and Dec (\*\*,  $p \leq 0.001$ ) (Figure 6).



**Figure 6 :** IC<sub>50</sub> of aqueous extracts from the aerial part of *Alkanna tinctoria* and EDTA, (values are the mean of three measurements ± SD) ( $p \leq 0.001$ ).

**DISCUSSION**

The estimation of the contents of phenolic compounds, flavonoids and tannins makes it possible to correlate the results obtained with the antioxidant activity of the extracts tested.

The Folin-Ciocalteu test gives a crude estimate of the total phenolic compounds present in an extract. It is not specific to polyphenols, but many interfering compounds can react with the reagent, giving apparent high phenolic concentrations. In addition, various phenolic compounds react differently in this test, depending on the number of phenolic groups they have <sup>17</sup>.

However, at high temperatures, water also extracts unwanted substances such as proteins, lipids and non-phenolic dyes that interfere with the determination of tannins. We can then conclude that the extraction of condensed tannins depends on their chemical nature, the solvent used and the operating conditions. However, the contents of condensed tannins can also be variable due to several factors such as: the sensitivity of the tannins to several degradation pathways (oxidation, light, etc.), cultural and climatic conditions<sup>18</sup>.

According to Tiwari *et al.*<sup>19</sup>, the amount and composition of secondary metabolites of an extract depend on: type of extraction, extraction time, temperature, nature of the solvent, concentration of solvent and polarity.

Antioxidants can exert their protecting effects by various mechanisms, radical scavenging or preventing the generation of ROS; they act by inhibiting the initiation and propagation steps leading to the termination of the reaction and delay the oxidation process. They also act by binding of transition metal ion catalysts, and subsequently, they retard the progress of many chronic diseases<sup>20</sup>.

In this context, Three methods are used to assess antioxidant activity, the DPPH scavenging, the  $\beta$ -carotene bleaching and the iron chelation tests.

The DPPH radical scavenging assay is a simple, fast and sensitive method for the antioxidant screening of plant extracts<sup>21</sup>. The anti-radical activity is measured by the discoloration of DPPH, which is a relatively stable free radical, from purple to a yellow color. The discoloration explains the power of the plant extract to scavenge this radical.

In this test, a correlation between the contents of phenolic compounds and the DPPH scavenging activity is observed in the case of the three extracts. In fact, Falleh *et al.*<sup>22</sup> have shown that there is a very significant correlation between the content of polyphenols (total polyphenols, flavonoids and condensed tannins) and the scavenging effect of DPPH radicals. On the other hand, Albano and Miguel<sup>23</sup> reported that this correlation is not always present.

Oxidation of linoleic acid generates peroxide radicals, these free radicals will subsequently oxidize  $\beta$ -carotene causing the disappearance of its red color, which is followed by spectrophotometry at 490 nm. However, the presence of an antioxidant could neutralize free radicals derived from linoleic acid and therefore prevent the oxidation and bleaching of  $\beta$ -carotene. In this test, the antioxidant capacity is determined by measuring the inhibition of the oxidative degradation of  $\beta$ -carotene (discoloration) by the oxidation products of linoleic acid<sup>24</sup>.

This method is widely used because  $\beta$ -carotene shows strong biological activity and is an important physiological compound<sup>18</sup>. Therefore, it is used in the evaluation of the antioxidant activity of AT extracts. An extract that inhibits  $\beta$ -carotene bleaching can be described as a free radical scavenger and primary antioxidant<sup>25</sup>.

AT extracts have the ability to react with free radicals to convert them into non-reactive species and interrupt the chain of radical reactions. Flavonoids and polyphenols in general have the ability to scavenge free radicals and therefore delay the auto-oxidation of lipids<sup>26</sup>. They inhibit lipid peroxidation at an early stage by scavenging peroxide radicals, as they can interrupt the chain of radical reactions by giving off hydrogen<sup>27</sup>.

This ability to modify lipid peroxidation induced by free radicals is related not only to the structural characteristics of antioxidant agents but also to their ability to penetrate and interact with lipid bilayers. The structure and lipophilicity of polyphenols have been shown to be important factors underlying the antioxidant property, possibly affecting the depth of incorporation of these compounds into the lipid phase of the membrane<sup>28</sup>.

Transition metals, such as ferrous ions, can stimulate lipid peroxidation by generating hydroxyl radicals by the Fenton reaction and accelerate lipid peroxidation by breaking down lipid hydroperoxides into peroxy and alkoxy radicals, thus driving the chain reaction of the lipid peroxidation. The  $Fe^{2+}$  ion is the strongest pro-oxidant among the various species of metal ions. Ferrozine can quantitatively form complexes with  $Fe^{2+}$ . In the presence of chelating agents, the formation of the complex is disturbed, which leads to a decrease in the red color of the complex. Measuring the reduction in color therefore makes it possible to estimate the chelating activity of the metals of the coexisting chelator. Lower absorbance indicates higher metal chelating activity<sup>29</sup>.

Dec and Mac AT extracts exhibit considerable chelating activity. However, the latter two are better than the Inf. This could be attributed to the solubility of chelating agents in water. Sahreen *et al.*<sup>30</sup> have shown that plant extracts chelating effects are directly proportional to the polarity of their solvents. The aqueous constituents are more able to inhibit the formation of the  $Fe^{2+}$  - ferrozine complex, capturing the ferrous ions before the ferrozine. Chelating agents have been reported to be effective as secondary antioxidants because they reduce the redox potential by stabilizing the oxidized form of metal ions<sup>31</sup>.

## CONCLUSION

According to the results of this study, aqueous extracts of *Alkanna tinctoria* possess significant antioxidant activity *in vitro*. They show a very significant inhibition vis-à-vis the DPPH radical, an excellent inhibitory effect on lipid peroxidation and / or scavenging of radicals resulting from the oxidation of linoleic acid and a strong chelating activity vis-à-vis ferrous iron. These activities may be due to phytochemical constituents present in these extracts.

## ACKNOWLEDGEMENTS

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