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Preliminary phytochemical screening, *in vitro* antioxidant activity and insecticidal activity of methanolic leaves extract of *Cedrus atlantica* from Belezma, Algeria

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Abstract

This work was conceived in the context of valorisation of the endemic forest species *Cedrus atlantica* (Pinaceae family), widespread in the mountainous massif of eastern Algeria. This study aimed to investigate the antioxidant and insecticidal activity of the methanolic extract of the aerial part (leaves) of *C. atlantica* (CAMeOH). The extraction made it possible to obtain a yield of approximately 7.49% from 350 g of dry powdered plant material. The results of the phytochemical screening revealed the presence of tannins, alkaloids, terpenoids and flavonoids as major components. *In vitro* antioxidant evaluation was carried out using two different methods. The reducing power assay test result revealed an optical density (DO) value of 1.7±0.02 at a dose of 600 µg/mL of CAMeOH. Concerning the iron chelation activity, the recorded IC50 value was 62.12 µg/mL and R^2=0.87. The insecticidal activity against the stored-food insect *Tribolium confusum* was evaluated using the contact treatment mode and showed a significant dose-dependent effect ($P \leq 0.05$) with a mortality rate of 98.32 ± 0.50 % at a dose of 50 mg/mL (after 96 hours of contact). The results of this study confirmed the potential antioxidant and insecticidal activity of the methanolic extract from the leaves of *C. atlantica.*

Introduction

Oxidative stress, defined as an imbalance between the pro-oxidant and antioxidant systems in the body, caused by an overproduction of free radicals, is a major public health problem, attracting enormous attention.[1]

Oxidative stress is implicated in the development of more than a hundred different human pathologies such as atherosclerosis, cancer, cardiovascular and neurodegenerative diseases, diabetes, rheumatism and premature ageing of the skin.[2] The use of synthetic antioxidants such as butyl hydroxyanisole (BHA) and propyl gallate (PG), have been suspected in recent years to possess some contraindications and to be responsible for liver damage and carcinogenesis.[3]

Currently, and with the revolution in science, bioactive molecules based on plant extracts have been considered by researchers to limit the complications of the use of synthetic substances.[4] Alternative methods of using bio-insecticides are attracting the attention of scientists, to avoid the disadvantages linked to the abusive use of synthetic pesticides such as pyrethroids, organophosphates and sulfuryl fluoride.[5] Faced with the harmful effects of chemical pesticides, plants constitute a source of a wide range of natural substances which have great potential for application against insects.[6]

The Atlantic cedar, *Cedrus atlantica* (Endl.) Manetti ex Carrière is a medicinal plant of the Pinaceae family, characterized by a wide biological activity. Its isolated secondary metabolites showed beneficial effects on health including anticancer, antioxidant, antimicrobial, and analgesic activities.[7] Recent researchers have studied the chemical characteristics of Atlantic cedar from different countries, such as Lebanon, France, Morocco and Algeria.[8,9] Many significant chemical variations have been reported and many results have been identified.[10,11] Several works have been carried out on *C. atlantica* phytochemical characterization, indicating the presence of oils, flavonoids, tannins, phenol acids (rosmarinic acid, caffeic acid) and saponins.[12,13] Additionally, previous studies have shown that aqueous extracts from *C. libani* and *C. deodara* have shown a fungicide activity, as they prevent fungal spoilage of some spices during storage.[14,15] Furthermore, results focusing principally on essential oils suggest that *C. atlantica* has therapeutic properties and insecticidal characteristics.[13]

Algeria, with its thousands of hectares of forests and pastures, is full of aromatic and medicinal plants; this great diversity of flora is related to the climate and the large surface area.[16] In Algeria, the surface covered with cedar is considered as a characteristic essence of the mountainous level.[17] However, in the Belezma massif, few studies have been carried out on the biological and phytochemical activities of cedar, such as antimicrobial, insecticidal and fungicidal activities[18,19]

Insect pests can cause partial or even total losses of stored cereal products. These losses are of the order of 5 to 10% in temperate regions of the world and up to 20 to 30% in tropical countries.[20] Algeria is not immune to this problem, and the greatest losses are inflicted by different species of beetles, lepidoptera and mites.[21] Among the beetles, the most common is *Tribolium confusum* (Jacquelin du Val) of the Tenebrionidae family.[22] This insect has long been considered one of the major destructive pests of grain and stored grain products.[23] Furthermore, in recent years this species has shown cross-resistance to many chemical insecticides such as phosphine, malathion, chlorpyrifos-methyl and dichlorvos.[22]

Given this background, the present study aims to provide the phytochemical screening of the methanolic leaf extract of *C. atlantica* from the Belezma massif and to evaluate the *in vitro* antioxidant activity as well as the insecticidal activity of the methanolic extract of *C. atlantica* leaves (CAMeOH) against *T. confusum.* To the best of our knowledge, there is no previous detailed report on the insecticidal activity of this species of the Belezma massif.

Materials and Methods

Plant material

The plant material (leaves) of *C. atlantica* was collected in April 2022 in the cedars of Belezma National Park Wilaya of Batna, located in northeast Algeria, northwest of Batna (Figure 1).

Procedures

Preparation of plant extracts

Fresh leaves of *C. atlantica* were washed in running water, dried at room temperature at 18°C, and placed in a ventilated place away from light for 40 days. Then, leaves were powdered using a blender. The obtained powder (350 g) was extracted by maceration in methanol (MeOH) 98% (10 g of powder/100 mL of methanol) under agitation for 3 days at room temperature and away from light. After each time interval (24h), the separated extracts were then filtered through Whatman No. 1 filter paper and the residue obtained was reserved for a second extraction. Finally, the methanol filtrate was evaporated to dryness using a rotary evaporator at room temperature (35°C). The fraction (26.22 g) was kept at 4°C in the dark until further analysis.[24] The extraction rate of each dry extract was calculated according to the formula below:

$$\text{Yield } \% = M / M_0 \times 100$$

Where M is the dry weight of extract (g) after evaporating the solvent and M_0 (g) is the weight of the soaked plant powder.[25]

Phytochemical analysis

The CAMeOH was tested to identify the major secondary metabolites existing in the leaves of this plant such as alkaloids, phenolics, tannins, flavonoids, saponins, steroids and terpenoids. The phytochemical screening was based on the observation of the characteristic coloring of the reaction mixture or the formation of a precipitate. In our investigational study, we used the standard techniques described by Trease and Evans,[26] Harborne[27] and Bruneton[28] with little modification. The presence of possible phytochemical constituents was evaluated qualitatively with the following tests.

Test for phenolic content

The total phenolics were assessed by the ferric chloride test. About 100 mg of extract was dissolved in 10 mL of solvent and then filtered. The filtrate (2 mL) was added to 1 mL of 3% v/v $FeCl_3$. The presence of a dark blue colour indicated the presence of polyphenols.

Test for alkaloids

The tested extract was mixed in 2% v/v H_2SO_4, warmed and filtered. The filtrate (1 mL) was mixed with 4-5 drops of Dragendorff reagent. The appearance of an orange-red precipitate indicated the presence of alkaloids.

Test for tannins

The diluted extract (2 mL) was added to 1 mL of 0.1% ferric chloride. After agitation, the appearance of a dark green colour showed the presence of tannins.

Test for flavonoids

About 1 mL of CAMeOH was added to 5 mL of 95% ethanol; then 2 mL of concentrated HCl was added through the test tube wall. Furthermore, 0.5 g of magnesium powder was added. The resulting mixture was agitated for 1 minute and the observation of a red deposit indicated the presence of flavonoids.

Test for saponins

About 1 mL of the extract of *C. atlantica* was mixed with 10 mL of distilled water and shaken vigorously for 15 min. The observation of a stable foam revealed the presence of saponins.

Test for steroids

An equivalent volume (2 mL) of the extract and chloroform ($CHCl_3$) were added to 500 µL of acetic anhydride and 3-4 drops of concentrated sulfuric acid. After agitation, the change in colour from purple to blue indicated the presence of steroids.

Test for terpenoids

A volume of extract (3 mL) was added to 1 mL of chloroform and a few drops of concentrated sulfuric acid were mixed carefully through the walls of the test tube. The formation of a reddish-brown colour indicated the presence of terpenoids.

In vitro *antioxidant activity*

Reducing power assay

The antioxidant activity of CAMeOH was determined by the method of reducing power assay. [29]A volume of plant extract (2.5 mL) at various concentrations (50 – 600 µg/mL) was added to 2.5 mL of sodium phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide (1 %). The mixture was incubated at 50°C in a water bath for 20 min. A blank was prepared without adding a standard or test compound. A volume of 2.5 mL of 10% (w/v) trichloroacetic acid was added, and then the mixture was centrifuged at 650 rpm for 10 min. A volume of 5 mL of supernatant was mixed with 5 mL of distilled water and 1 mL of 0.1% ferric chloride. The absorbance was measured at 700 nm; ascorbic acid at various concentrations was used as standard. Increased absorbance of the reaction mixture indicates an increase in reducing power.

The per cent increase in reducing power was calculated using the following equation:

$$(1)\ \text{increase in reducing power \%} = \frac{\text{A test} - \text{A blank}}{\text{A blank}} \times 100$$

Where A test is the absorbance of the test solution; A blank is the absorbance of the blank.

Iron chelation activity

The chelating effect of ferrous ions Fe^{2+} was estimated using the method described by Dinis et al.[30] A volume of 1 mL of the extract solution (0-600 µg/mL) or the reference chelator EDTA (0-200 µg/mL) was previously mixed with 50 µL of iron chloride ($FeCl_2$) (2 mM). After 1 min, 200 µL of Ferrozine (5 mM) was added to the reaction. The mixture was vigorously stirred and allowed to stand at room temperature for 10 min, thus allowing the complexing of the residual iron and the formation of a red chromophore (Fe(II)-Ferrozine) having a maximum absorption at λ=562 nm. Quercetin at various concentrations was used as standard. The per cent inhibition of the ferrozine-Fe^{2+} complex was calculated according to equation (1).

The IC_{50} value, indicating the concentration at which 50% chelating activity is inhibited, was calculated from the graph of inhibition percentage against different extract concentrations.

Evaluation of bioinsecticidal activity

Collection and rearing in the laboratory

Specimens of *Tribolium confusum* Jacquelin du Val, 1868 (Coleoptera: Tenebrionidae) were collected in a regional Cereals station in Batna. The examination was carried out using a 2 mm sieve with a white filter paper and identified by the naked eye. The specimens were reared in laboratory conditions (temperature: 27 ± 1°C and relative humidity: 65 to 70%), in plastic bottles containing wheat grains.

Insecticidal activity

The different doses of the extract were prepared by dilution in distilled water. The dilution of the extract was prepared at the following concentrations: 0.5mg/ mL, 2.5mg/mL, 5mg/mL, 25mg/mL and 50 mg/mL. Each dose of the test extract was uniformly deposited on filter paper (Whatman No. 1). In each Petri dish, 20 adult insects were placed directly on the treated filter paper. After 24 h, the insects were placed in 40g of flour grains and kept in optimal conditions. Three replicates were performed for each concentration. The number of dead insects was recorded at 24, 48, 72 and 96 hours. Distilled water was used as a control. The insecticide test procedure used in this study is that described by Tapondjou *et al*.[31] The mortality rate was calculated and corrected according to the formula:

$$M\% = \left(\frac{M_I - M_C}{100 - M_C} \right) \times 100$$

Where *M*: Mortality corrected, MI: Mortality observed in insects and *MC*: Mortality observed in controls.

Data analysis

All data were expressed as means ± standard deviation of triplicate measurements. Linear regression analysis was performed to find out the correlation that existed between the per cent inhibition of iron chelating activity and the different CAMeOH concentrations.

Statistical software (SPSS), version 25.0, was used to determine the level of significance of the recorded differences. The analysis of variance ANOVA between the different groups followed by the least significance difference (LSD) post-hoc test was used to assess the antioxidant activities *in vitro* and to estimate the lethal concentrations of CAMeOH in adults of *Tribolium confusum* after 24, 48, 72 and 96 h. The level of significance was set at $p<0.05$.

Results

Percentage yield

The yield of the fraction from the MeOH extraction was calculated according to the dry plant matter of the *C. atlantica* leaves (350 g of dry powdered). The percentage yield of plant extract was 7.49 %.

Phytochemical analysis

The summary of the results of the phytochemical screening of CAMeOH is presented in Table 1. Tests indicated the presence of tannins, alkaloids, terpenoids, flavonoids and phenolics while steroids and saponins were detected in traces.

Reducing power assay

Figure 2 shows reduced activity of crude methanolic extract of *C. atlantica* leaves in comparison with ascorbic acid as standard. The higher absorbance of the reaction mixture indicates an increase in reducing power. The reducing power of the reference compound was found to be higher than the CAMeOH. It has been reported that the reducing power of substances is probably due to of their hydrogen-donating ability.[32] The reducing power of the extract increased with an increase in concentration. At a dose of 600 μg/mL of CAMeOH the optical density (DO) is =1.7±0.02. The result of the reducing power assay showed that the tested CAMeOH exhibited moderate power.

Iron chelation activity

Figure 3 shows that the chelating capacity of CAMeOH at 600 µg/mL is 85.00 %. At equal rank, the results presented in Figure 4, which correspond to the calculation of the linear regression equation, showed that the IC50 value of CAMeOH was of the order of 62.12 µg/mL. The chelating power dependent on the presence of the reducing principles appears to be consistent with the qualitative content of these fractions as described in Table 1. Our results revealed a high correlation coefficient (r =0.87) of the iron chelation activity of CAMeOH (Figure 4).

Insecticidal activity

The statistical analysis of the results showed that the insect mortality rates recorded in the treated batches are proportional to the times of exposure and the different doses of the CAMeOH used. Consequently, as shown in Table 2, the plant extract with a concentration of 50 mg/mL has a significant power to induce insect mortality (68.23 ± 2.15 % after 24 h and 98.32 ± 0.50 % after 96 h, respectively). On the fourth day, all used CAMeOH doses revealed significant differences in the recorded different mortalities (65.23 ± 1.46% to 98.32 ± 0.50%). The mortalities recorded in the treated group at the lowest concentrations (0.5 and 2.5 mg/mL) revealed significant differences compared to those of the negative control ($p > 0.05$) (Figure 5).

Discussion

The use of synthetic antioxidant molecules, including butyl-hydroxy-toluene (BHT), butyl-hydroxy-anisole (BHA) and tert-butylhydroquinone (TBHQ) was currently questioned because of the potential toxicological risks. New plant sources of natural antioxidants are being sought, and they are used as additives in the food industry.[33] In this context, the Algerian forest cedar (*C. atlantica*) has been the subject of studies about its antioxidant and antibacterial activities.[7,12] Our research work has expanded the preliminary phytochemical characterization of the *C. atlantica* extracts and showed the antioxidant and insecticidal activity of plants from the Belezma Massif.

Our *C. atlantica* extract resulted in a considerable extraction yield of 7.49%. This yield was much lower than that obtained by Belkacem *et al.*[7] These authors declared a yield of 19.70±2.68%, produced by the methanolic extract of the leaves of *C. atlantica* from the region of Adekar, in Algeria. This disparity in extraction yields can be attributed to various factors, such as the environmental and

climatic conditions, the season of the collection of the plants, the storage conditions, and the method used for the extraction.[34]

The preliminary phytochemical investigation revealed various secondary metabolites which were previously isolated by other researchers.[19] In our investigation, these secondary metabolites contain significant amounts of tannins, alkaloids, terpenoids, flavonoids and phenolic compounds. Equally, other research on *C. atlantica* in Algeria revealed that its chemical composition presents bioactive compounds including oxygenated sesquiterpenes and monoterpenes.[7,18] In addition, Benouaklil *et al*.[35] revealed the presence of β-himachalene as a major compound in the essential oil of *C. atlantica* leaves.

In addition, we studied the *in vitro* antioxidant properties of CAMeOH using two different methods: reducing power assay and iron chelation activity. Our results show that CAMeOH exhibits strong antioxidant activity. According to Agrawal et al.,[36] the needles of the Atlas cedar are known for their content of taxifolin (flavanone), whose effectiveness as an antioxidant agent has been approved. Several works have also shown that the reducing and chelating power of a compound can be considered an indicator of its potential antioxidant effect.[32] Regarding the chelating power, our results revealed an IC50 value of 62.12 µg/mL in comparison with quercetin. This may be explained by ferrous ions which are considered the most effective pro-oxidants. Chelating agents which form a σ-type bond with metals have been reported to be active as secondary antioxidants as they decrease redox potential and stabilize the oxidized form of the metal ion.[37] A few previous studies have also reported that species belonging to the *Cedrus* genus, such as *C. brevifolia* and *C. deodara*, have reducing properties correlated with recovered phytoconstituent levels (terpenoids, flavonoids), thus explaining their antioxidant role.[15,37] Furthermore, other authors,7 in another similar study, confirmed that the organic extract of the aerial parts of *C. atlantica* growing in Algeria has a good reducing power of ferric ions with a value of 325.95±5.05 mg Eq vitamin C. However, the study of the antioxidant activities of organic extracts of the plant such as 2, 2-diphenyl-2-picryl-hydrazyl (DPPH), ferric reducing antioxidant power (FRAP) and scavenging 2, 2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) revealed variable results.[7,19,38] This variability can be explained by the fact that the organic extract of Atlas cedar needles has an appreciable antioxidant activity which differs from one region to another. This activity is associated with the presence of phenolic compounds that characterize this part of the plant.[38]

Moreover, *C. atlantica* secondary metabolites have been studied for different effects on insects: repellency, attraction, interference with development, inhibition of reproduction, etc.; their activity

can directly or indirectly affect the target organs (sensory organs, nervous system, endocrine system, gastrointestinal tract, reproductive apparatus, etc).[39]

In addition, the studies by Ainane *et al.*[13] confirmed the effectiveness of the insecticidal power of *C. atlantica* essential oil on *T. confusum*. Our results are in agreement with those reported in the literature.[40] Indeed, Ainane *et al.*[13] recorded total mortality of *T. confusum* after five days of contact at a dose of 3.5×10^{-2}µL/cm^3 of the essential oil of *C.atlantica*. In our study, the same mortality result (98.32 ± 0.50%), was obtained at a dose of 50 mg/mL after the 5th day of contact.Based on the lack of data on the effect of C. *atlantica* extracts on *T. confusum* we tried to compare the action of *Cedrus* sp. extracts on other insects and larvae previously studied. A Moroccan study was carried out on the insecticidal activity of the essential oil of the aerial parts of *C. atlantica* against *Culex pipiens* with a larval mortality rate of 90% at the concentration of 1253.93 ppm.[39] Another investigation carried out by Huseyin *et al.*[41] demonstrated the larvicidal activity of *C. libani* on *C. pipiens* with LC50 values ranging from 47.8 to 116.0 ppm. Another research showed the insecticidal activity of *C. deodara* against *Plutella xylostella*.[42] Furthermore, Naples *et al.*[43] reported that the essential oil of cedarwood has high toxicity against the parasite *Schistosoma mansoni* which affects humans.

In our research, the insecticidal mechanism of the CAMeOH was probably due to the biological activity of terpenoids and alkaloids which have a toxic effect. According to research by Carpinella et al.,[44] terpenes inhibit the food intake of phytophagous insects and cause death and malformations in future generations. According to several investigations, alkaloid compounds are capable of causing toxicity which will allow the epicuticular barrier to be crossed. These toxins act as blockers of the insect's nervous system.[45]

Conclusions

The present study revealed significant antioxidant and insecticidal activities of crude methanolic extract prepared from *C. atlantica* species. Our results showed that the MeOH extract from the leaf fraction of this species is very rich in alkaloids, tannins, flavonoids and terpenoids, and exhibits a high level of insecticidal activity against *T. confusum*. Finally, this endemic plant with these secondary metabolites requires other in-depth phytochemical investigations in order to know the underlying biological mechanisms associated with its use.

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Table 1. Phytochemical test of the methanolic extract of *C. atlantica* (CAMeOH).

Test	**Observation**	**results**
Phenolics (ferric chloride test)	dark blue colour	++
Alkaloids (Dragendorff test)	appearance of a orange-red precipitate	+++
Tannins (ferric chloride test)	dark green color	+++
Flavonoids (Shinoda test)	red color	+++
Saponins (foaming test)	appearance of foam	+
Steroids (Salkowski's test)	change in color from purple to blue	+
Terpenoids (Salkowski's test)	reddish brown color	+++

Note: a key +++ indicates presence in high concentration; ++ indicates presence in moderate concentration; + indicates presence in trace concentration

Table 2. Percentage of corrected mortality of *T. confusum* treated with CAMeOH expressed as mean ± SD.

time	D1 **(0.5 mg/mL)**	D2 **(2.5 mg/mL**	D3 **(5 mg/mL)**	D4 **(25 mg/mL)**	D5 **(50 mg/mL)**	Control
24h	2.62 ± 0.20	03.60 ± 0.20	06.25 ± 0.92*	51.00 ± 2.50**	68.23 ± 2.15**	0.00 ± 0.00
48h	4.30 ± 1.27*	07.56 ± 0.66**	14.62 ± 1.19**	70.41 ± 1.62**	83.52 ± 0.56**	0.00 ± 0.00
72h	31.62 ± 3.45**	55.79 ± 1.47**	63.96 ± 3.94**	77.98 ± 2.00**	96.60 ± 1.44**	2.00 ± 2.89
96h	65.23 ± 1.46**	70.23 ± 1.50**	81.03 ± 1.56**	85.92 ± 1.80**	98.32 ± 0.50**	2.00 ± 0.45

D=dose used, *Denote a significant difference ($p<0.05$), **Denote a highly significant difference ($p<0.01$).

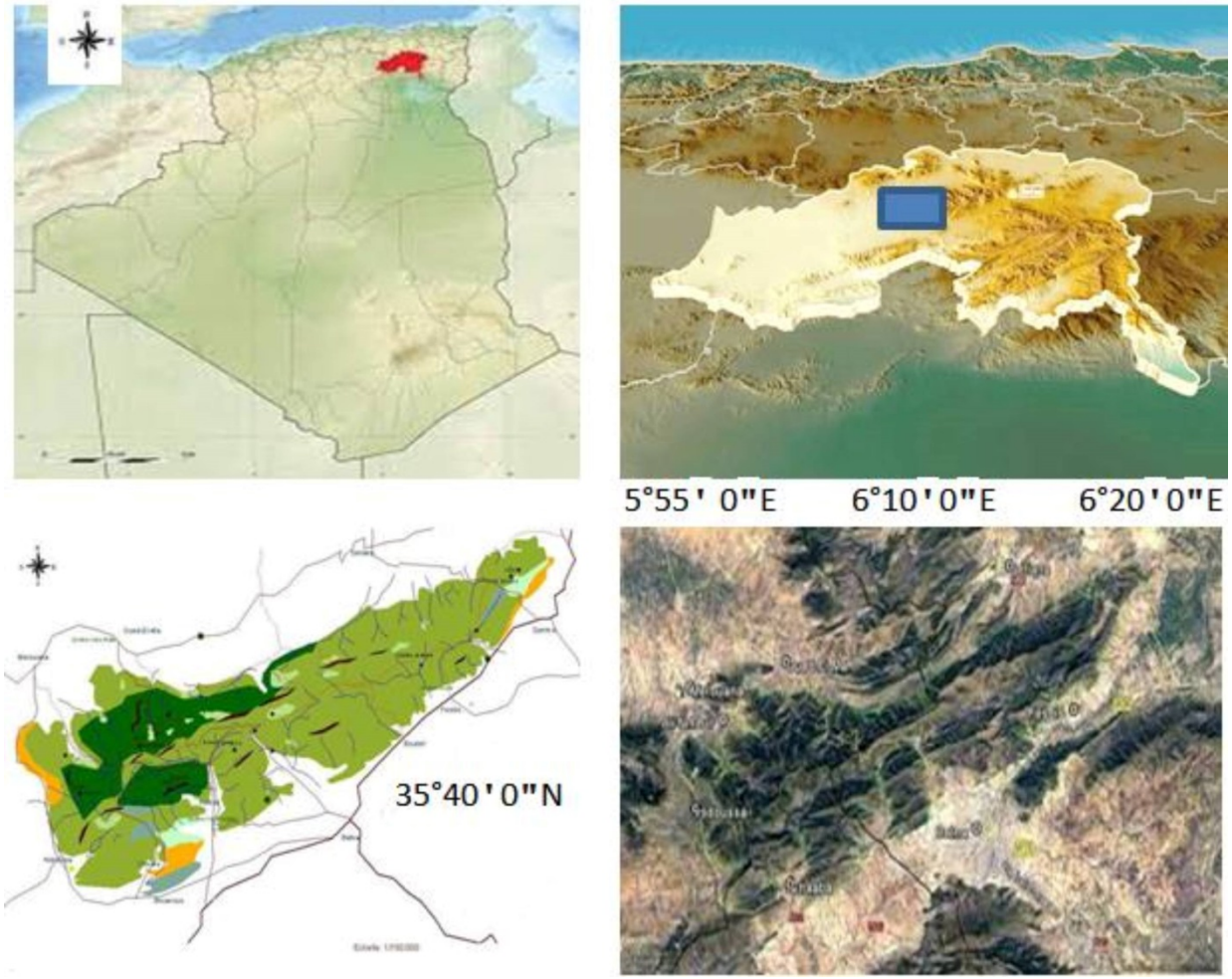

Figure 1. Location of Belezma, indicating the study area (35° 30' and 35° 40' N; 5° 50' and 6° 20' E).

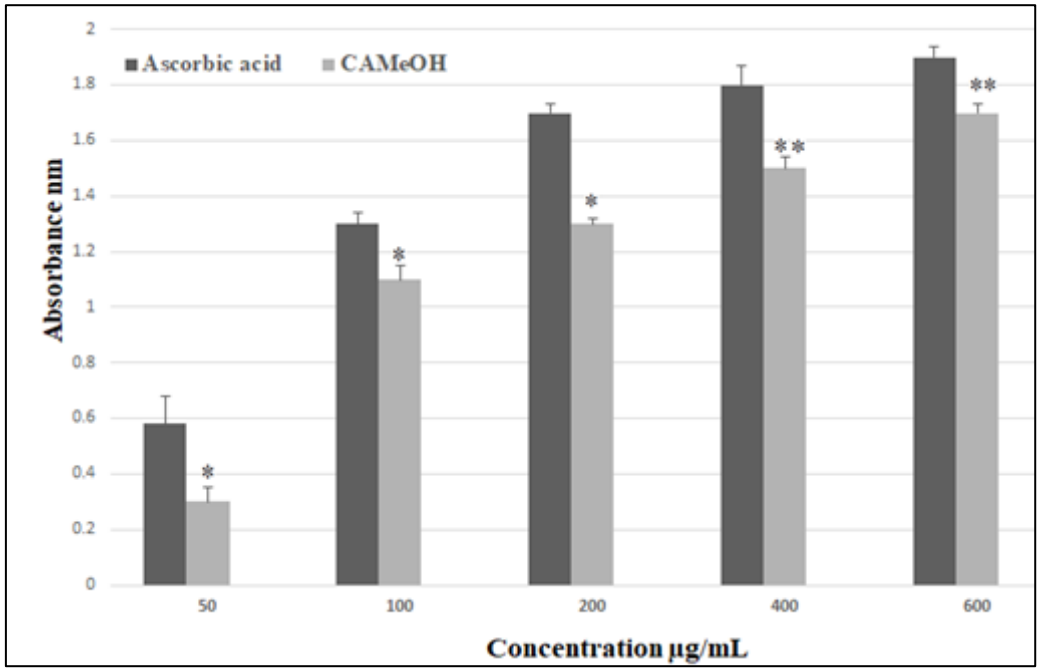

Figure 2. Reducing power of the methanolic extract of *C. atlantica* (CAMeOH) and ascorbic acid. Values are expressed as means ± SD of triplicate samples. * $p<0.05$ and ** $p<0.01$ are considered significant compared to the standard.

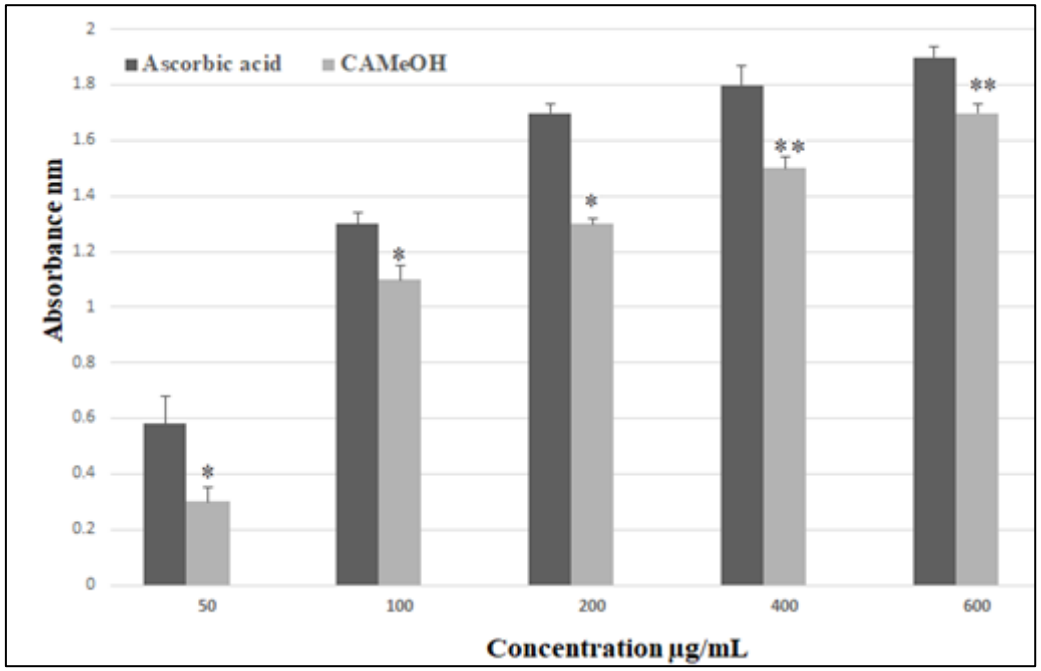

Figure 3. Chelating effect of the methanolic extract of *C. atlantica* (CAMeOH) and quercetin. Values are expressed as means ± SD of triplicate. * $p<0.05$ and ** $p<0.01$ are considered significant compared to the standard.

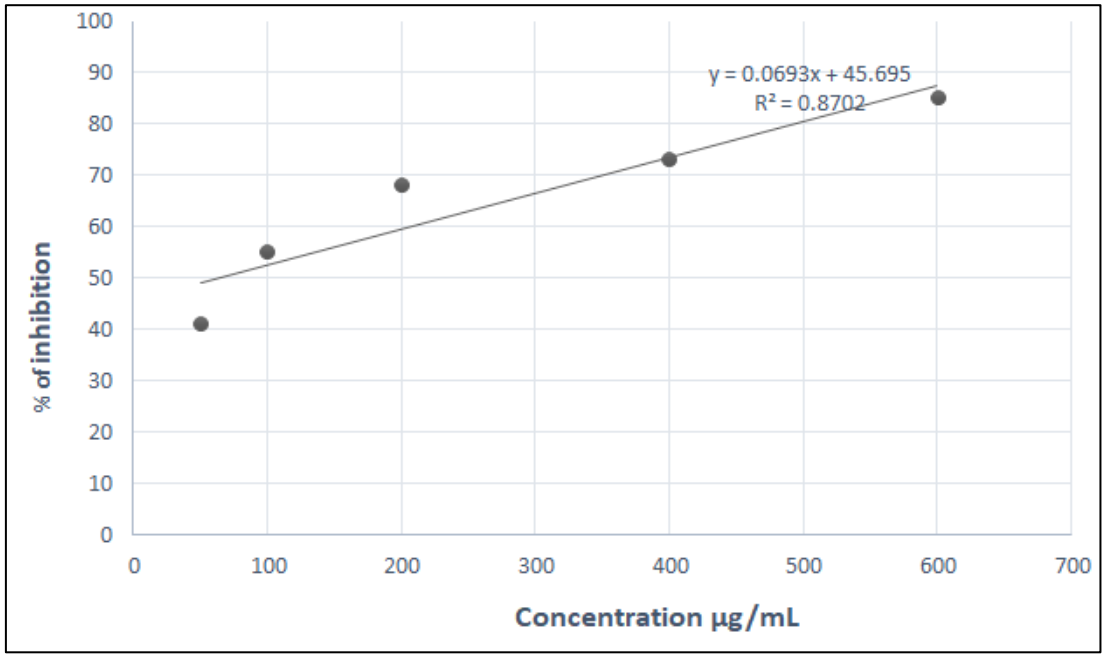

Figure 4 Percent inhibition of iron chelation activity as a function of the concentrations of the methanolic extract of *C. atlantica* (CAMeOH).

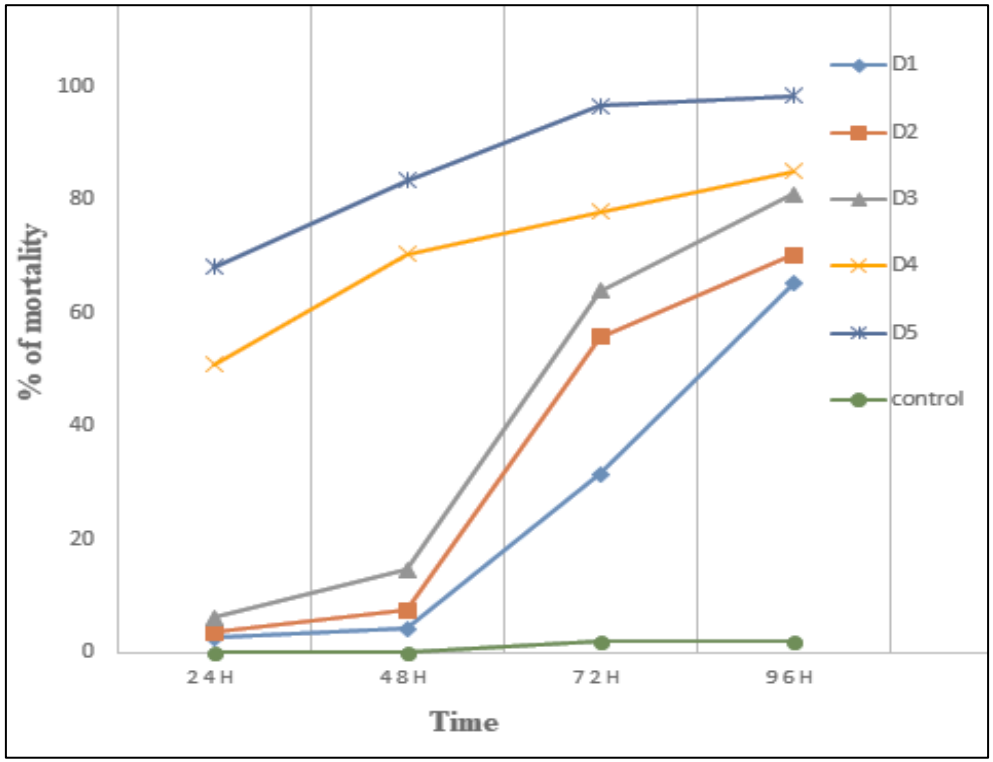

Figure 5. Evolution of *T. confusum* adult mortality as a function of exposure duration and CAMeOH doses.