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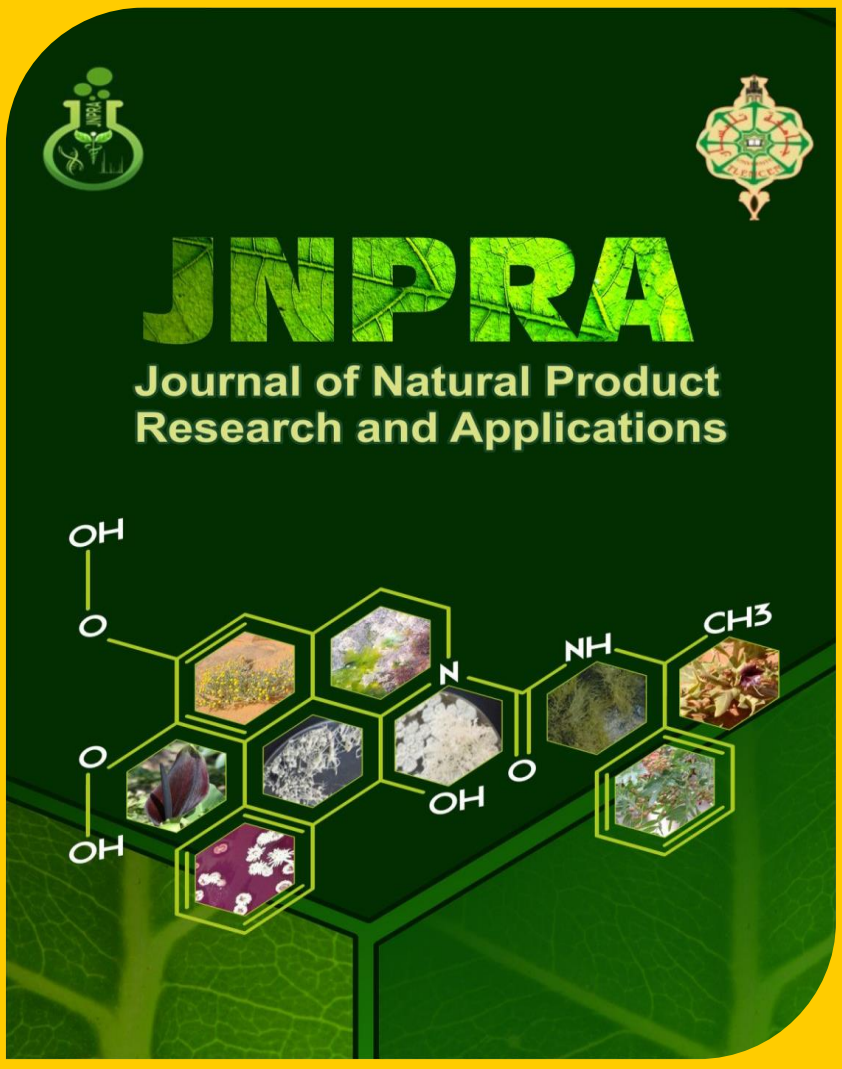

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Phytochemical study and evaluation of α-Amylase inhibitory effect of extracts from *Cistanche violacea* Desf.

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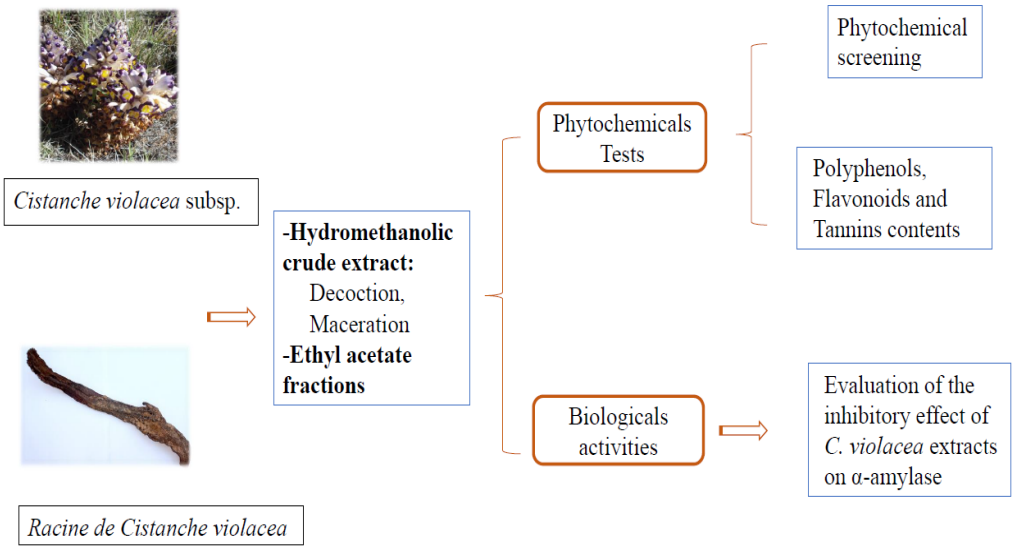
Highlights

- Diabetes mellitus is a public health issue;
- Inhibitors of α-amylase result in a prolonged carbohydrate digestion;
- *Cistanche violacea* is a parasitic species on several hosts;
- *C. violacea* is an angiosperm dicotyledonous holoparasite.

Graphical Abstract



Abstract

Cistanche violacea Desf. is a species of the family Orobanchaceae. It is an angiosperm dicotyledonous holoparasitic plant, which lacks chlorophyll. This work focuses on the search for an inhibitory effect of the enzymatic activity of α-amylase, in the presence of the crude water-methanol extract, of the underground part of *C. violacea*, prepared by maceration or decoction and its ethyl acetate fractions. Qualitative phytochemical tests and quantitative assays of phenolic compounds of the prepared extracts were performed. The effect of each extract on the activity of the α-amylase enzyme was evaluated under optimal conditions (pH 6.9 and T° 37°C). The results of phytochemical tests revealed the presence of tannins, flavonoids, free quinones, terpenoids and reducing compounds. The determination of phenolic compounds showed that the ethyl acetate extract prepared by maceration was the richest in total polyphenols and flavonoids with contents of the order of 82.9±6.8 µg EGA/mg DW and 112.4±25.7 µg EC/mg DW respectively. While the crude hydromethanolic extract prepared by maceration presented the highest content of condensed tannins (525.5±77.5 µg EC/mg DW). On the other hand, the ethyl acetate extract prepared by decoction of the underground part of *C. violacea* showed a better inhibitory activity of the α-amylase enzyme, with IC_{50} value of 1.88 mg/mL, compared to the other extracts tested. This activity remains low compared to acarbose (IC_{50} = 0.132 mg/mL). We concluded that the roots of *C. violacea* exerts an inhibitory effect on α-amylase activity and thus on the regulation of hyperglycemia through a decrease in intestinal glucose absorption.

Keywords : Diabetes mellitus, *Cistanche violacea*, α-amylase, phenolic compounds, , inhibitory effect.

1. Introduction

Diabetes mellitus is a public health issue. It is a chronic progressive endocrine disorder characterized by a high level of blood glucose. It results from a decrease in insulin production and/or resistance to its activity (WHO, 2002). One of the objectives of diabetes treatment is to delay or reduce the ability of the digestive process to break down and absorb carbohydrates ingested by digestive enzymes (α-amylase and/or α-glucosidase). Inhibitors of these enzymes result in a generally prolonged carbohydrate digestion, slowing down the rate of glucose absorption and lowering postprandial blood sugar levels (Rhabasa-Lhoret et Chiasson, 2004). Human pancreatic α-amylase (HPA) catalyses the initial step in the hydrolysis of α-1,4 glycosidic bonds in amylose, amylopectin, glycogen, maltodextrins, or starch, yielding maltose, which is subsequently broken down into glucose by glucosidases (Ponnusamy et al., 2015; Etxeberria et al., 2012).

C. violacea is a species belonging to the family Orobanchaceae. It is an angiosperm dicotyledonous holoparasite, lacking chlorophyll, which makes it a group of root parasites on various hosts: *Haloxylon articulatum* and *Limonistrum guyonianum*. It is a perennial plant with a fleshy stem, mostly underground, with the above-ground portion ranging in size from 20 to 40 cm (Quezél and Santa, 1963)**.** It is found on the salty and arid soils of the high plateaus up

to the Sahara. It is a native species of North Africa (especially Tunisia, Algeria, and Morocco). It is also found in Saudi Arabia, Libya, Western Sahara, and Egypt (Baba Aissa, 2011).
The aerial part of *C. violacea* is used in decoction to treat diabetes, stomachaches, and diarrhea. Meanwhile, the underground part of this plant is used as flour for its aphrodisiac properties (Hammiche and Maiza, 2006; Baba Aissa, 2011).
In this study, we are interested in the phytochemical analysis and evaluation of the inhibitory effect of hydromethanolic crude extracts and their ethyl acetate fractions, prepared through maceration or decoction of *C. violacea* roots, against α-amylase.

2. Material and Methods

2.1 Plant Materials and extraction

The plant material used in our study is the roots of *C. violacea*, harvested during the month of March 2022, in the Moghrar wilaya of Naâma (Algeria).
The plant material was dried away from light in a dry place and at room temperature in order to preserve molecular integrity as much as possible. After drying, the roots were crushed and stored carefully for subsequent analysis.

2.1.1 Hydro-methanolic extract prepared by maceration

10 g of dried and ground plant material from our plant are combined with 150 mL of the water/methanol mixture (30:70, v/v) at room temperature for 48 h, protected from light. After filtration, the hydro-methanolic filtrate is subjected to methanol evaporation using a rotary evaporator at 60°C, followed by drying in an oven at 37°C. The resulting dried extract is stored in a moisture-free environment until its use (Abbou et al., 2022).

2.1.2 Hydro-methanolic extract prepared by decoction

10 g of dried and ground plant material from our plant is brought into contact with 200 mL of a water/methanol mixture (30:70, v/v). The setup is subjected to reflux using a heating mantle for 45 minutes at a constant boiling temperature. After filtration, the hydro-methanolic filtrate undergoes methanol evaporation using a rotary evaporator at 60°C, followed by drying in an oven at 37°C. The resulting dry matter is stored away from moisture until its use (Abbou et al., 2022).

2.1.3 Ethyl acetate extract prepared by fractionation (liquid/liquid)

After the evaporation of methanol from the hydro-methanolic extract, we proceeded with a liquid-liquid extraction using ethyl acetate, using a separating funnel.
The crude hydromethanolic extracts prepared by maceration or decoction and their respective ethyl acetate fractions are stored protected from light, in order to conduct phytochemical and biological tests (Abbou et al., 2022).

2.2 Phytochemical screening

The prepared extracts were subjected to phytochemical and qualitative tests in order to detect the presence or absence of certain chemical families. These tests were carried out using the methods described by Bruneton (1999) and Harbone (1998).

2.3 Quantification of Phenolic Compounds

The crude hydromethanolic extracts are solubilized in distilled water at a concentration of 1mg/mL, and the organic ethyl acetate fractions are solubilized in methanol at a concentration of 1 mg/mL for the determination of total polyphenol and flavonoid contents.

2.3.1 Determination of total polyphenols

The total polyphenol content of the different extracts prepared was determined using the Folin-Ciocalteau method described by (Vermerius and Nicholson, 2006). The Folin-Ciocalteu reagent is a yellow mixture of phosphotungstic acid and phosphomolybdic acid. It is reduced during phenol oxidation to a mixture of blue tungsten oxide and molybdenum oxide. The intensity of the blue coloration is proportional to the quantity of phenolic compounds present in the sample, with an absorption maximum at 725 nm. Total polyphenol contents are calculated from the linear regression equation (y = 0.0016x) of the gallic acid calibration curve. Results are expressed in equivalent micrograms of gallic acid per milligram of dry extract (µg Eq GA/mg DW).

2.3.2 Determination of Flavonoid contents

The flavonoid content of the various extracts prepared was determined using the method described by Zhishen et al. (1999). The method is based on the oxidation of flavonoids in an alkaline medium by sodium nitrite ($NaNO_2$) and Aluminium chloride ($AlCl_3$), resulting in a pink complex that absorbs light at 520 nm. Flavonoid contents are calculated from the linear regression equation (y = 0.0027x) of the catechin calibration curve.

The results are expressed in micrograms of catechin equivalent per milligram of dry extract (µg Eq C/mg DW).

2.3.3 Determination of condensed tannins

Condensed tannins are determined using the vanillin reagent, according to the method described by Sun et al. (1998). Condensed tannins depolymerize in an acid medium and react with vanillin to form red anthocyanidols which absorb at 550 nm. This color is proportional to the quantity of condensed tannins present in the extracts.

Condensed tannins contents are calculated from the linear regression equation (y = 0.0002x) of the catechin calibration curve. The results are expressed in micrograms of catechin equivalent per milligram of dry extract (µg Eq C /mg DW).

2.4 Evaluation of the inhibitory effect of *C. violacea* extracts on α-amylase

The principle of this method is to evaluate the inhibitory effect of extracts of the plant studied on the activity of pancreatic α-amylase. The substrate used is starch. Acarbose, a reference molecule in the inhibition of this enzyme, is used as a positive control (Worthington, 1988).
Under alkaline conditions and at elevated temperatures, oxidation of the free aldehyde and ketone groups on the sugars simultaneously reduces yellow-orange 3,5-dinitrosalicylic acid (DNSA) to orange-red 3-amino 5-nitrosalicylic acid, which absorbs at 540 nm. The intensity of the coloration is proportional to the quantity of reducing sugars present in the reaction medium.
Percentage inhibition (I%) is calculated using the following equation:

$$I\% = (AA - AB / AA) * 100$$

AA: absorbance of negative control. **AB:** absorbance of the test sample.
IC_{50} (50% inhibitory concentration) for each extract are calculated from logarithmic regression curves of inhibition versus concentration.

2.5 Statistical analysis

In all experiments of the dosage and evaluation of the inhibitory effect. The experimental data obtained were expressed as mean ± standard deviation. Each test was repeated 3 times.

3.Results

3.1 Extraction

The extracts obtained from the roots of *C. violacea* through different extraction methods exhibit varying aspects, colors, and yields **(**Table 1**).** We noticed that the crude hydromethanolic extracts are recovered in a viscous form, with a dark brown color. In contrast, the ethyl acetate fractions exhibited a light brown color and a caramelized appearance. The crude hydromethanolic extracts are soluble in distilled water, unlike their ethyl acetate fractions which are insoluble in distilled water and become soluble in methanol. The yields of hydromethanolic crude extracts are similar; the extract obtained by maceration has the highest yield (21.61%), followed by the extract obtained by decoction (21.31%), while the two ethyl acetate fractions of the extract prepared by decoction and maceration show the lowest yields of 1.25% and 0.69%, respectively.

Table 1. Characteristics of hydromethanolic crude extracts and their ethyl acetate fractions from the roots of *C. violacea*.

The extracts	*Extraction Method*	*Yield (%)*	*Appearance*	*Color*	*Solubility*
Crude Extracts	Maceration	21.61	Viscous	Dark brown	Distilled water
	Decoction	21.31	Viscous	Dark brown	Distilled water
Ethyl acetate fraction	Maceration	0.69	Caramelized	Light brown	Methanol
	Decoction	1.25	Caramelized	Light brown	Methanol

3.2 Phytochemical tests

The results of the phytochemical screening conducted on the various hydro-methanolic crude extracts, prepared by maceration or decoction, of *C. violacea* roots are presented in Table 2**.** The results revealed the presence of tannins, free quinones, terpenoids, and reducing compounds. While flavonoids are only present in the hydromethanolic extract prepared by decoction. We also noted the absence of anthraquinones, anthocyanins, alkaloids, and saponins.

Table 2. Results of phytochemical tests conducted on the two hydromethanolic crude extracts, prepared by maceration or decoction, of *C. violacea* roots.

Phytochemical tests	Maceration	Decoction
Alkaloids	-	-
Flavonoids	-	+
Tannins	+++	+++
Free Quinones	+	+
Anthraquinones	-	-
Terpenoids	+++	++
Saponins	-	-
Reducing Compounds	++	++

(+++): Strongly present; (++): Moderately present; (+): Weakly present; (-): Negative test.

3.3 Contents of total polyphenols, flavonoids and condensed tannins

The contents of polyphenols, total flavonoids, and tannins in the crude hydromethanolic extracts prepared by maceration or decoction (EBM and EBD) and their ethyl acetate fractions (FAEM and FAED) are grouped in Table 3**.** For the total polyphenol content determination, the two ethyl acetate fractions of the crude extract prepared by maceration and decoction contain the highest levels (82.9 µg EGA /mg DW) and 56.9 µg EGA/mg DW), respectively), followed by the crude extract prepared by decoction (47.9 µg EGA/mg DW), and the crude extract prepared by maceration (44.3 µg EGA/mg DW).

Table 3. Polyphenol, flavonoid, and tannin contents of the hydro-methanolic crude extracts and their ethyl acetate organic fractions from *C. violacea* roots.

Extraction method	Total polyphenols (µg EGA /mg DW)	Flavonoids (µg EC/mg DW)	Condensed tannins (µg EC/mg DW)
Maceration **(EBM)**	44.3± 2,9	56.6± 11.6	525.5± 77.5
Decoction **(EBD)**	47.9± 5.6	24.8± 2.5	326.3± 23.9
Maceration **(FAEM)**	82.9± 6.8	112.4± 25.7	96.7± 55.4
Decoction (**FAED)**	56.9± 4.7	50.6± 5.9	96.2± 38.0

EBM: Crude hydromethanolic extract prepared by maceration; **EBD**: Crude hydromethanolic extract prepared by decoction; **FAEM:** Ethyl acetate fraction of the hydromethanolic crude extract prepared by maceration; **FAED:** Ethyl acetate fraction of the hydromethanolic crude extract prepared by decoction.

Regarding the total flavonoid contents, the ethyl acetate fraction of the crude hydromethanolic extract obtained by maceration recorded the highest content (112.4 µg CE/mg DW). Meanwhile, the crude extract prepared by decoction exhibits the lowest content (24.9 µg CE/mg DW). The results of condensed tannins assay indicate that the crude hydromethanolic extract prepared by maceration has the highest content of condensed tannins (525.5 µg CE/mg DW), followed by the crude extract obtained by decoction with a content around 326.3 µg CE/mg DW. The other organic fractions from maceration and decoction exhibit similar contents, with levels of 96.7 µg CE/mg DW and 96.2 µg CE/mg DW, respectively.

3.4 Effect of *C. violacea* extracts on α-amylase activity

In the present study, we evaluated the inhibitory effect of the crude hydromethanolic extracts of *C. violacea* roots and their fractions against α-amylase, using acarbose as a positive control. The logarithmic curves of inhibition percentages of the extracts as a function of different concentrations of crude extracts and their fractions, as well as acarbose, are respectively presented in Figures 1 and 2, and the results are expressed as IC_{50} values (Table 4).

The obtained results show that the hydro-methanolic crude extracts prepared by maceration or decoction and their ethyl acetate fractions exert an inhibitory effect towards α-amylase. This inhibitory effect is proportional to the concentration of each extract.

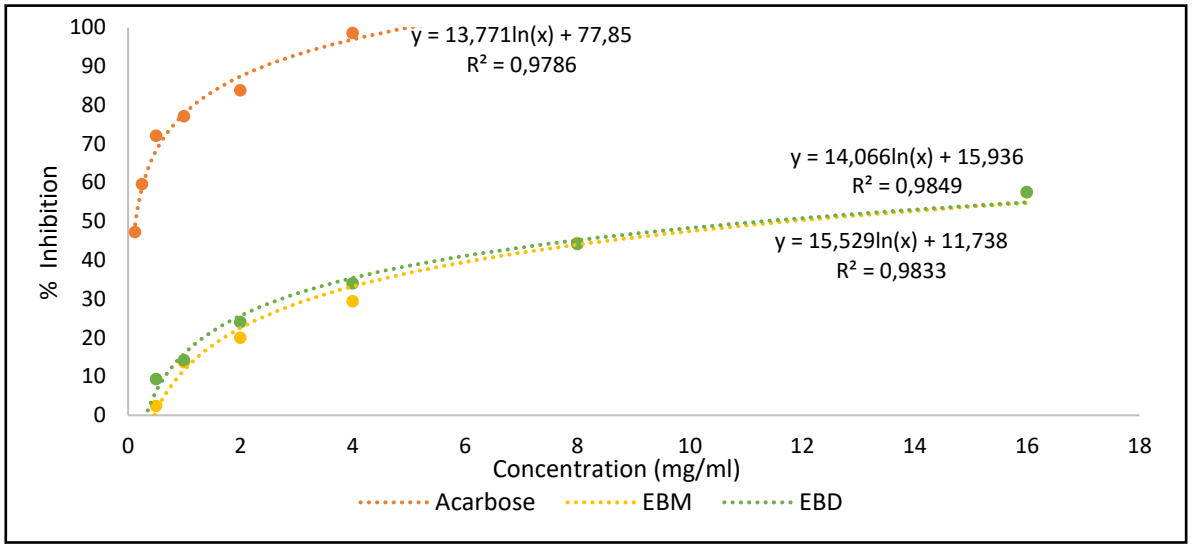


Figure 1. Evolution of inhibition percentages based on concentrations of acarbose and hydro-methanolic crude extracts prepared by maceration or decoction from *C. violacea* roots.

EBM: Crude hydromethanolic extract prepared by maceration; **EBD**: Crude hydromethanolic extract prepared by decoction.

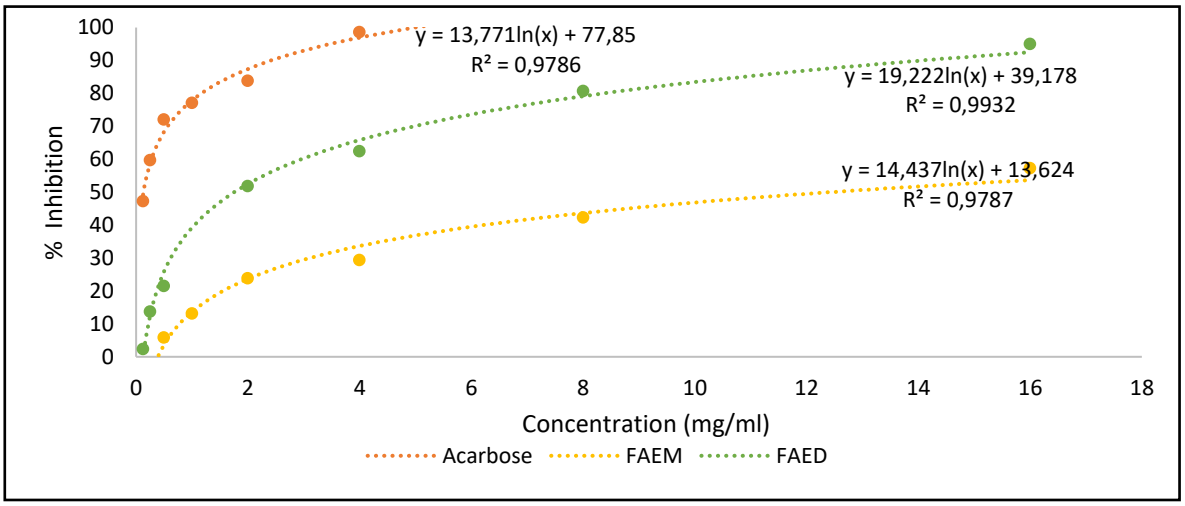


Figure 2. The evolution of inhibition percentages as a function of the concentrations of acarbose and ethyl acetate fractions from *C. violacea* roots.

FAEM: Ethyl acetate fraction of the hydromethanolic crude extract prepared by maceration; **FAEM:** Ethyl acetate fraction of the hydromethanolic crude extract prepared by decoction.

Table 4. IC_{50} values of acarbose and extracts from *C. violacea* roots.

Extracts	***Extraction method***	***IC_{50} (mg/mL)***
Acarbose	-	0.13 ± 0.004
Crude water-methanol extract	Maceration **(EBM)**	11.8 ± 2.5
	Decoction **(EBD)**	11.2 ± 0.7
Ethyl acetate fraction	Maceration **(FAEM)**	12.4 ± 2.4
	Decoction **(FAED)**	1.9 ± 0.13

4. Discussion

The valorization of medicinal and aromatic plants is generating increasing interest in scientific research on a global scale, and they are being increasingly utilized in the pharmaceutical field for therapeutic and cosmetic purposes. In this context, the present study focused on the phytochemical analysis and evaluation of the inhibitory effect of extracts prepared from the roots of *C. violacea* (belonging to the *Orobanchaceae* family) against α-amylase.

C. violacea is a parasitic species on several hosts, including *Haloxylon articulatum* and *Limonistrum guyonianum*. Parasitism is a highly effective life strategy and a biological mechanism connecting several plant species. Upon contact with the hosts, the parasitic plant grows towards them and selectively penetrates their tissues (Alia et al., 2021).

The hydromethanolic crude extracts and their fractions (EAFM and EAFD) are obtained in a viscous or caramelized form with a brown color, and their extraction yields vary depending on the solvent used for extraction. It is noteworthy that the crude extracts show higher extraction yields compared to the fractions, with values of approximately 21.61% and 21.31% for EBM and EBD, respectively. Bouchouka (2016) obtained a yield of approximately 37.60% for the methanolic extract of the aerial part of *C. violacea* prepared using the Soxhlet apparatus. The methanolic extract of *C. violacea* leaves, prepared by maceration, showed a yield of approximately 18.89% (Kacimi and Ammam Nouas, 2020). Ben Attia et al. (2020) recorded yields of approximately 27.3% and 26.4% for the aqueous and methanolic extracts of the aerial part of *C. violacea*, respectively.

The phytochemical tests we conducted on the hydromethanolic crude extracts of *C. violacea* revealed the presence of tannins, flavonoids, free quinones, terpenoids, and reducing compounds, and the absence of alkaloids, anthraquinones, and saponins.

The study conducted by Bouchouka (2016) demonstrated the presence of alkaloids, flavonoids, tannins, and anthocyanins in the methanolic extract of the aerial part of *C. violacea* prepared using the Soxhlet apparatus. The quantitative analysis of phenolic compounds, performed on the same plant extracts, allowed for the highest levels of total polyphenols and flavonoids to be recorded in the ethyl acetate fraction of the crude extract prepared by maceration (EAFM), with content values of approximately 82.9±6.8 µg EGA/mg DW and 112.4±25.7 µg CE/mg DW, respectively. As for tannin levels, the crude extract prepared by maceration (CEM) exhibited the highest content, with a value of around 525.5±77.5 µg CE/mg DW. Ben Lacheheb and Houamed (2019), found contents of around 116 mg GA Eq /g E in total polyphenols and

11.891 mg CE/ g E in flavonoids for the methanolic extract of *C. violacea* flowers. Furthermore, the study conducted by Chenguel (2019), recorded polyphenol and flavonoid contents of approximately 167.74 ± 22.36 mg EGA/ g DW and 26.76 ± 2.69 mg CE/ g DW for the methanolic extract of *Cistanche tinctoria* flowers, respectively. The variations observed in qualitative and quantitative analysis can be attributed to several factors such as climatic and environmental conditions, polarity of the solvent used, harvesting period, plant part utilized, developmental stage of the plant, and the extraction method and duration.

The antidiabetic potential of hydromethanolic crude extracts (CME and CDE) from the roots of *C. violacea* and their ethyl acetate fractions (AEFM and AEFD) was studied by evaluating their inhibitory effects against α-amylase. The obtained results showed that the ethyl acetate fraction obtained from the crude decoction extract (AEFD) exhibited the best inhibitory activity with an IC_{50} of approximately 1.88 mg/mL. Meanwhile, the ethyl acetate fraction obtained from the crude maceration extract (AEFM) presented the weakest inhibitory activity (IC_{50} = 12.42 mg/mL). These results remain lower compared to that of acarbose, which exhibited an IC_{50} of around 0.13 mg/mL. According to our bibliographic research, we did not find any published studies on the inhibitory effect of *C. violacea* on α-amylase. Certain families of secondary metabolites have demonstrated an inhibitory effect on α-amylase, such as terpenoids, alkaloids, and phenolic compounds (Sales et al., 2012). Notably, phenolic compounds, especially flavonoids, are the most effective inhibitors (Sun et al., 2019).

The inhibitory effect of hydromethanolic crude extracts of *C. violacea* and their fractions against α-amylase could be attributed to their contents of polyphenols, flavonoids, and tannins. The ethyl acetate fraction of *Salvia officinalis* L prepared by decoction, the ethyl acetate fraction of *Clausena indica* prepared by maceration, and the fraction of *Prosopis cineraria* prepared by maceration showed significant inhibitory activity against α-amylase with IC_{50} values of approximately 46.50 ± 2.68 µg/mL, 860 µg/mL, and 40.29 µg/mL, respectively (Mahdi et al., 2020; Hoang Anh et al., 2020; Soni et al., 2018).

5.Conclusion

The results obtained during this study have shown that the roots of *C. violacea* contain polyphenols (flavonoids, tannins, and free quinones), reducing compounds, and terpenoids.

The ethyl acetate fraction recovered from the crude extract prepared by maceration is the richest in total polyphenols and flavonoids, with contents in the range of 82.9±6.8 µg EGA/mg DW and 112.4±25.7 µg EC/mg DW, respectively, followed by the ethyl acetate fraction of the crude extract prepared by decoction. The latter exhibits the most potent inhibitory effect, with an IC_{50} of approximately 1.88 mg/mL.

Our final result shows that the prepared extracts from *C. violacea* roots can be considered as inhibitors towards α-amylase, rich in bioactive components.

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Author Contribution Statement
Rachid AZZI supervised the findings of this work; **Ibrahim BOUCHEKIF**,: Carried out the survey; **Brahim BABALI**: plant identification, **Kouider BOUDAOUD**: plant harvest, **Fayza ABBOU**: discussed the results and contributed to the final manuscript.

Conflict of interest
Authors declare no conflict of interest.

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