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RESEARCH ARTICLE

Assessment of Chemical Composition and Investigation into the Antioxidant, Anti-inflammatory, and Hemolytic Properties of Hexane Extracts from *Cynara cardunculus* subsp. *Cardunculus* and *Cynara cardunculus* subsp. *sylvestris*

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Abstract: ***Background*:** *Cynara cardunculus* is frequently used in Mediterranean cuisine and is known for its possible medicinal properties. These properties are usually related to the presence of specific bioactive compounds present in the leaves of the artichoke. On the other hand, the root parts of the artichoke have not been subjected to extensive studies so far.

***Objective*:** The main objective of this study was to conduct a chemical analysis of the root part of the hexane extract of *Cynara cardunculus* subsp. *cardunculus* and *Cynara cardunculus* subsp. sylvestris, while exploring their antioxidant, anti-inflammatory, and hemolytic effects.

***Methods*:** The chemical composition of the extracts of both species was analyzed using gas chromatography (GC) and gas chromatography coupled with mass spectroscopy (GC/MS). The antioxidant properties were evaluated using the DPPH radical scavenging method. The anti-inflammatory activity was evaluated through the protein denaturation method using diclofenac as a positive control. The hemolytic effect was examined on a suspension of erythrocytes in human blood.

***Results*:** The main constituents of the hexane extract of *C. cardunculus* and *C. sylvestris* were aplotaxene (70.5% and 56.3%, respectively) and hexadecanoic acid (10.2% and 13.2%, respectively). The hexane extracts of *C. sylvestris* and *C. cardunculus* showed positive antioxidant activity with the DPPH test by comparing them with the BHT control. However, it should be noted that the extract of *C. cardunculus* showed the best performance, with an IC_{50} of 4.3 µg/mL, while the extract of *C. sylvestris* presented an IC_{50} of 5.6 µg/mL. The hexane extracts of *C. cardunclus* and *C. sylvastris* showed good anti-inflammatory activity with IC_{50}s of 17.3 µg/mL and 23.8 µg/mL compared to diclofenac (IC_{50}= 13.3 µg/mL), respectively. The toxicity assessment on human erythrocytes shows that both extracts of roots of *C. cardunculus* and *C. sylvestris* have a very low hemolysis rate (1.4% and 11.1%, respectively), even at high concentrations (2000 µg/mL).

***Conclusion*:** The extracts obtained from hexane from the plants *C. cardunculus* and *C. sylvestris*, during the tests, revealed particularly promising antioxidant, anti-inflammatory, and hemolytic properties. These results offer an interesting perspective for the creation and development of new antioxidant and anti-inflammatory agents for the pharmaceutical and cosmetic industry.

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1. INTRODUCTION

Some diseases may benefit from an anti-inflammatory and antioxidant approach, as chronic inflammation and oxidative stress are involved in many diseases such as cardiovascular disease, autoimmune diseases, type 2 diabetes, and cancer [1, 2].

It has been proven that synthetic drugs may present side effects and potential health risks, depending on the treated disease, dosage, and individual reaction of each person [3]. Herbal medicine is an ancient approach used in many cultures around the world. Plant extracts contain a variety of

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bioactive compounds, such as flavonoids, terpenes, and alkaloids, which can have beneficial effects on health. These compounds are endowed with many biological activities and are an excellent source for the search for a new compound for therapeutic use [3].

Cynara cardunculus l., which belong to the family Asteraceae and commonly called "cardon", is abundant in the Mediterranean region [4]. This plant includes two botanical varieties: *C. cardunculus* var. *altilis*, syn. = *C. cardunculus* L. *subsp. cardunculus* (domestic cardon) and *C. cardunculus* l. var. *sylvestris* (lamk) fiori (wild cardoon). Additionally, *C. cardunculus* is considered the natural ancestor of the globe's artichoke [5, 6]. The cardoon (*C. cardunculus*) is a perennial plant that shares botanical ties with the artichoke. It is frequently cultivated for both its aesthetic appeal as an ornamental plant and its culinary uses. Domesticated cardoons were developed from wild cardoons by selective breeding. This plant boasts a nutritional profile characterized by high fiber content, antioxidants, and various vitamins and minerals. Beyond its culinary applications, cardoons have a history of use in traditional medicine. They are reputed for their potential in treating liver diseases and serving as an antidiabetic, cardiotonic, choleretic, and anti-hemorrhagic agent [7-11]. Indeed, numerous studies highlight the diverse array of bioactive compounds found in various plant tissues of *C. cardunculus*. The plant is a valuable source of essential dietary components, including fiber, minerals, and inulin. Moreover, its phenolic compounds, predominantly originating from caffeoylquinic and dicaffeoylquinic acids, contribute to its nutritional richness. Additionally, *C. cardunculus* contains anthocyanins, sesquiterpene lactones, and flavonoids, such as luteolin and apigenin derivatives [7, 11, 12].

Scientific evidence has associated *C. cardunculus* with a range of health-promoting effects, including lipid-lowering, antioxidant, and anti-inflammatory effects [4, 13, 14]. In this study, the main objective was to deepen our knowledge of the chemical composition of the roots of these two species, considering them as valuable sources of beneficial bioactive compounds to mitigate oxidative stress and inflammation. For this study, the root parts of *C. cardunculus* subsp. *cardunculus* and *C. cardunculus* subsp. *sylvestris* grown in Algeria were used. The hexane extracts have been prepared and tested for their antioxidant and anti-inflammatory properties for the first time. The chemical compounds were characterized by analyses of GC and GC-MS. In addition, the hemolytic potential of these two extracts was evaluated using appropriate tests.

2. MATERIALS AND METHODS

2.1. Plant Materials and Extraction

The plant material used in this study comes from the roots of two plants, namely *C. cardunculus* and *C. sylvestris*. These plants were harvested in March 2021. The roots of *C. cardunculus* were collected in the Sebdou region, located 38 km from Tlemcen (Algeria), while those of *C. sylvestris* were collected at Bouhenak (Tlemcen, Algeria). The botanical identification of the two plants was carried out at the level of the Department of Ecology and Ecosystem Management of the University of Tlemcen. An amount of 91g of the dried roots of *C. sylvestris* and 115 g of *C. cardunculus* were placed in a well-sealed Erlenmeyer containing 350 ml of hexane. The mixture was kept in maceration for a week at a temperature of 3C°. The mixtures were filtered and evaporated using a steam burp to remove the hexane. The extracts obtained were stored in opaque glass pillboxes at a temperature of 4°C away from light.

2.2. Identification of the Extract Components

Gas chromatography (GC) analysis was performed using a Perkin Elmer Auto system XL GC apparatus equipped with two capillary columns (60 m x 0.22 mm i.d. 0.25 µm film thickness). The oven temperature was regulated for an increase from 60°C to 220°C at 2°C/min and then kept isothermally for 35 min at 230°C. The temperatures of the detector and injector continued at 280°C. The injection volume was 0.1 µL. Gas chromatography-mass spectrometry (GC/MS) was carried out using a Perkin Elmer Turbo mass detector coupled to a Perkin Elmer Autosynstem XL equipped with dual fused silica capillary columns, which functioned with the same Gas chromatography mentioned above except for the split, which was 1/80. The ion source temperature was 150°C, the ionization energy (IE) was 70 eV, and the mass spectra were acquired with a mass range of 35 to 350 Da. The identification of components was conducted using two main approaches. Firstly, it involved comparing their GC retention indices (RI) on nonpolar and polar columns, which were determined in relation to the retention time of a series of n-alkanes through linear interpolation. This comparison was made with reference to authentic compounds or literature data [15, 16]. Secondly, computer matching was employed, utilizing commercial mass spectral libraries [17, 18], and spectra were compared with those stored in our in-house laboratory library.

2.3. Determination of Antioxidant Activity

2,2-diphényl 1-picrylhydrazyle (DPPH) is a relatively stable free radical that absorbs in the UV-visible range at 515-520 nm. In order to measure or verify the free radical scavenging capacity of molecules called antioxidants, we use DPPH, which turns yellow when it is reduced. According to the protocol described by Que *et al.* [19], 1 mL of various concentrations of samples ranging from (0.5 mg/mL to 10 mg/mL)) was mixed with 1 mL of the ethanolic solution of DPPH (0.1 mM). After 30 minutes of incubation at room temperature and in the dark, the antioxidant activity was measured at 517 nm against blank and the standard antioxidant (BHT). The percentage of anti-radical activity was calculated by the following formula:

(%) = [(Abcontrol – Absample)/Abcontrol]*100

Where: Ab: absorbance

2.4. Iron Reduction Method (FRAP)

The method used for the samples was as described by Oyaizu [20] with some modifications. One milliliter of each extract at different concentrations (ranging from 0 to 50 µg/mL) was mixed with 2.5 mL of phosphate buffer (0.2 M, pH = 6.6) and 2.5 mL of a 1% potassium ferricyanide solution, $K_3Fe(CN)_6$. The mixture was then centrifuged at 3000 rpm for 10 minutes. Subsequently, 2.5 mL of the supernatant from each concentration was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% $FeCl_3$. After a 30-minute incubation, the absorbances were measured at 700 nm using a double-beam visible spectrophotometer against ethanol (80%) under vacuum. A solution of a standard antioxidant, BHT, was used as a positive control, and its absorbance was also measured under the same conditions as the samples.

2.5. *In-vitro* Anti-inflammatory Activity

According to the method of protein denaturation, the activity *in vitro* was assessed. A mixture of 0.2 ml of fresh egg albumin, 2.8 ml of phosphate-buffered saline PBS (ph= 6.4), and 2 ml of different concentrations of samples was incubated for 15 minutes at 37°C and then heated in a hot water bath at 70°C for 5 minutes. After cooling, the absorbance was measured at 660 nm [21, 22]. Additionally, diclofenac sodium was used as the reference drug [21]. The percentage inhibition of protein denaturation was determined by using the following formula:

$$\text{Inhibition (\%)} = [(A_{bt} / A_{bc}) - 1)] * 100$$

where: A_{bt}: the absorbance of the sample, A_{bc}: the absorbance of control.

2.6. Evaluation of the Hemolytic Activity

Hemolytic activity was evaluated as described previously by Andra *et al.* [23] with a slight modification [20]. Red blood cells (erythrocytes) were centrifuged and washed with phosphate-buffered saline solution (PBS: 1.5 mM KH_2PO_4, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 135 mM NaCl, pH-7.4) to remove residual plasma. The suspended red blood cells were washed in saline PBS to obtain a concentration of 2%. A series of concentrations of the extract with the héxane was prepped. One hundred and fifty µL of extract mixed with PBS were incubated with 150 µL suspension of human erythrocytes for 60 minutes at 37°C. After 10 minutes of incubation, the solution was centrifuged to separate the lytic red blood cells from the plasma. The release of hemoglobin in plasma was achieved by spectrophotometry at a wavelength of 540 nm. Hemolytic activity was determined using the following formula.

$$\text{Hemolysis (\%)} = \frac{A_{(\text{extract})} - A_{(\text{Negative control})}}{A_{(\text{Positive control})}} \text{X } 100$$

2.7. Statistical Analysis

An analysis of variance (ANOVA) was employed to statistically evaluate the means of the data. The significance values were assessed based on the *p*-values obtained ($p<0.05$ = significant, $p<0.01$ = moderately significant, $p<0.001$ = highly significant). Each test was conducted three times.

3. RESULTS AND DISCUSSION

3.1. Chemical Composition of *C. cardunculus* and *C. sylvestris* Hexane Extracts

The yield of *C. sylvestris* and *C. cardunculus* hexane extracts was 2.3% and 3.0%, respectively. Hexane extracts of *C. sylvestris* and *C. cardunculus* were analyzed by CPG and CPG-SM. Five majority elements represent more than 93.2% of the total chemical composition of the *C. sylvestris* hexane extract mainly including aplotaxene (56.3%), hexadecanoic acid (13.2%), 6-methyl-hept-5-in-2-one (7.6%), tetradecene (6.3%) and 9-oxabicyclo nonane (5.6%) were identified (Table **1**). On the other hand, six components were identified in the extract of *C. cardunculus*, namely aplotaxene (70.5%), hexadecanoic acid (10.2%), nonal (5.2%), tetradecane (3.2%), 9-oxabicyclo nonane (1.2%), eudesma-7,11-en-4-α-ol (0.2) and nomadendrene (0.1%) representing 90.4% of the total composition (Table **1**).

Previous work on volatile flavours of *C. cardunculus* has reported the presence of hexadecanoic acid, methyl hexadecanoate, methyl octadecadiene, tetradecanoic acid, and dodecanoic acid. Chemical studies of *C. cardunculus* subsp. *cardunculus* showed the appearance of sterols and triterpenoids [6, 24, 25], coumarins [26], flavonoids [27-29], triterpenoid saponins [20], and acids [7]. On the other hand, *C. cardunculus* subsp. *sylvestris* was composed mainly by the presence of several phenolic acids, such as caffeoylquinic acid, including cynarin, and flavonoids, such as derivatives of luteolin and apigenin [6]. Their leaves are high in polyphenols [30] and flavones [31].

Table 1. Chemical composition of the hexane extract of the root part of *C. cardunculus* and *C. sylvestris*.

Compounds	RI apol	RI pol	*C. s*	*C. c*	Identification
6-Methyl-hept-5-en-2-one	961	1337	7,6	-	RI,MS
9-Oxabicyclo nonane	963	1343	5,6	1,2	RI,MS
Nonal	1082	1083	0,6	5,2	RI,MS
Nomadendrene	1452	1450	1,3	0,1	RI,MS
Tetradecene	1487	1809	6,3	3,2	RI,MS
Aplotaxene	1657	1871	56,3	70,5	RI,MS
Eudesma-7,11-en-4-α-ol	1683	2300	2,3	0.2	RI,MS
Hexadecanoic acid	1942	2890	13,2	10,2	RI,MS
TOTAL			**93,2**	**90,6**	RI,MS

Abbreviations: C.S: *C. sylvestris*: C.c: *C. cardunculus*. RI. Retention index; MS. Mass spectrometry in electronic impact mode.

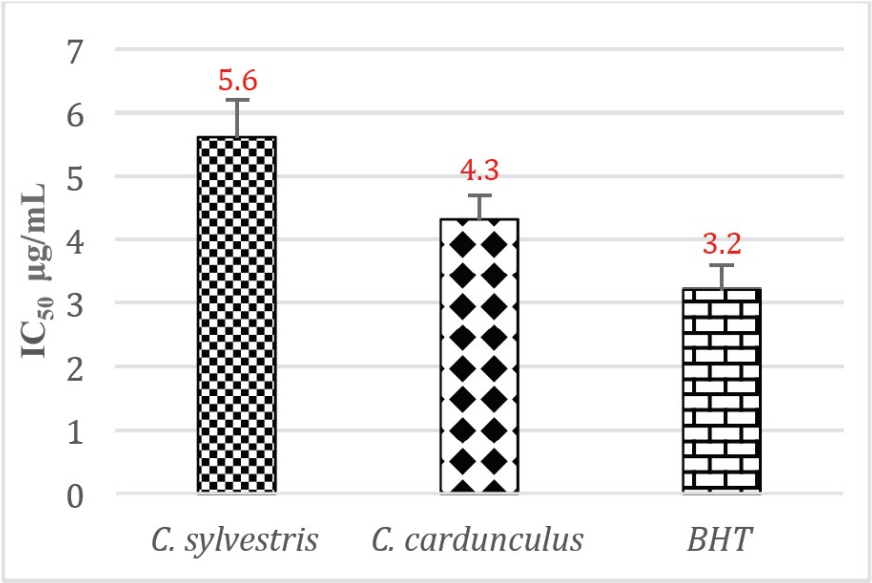

Fig. (1). IC_{50} of hexane extracts determined by the DPPH method. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

3.2. Evaluation of the Antioxidant Properties of Hexane Extract by DPPH Method

The DPPH method was used to evaluate the antioxidant efficacy of two extracts, thus obtaining a rapid evaluation of the ability of these extracts to neutralize free radicals. According to the results presented in Fig. (**1**), it was observed that the hexane extract of *C. sylvestris* exhibits the highest antioxidant potential, with an IC_{50} of 4.3 mg/mL. Then, the hexane extract of *C. cardunculus* also showed significant antioxidant activity with an IC_{50} of 5.6 mg/mL, although this remains lower than the control BHT (IC_{50} = 3.2 mg/mL).

The antioxidant activity of the extracts of both *Cyanara* species can be attributed to the presence of various compounds, such as aplotaxene and hexadecanoic acid. Indeed, previous studies have effectively suggested that aplotaxene has antioxidant activities [32, 33]. Hexadecanoic acid has been reported to possess antioxidant activities [34, 35]. It is important to note that antioxidant activity can result from the complex interaction of several compounds present in the extract. Further investigation of isolated compounds may be necessary to accurately identify the compounds responsible for antioxidant activity.

3.3. Evaluation of the Antioxidant Properties of Hexane Extract by FRAP Method

The FRAP method is often used in conjunction with the DPPH method to assess the antioxidant activity of a sample. At a concentration of 50 µg/mL, the extracts of *C. cardunculus* and *C. sylvestris* have a reducing power with an optical density (OD) of 2.9 and 2.71, respectively. In comparison, the synthetic antioxidant BHT has a DO of 3 at a concentration of 13 µg/mL. The absorbances measured at 700 nm indicate that *C. sylvestris* and *C. cardunculus* hexane extracts were able to reduce ferric ions to ferrous ions. These two extracts showed a reducing power, but relatively low to that of BHT, was used as a reference (Fig. **2**).

The hexane extracts of these two species are endowed with very interesting antioxidant activity and could play a crucial role thanks to their very interesting antioxidant activity by neutralizing free radicals, thus reducing oxidative stress, which can damage the cells, thus promoting the appearance of various chronic diseases. For example, *Thymus vulgaris* oil, rich in thymol and carvacrol, showed powerful antioxidant activity by trapping free radicals [36]. In addition, flavonoids in olive oil inhibit xanthine oxidase, reducing oxidative stress [37]. Finally, clove essential oil, thanks to its eugenol content, can increase the activity of antioxidant enzymes in the body. These combined mechanisms explain the ability of these extracts to reduce oxidative stress and protect cells from oxidative damage [38].

3.4. Evaluation of the Anti-inflammatory Property

The anti-inflammatory activity of the extracts of *C. cardunculus* and *C. sylvestris*, as well as that of the standard anti-inflammatory drug (Diclofenac sodium), was evaluated by the egg albumin denaturation method. The results indicate an inhibition of denaturation of proteins, including albumin, depending on the concentration of the samples (5.0 To 30 µg/ml). The observations reveal that the samples of *C. sylvastris* and *C. cardunclus* have a remarkable inhibitory effect, with inhibition rates of 84.9% and 75.56%, respectively, at a concentration of 30 µg/ml.

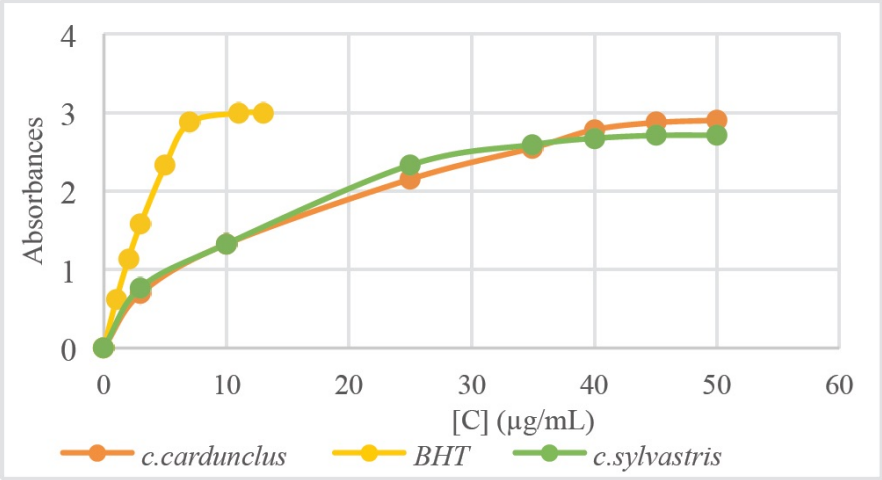

Fig. (2). Evaluation of antioxidant power by the FRAP method. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Table 2. Chemical composition of the hexane extract of the root part of *C. cardunculus* and *C. sylvestris*.

Samples	-	Anti-inflammatory Activity						IC_{50} (µg/mL)
C. sylvastris	Concentration (µg/mL)	5.0	10	15	20	25	30	**23.8**
	Inhibition of denaturation (%)	5.4 ± 0.4	15.4 ± 1.2	20.1 ± 0.4	33.2 ± 1.4	52.3 ± 1.4	75.6 ± 2.3	
C. cardunclus	Concentration (µg/mL)	5.0	10	15	20	25	30	**17.3**
	Inhibition of denaturation (%)	20.7 ± 1.2	26.1 ± 0.8	47.1 ± 0.4	55.2 ± 2.2	62.1 ± 2.1	84.9 ± 2.6	
Diclofenac	Concentration (µg/mL)	5.0	10	15	20	25	30	**13.3**
	Inhibition of denaturation (%)	29.9 ± 0.9	40.6 ± 1.6	55.7 ± 0.9	65.7 ± 3.2	80.4 ± 3.2	86.12 ± 3.4	

Abbreviations: Samples and positive control were done in triplicates (n=3). Values expressed are means ± S.D

These performances are comparable to that of diclofenac, which achieves an inhibition rate of 86.12% at the same concentration (Table **2**). The effective concentration at which the sample or drug achieves a 50% inhibition (IC_{50}) was determined by following the percentage inhibition relative to the treatment concentration. A comparison of the results of the anti-inflammatory activity revealed that *C. cardunclus* (IC_{50} = 17.3 µg/mL) showed the best activity close to reference diclofenac (IC_{50} = 13.3 µg/mL), while *C. sylvastris* showed the lowest activity with an IC_{50} of 23.8 µg/mL. The GC-MS analysis of hexane extracts identified a significant concentration of aplotaxene and hexadecanoic acid.

According to various reports, saturated fatty acids such as hexadecanoic acid have been associated with the regulation of the anti-inflammatory pathway [39, 40]. At the same time, aplotaxene has already demonstrated well-established anti-inflammatory properties, according to Benhamidat *et al.* [32, 33]. The hexane extracts of *C. cardunculus* and *C. sylvestris* have shown anti-inflammatory potential; however, these extracts can inhibit the production of pro-inflammatory mediators, which are essential in inflammatory processes. For example, the essential oil of *Zingiber officinale*, rich in gingerols, is known to selectively inhibit cyclooxygenase-2 (KO-2) [41]. In addition, the bioactive compounds present in these extracts can regulate cell signaling pathways, particularly by inhibition. On the other hand, the essential oil of *Lavandula angustifolia* has shown the ability to inhibit the activation of NF-B [42].

3.5. Hemolytic Activity

According to the data in Table **3**, it can be observed that hexane extracts of *C. cardunculus* and *C. sylvestris* induce dose-dependent hemolysis. After 60 minutes of incubation, the hemolysis rate for *C. cardunculus* and *C. sylvestris* extracts varies between 1.4% and 11.1%. This level remains relatively low compared to gallic acid, whose maximum concentration of 2000 µg/mL causes a very powerful hemolytic effect, reaching about 80%. Notably, at the maximum concentration of 2000 µg/mL, the *C. cardunculus* extract has the lowest hemolytic effect, with a percentage of 10.2%, followed closely by the *C. sylvestris* extract, which has a percentage of 11.1% (Table **3**). These results show very limited toxicity of these extracts, even at higher concentrations and after an incubation period of 60 minutes, with respect to isolated human erythrocytes (red blood cells).

Table 3. Results of the hemolytic activity of the hexane extract of the root part of *C. cardunculus* and *C. sylvestris*.

Concentration (µg/mL)	% Hemolysis of *C. cardunclus*	% Hemolysis of *C. sylvastris*	% Hemolysis of Gallic Acid
100	1.4 ± 0.6	1.4 ± 0.3	38.9 ± 0.6
300	1.8 ± 0.6	1.9 ± 0.2	48.9 ± 0.5
500	2.9 ± 0.4	2.7 ± 0.2	58.9 ± 0.6
1000	8.9 ± 0.5	7.7 ± 0.1	78.9 ± 0.5
2000	10.2 ± 0.2	11.1 ± 0.3	80.1 ± 0.8

Abbreviations: Samples and positive control were done in triplicates (n=3), Values expressed are means ± S.D

CONCLUSION

To date, no previous publication appears to have addressed the antioxidant, anti-inflammatory, and hemolytic characteristics of hexane extracts from the roots of *species C. cardunculus* and *C. sylvestris*, making our results an unprecedented contribution to the search for new molecules endowed with biological activities. The results indicated that aplotaxene predominated in both extracts. The findings of this study also showed that both extracts have significant antioxidant and anti-inflammatory properties.

The toxicity test for human erythrocytes reveals that both extracts have a very low hemolysis rate, even at high concentrations and after one hour of administration, in comparison with gallic acid. These properties position the roots of *C. cardunculus and C. sylvestris* species as potential natural alternatives to be considered in the food and pharmaceutical industries for the treatment of diseases involving oxidative stress as well as for the treatment of inflammation.

However, prior to practical application, extensive research, clinical trials, and safety assessments are required to ensure the efficacy and safety of these extracts.

AUTHORS CONTRIBUTION

N.M., R.A., M.A.D., and A.M. contributed to the research design and implementation, as well as the data analysis and manuscript writing.

LIST OF ABBREVIATIONS

ANOVA	=	Analysis of Variance
GC	=	Gas Chromatography
IE	=	Ionization Energy
OD	=	Optical Density
RI	=	Retention Indices

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable

HUMAN AND ANIMAL RIGHTS

Not applicable.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The authors confirm that the data supporting the findings of this research are available within the article.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Declared none.

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