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Research Article

Antioxidant, anti-inflammatory effects of *Centaurium erythraea* Rafn. aerial part extracts and identification of its bioactive constituents by LC-MS/MS analysis

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Abstract:

Centaurium erythraea Rafn. (CE) is a medicinal plant used in traditional medicine to cure several illnesses. This study aimed to evaluate the LC-MS phytochemical analysis, antioxidant, *in vivo* topical anti-inflammatory effects and acute oral toxicity of the methanolic (MethE) and the decoction extract (DecE) of this plant. A qualitative and quantitative analysis were performed. The qualitative analysis indicated the presence of phenolic compounds: phenolics, flavonoids, tannins, quinones, terpenes and alkaloids while the anthraquinone, coumarins and proteins were absent. For the quantitative analysis, LC-MS analysis revealed the presence of ascorbic acid, rutin, synergic acid, luteolin and other phenolic compounds in the extracts. Furthermore, the results showed that the DecE present high amounts in polyphenols, flavonoids and condensed tannins with highest values of 77.61 µg gallic acid equivalent/ mg of extract; 19.17 µg quercetin equivalent/ mg of extract and 81.70 µg catechin equivalent/mg dry extract, respectively. For the antioxidant activity, DecE showed an IC_{50} of 119.00 and 85.32 µg/mL for DPPH radical scavenging and the reducing power, respectively. This extracts showed a percentage of inhibition of 86.11 % in the anti-inflammatory activity. CE was not toxic even at 2 g/kg body weight. This suggests that this plant confirms the purposes of the plant in folk medicine and is a potential source of organic anti-inflammatory and antioxidant agents.

1. Introduction

Medicinal plants are directly employed in traditional medicine and phytotherapy after being prepared in various forms such as infusions, tisanes, or macerations, and are then used for either external or internal consumption [1]. Their effects on the human body are attributed to their chemical composition, which determine their pharmacological activity through different mechanisms associated with phenolic compounds [2]. The results of related studies may contribute to advanced research, particularly in drug design and discovery, where medicinal plants serve as essential sources of therapeutic agents [3-6]. Recently, many studies have focused on identifying and evaluating the bioactivities of chemical constituents of plants commonly known as phyto-compounds in order to explore new pharmaceutical sources. This includes investigating their roles and mechanisms of action within the human body [7]. In addition to their efficacy, medicinal plants generally lack harmful side effects, which is why there is increasing global interest in replacing synthetic drugs with herbal formulations [2]. Furthermore, their biological activity forms the foundation for numerous modern medicines [3]. Hence, scientific research should

increasingly focus on plant-based drug discovery. Phenolic compounds exhibit potent antioxidant activity, attributed to their chemical structure and functional groups [8]. Therefore, they help prevent oxidative stress-related damage in biomolecules [9] depending on their capacity of scavenging reactive oxygen species and suppressing their formation. This antioxidant activity is associated with antidiabetic, anti-inflammatory, antitumor, neuroprotective, cardioprotective, and antiaging effects [10]. Polyphenols modulate the activity of the enzymes that are involved in arachidonic acid metabolism [11]. During the onset of inflammation, immune cells produce more free radicals to eliminate pathogens, leading to chronic inflammation and cellular damage, thereby linking the two processes [12, 13].For example, polyphenols modulate the activity of the enzymes that are involved in arachidonic acid metabolism [11]. *C. erythraea* Rafn. is a small centaury [14] and an annual or biennial herbaceous medicinal plant [15] from the Gentianaceae family. This plant is distributed throughout the Mediterranean regions [16] and is also known as *Centaurium umbellatum* or *Centaurium latifolium* [17]. In Arabic, it is known as Kantaroiun, Marrarat Lahnech, Kina, and Klilwa. It is now one of the 28 species of *Centaurium*. It grows in wet meadows, woodlands and sandy terrains. It is generally harvested during its blooming season, from May to August [1]. Traditionally, it is used to treat dyspepsia, loss of appetite and digestive problems [18], atopic dermatitis, cancer [19], pneumonia, cardiovascular disorders asthma, gastric pain, kidney diseases, and skin problems. Moreover, it can be used as an antiapoplectic and anticoagulant [15], anthelmintic, hypotensive, antipyretic, antidiabetic, and externally as a wound healer [20]. Based on the literature review, there is no available information regarding the biological activity or therapeutic uses of this plant in Algeria. Moreover, limited research has been conducted on this plant. Therefore, this study aimed to examine the toxicity, total phenolic content, antioxidant properties, and *in vivo* topical anti-inflammatory activity of various extracts of *Centaurium erythraea* Rafn., as well as to identify potential therapeutic applications and directions for future research.. In addition, the phenolic compounds in the phenolic-rich extracts were identified and quantified using LC-MS/MS analysis. To the best of our knowledge, no previous research have investigated the topical *in vivo* anti-inflammatory properties of these extracts for treating skin inflammation. These experiments were also conducted to characterize the anti-inflammatory and antioxidant effects, assessing the validity of their use in traditional medicine for treating inflammation.

2. Material and Methods

2.1. Animals

Swiss albino mice, purchased from the Pasteur Institute in Algiers, weighing 25 and 30 g, were used for *in vivo* studies. A 12/12 h light/dark cycle and a consistent temperature of 25 to 30°C are maintained in their animal facility. The mice were returned to their original cages, with six mice per cage. The National Office of Cattle Feed (ONAB) in Bejaia provided these mice with a regular diet (ad libitum) as well as unfettered access to water and standard food after 14–20 days of acclimatization. In accordance with Animals By-Laws N° 425-2008, mice were handled. Testing procedures were developed by the "Algerian Association of Sciences in Animal Experimentation".

2.2. Plant Collection and Drying

The fresh aerial part plant of *C. erythraea* (CE) was collected from Ouled-Tebban, located in the northeastern part of Algeria (Figure 1). This region is known for its diverse flora and significant traditional medicinal knowledge. The plant was identified and confirmed by a botanist Prof. H. Laouer at the Laboratory of Valorization of Natural Biological Resources, University Setif-1, Algeria. The Department of Vegetal Biology and Ecology herbarium at the University Setif 1, Algeria, received a voucher specimen (025/DBEV/UFA/23). The plant material was cleaned and air-dried in the shade after harvest to maintain its quality. An electric grinder was then used to powder it.

Figure 1. *Centaurium erythraea Rafn. from Algeria*

2.3. Extracts Preparation

2.3.1. Preparation of Decoction Extract

Approximately 50 g of dried plant materials were boiled for 20 minutes in 500 mL of distilled water to create the decoction extract [21]. After passing through muslin fabric, the fluid was centrifuged for 20 minutes at 4000 rpm. Before being assessed for pharmacological activity, the dried decoction extract (DecE) was obtained at 4°C.

2.3.2. Preparation of Methanolic Extract

A defatting was performed by hexane: approximately 50 g of dried plant material were soaked in 500 mL hexane for 2 days at 4°C with occasional stirring. Then, the mixtures was filtered and residues were extracted with 500 mL of methanol for 2 days at 4°C with occasional stirring. Then the solutions were filtered through muslin cloth, and the solvents were evaporated under reduced pressure to produce an initial methanolic extract (MethE). The solution was filtered then the dried extract was stored at 4°C.

2.4. Phytochemical Evaluation

2.4.1. Qualitative Phytochemical Analysis

The presence of potential phytochemical components such as polyphenols, flavonoids, terpenoids, condensed and hydrolysable tannins, saponins, alkaloids, coumarins, quinones, anthraquinones, proteins was detected in two extract (DecE and MethE), [21].

2.4.2. Quantitative Phytochemical Analysis

2.4.2.1. LC-MS/MS Analysis

Analysis of some selected phenolic acids and flavonoids was performed on AB Sciex QTRAP 4500 LC MS/MS (Japan), utilizing C-18 column (100mm × 4.6 mm, 5 µm, ambient temperature). The analyzed ethanolic and decoction extracts were freshly prepared (10 mg/1.5 mL of each sample separately) and dissolved with 1.5 mL of acidified methanol (0.1% FA) and deionized water, respectively. The samples filtrated and 5 µL of the samples were injected over the developed method in QTRAP 4500. The flow rate was 0.3500 mL/min. The sample solutions were prepared and immediately analyzed for their phenolic acids and flavonoid compounds using HPLC-MS/MS. A stock solution of the phenolic acids and flavonoids (26 Std) was prepared in HPLC-grade methanol. Quantitative data of phenolic compound refer to the injected reference standard of mixed phenolic standards.

2.4.2.2. Total Phenolics, Flavonoids and Condensed Tannins Contents Determination

The total phenolic content of the extracts was determined using the Folin-Ciocalteu method. The polyphenolic content was expressed as µg gallic acid equivalent (GAE) per mg of extract. A gallic acid standard curve, with concentrations ranging from 0.00 to 160 µg/mL, was used to quantify the polyphenol content in the various extracts [23]. Simultaneously, the total flavonoid content was determined using the aluminum chloride method. The flavonoid content in the various extracts was quantified using a standard curve of quercetin concentrations ranging from 0.00 to 40 µg/mL [23]. Additionally, condensed tannins were measured using the vanillin method in an acidic medium [24]. This technique exploits the reaction between vanillin and condensed tannin units in the presence of hydrochloric acid (HCl), resulting in a complex that absorbs at a wavelength of 500 nm. The tannin content was expressed as µg catechin equivalent (CE) per mg of extract.

2.5. Antioxidant Activity

2.5.1. DPPH Radical Scavenging Assay

The extracts' capacity to scavenge free radicals was assessed using the 2,2'-diphenyl-1-picrylhydrazyl (DPPH) assay by looking for a decline in the DPPH maximum absorbance at 517 nm [21]. The scavenging capacity was computed using the formula: $I\ \% = (A_{test} – A_{blank} / A_{llank}) \times 100\%$.

A_{test} is the absorbance of the tested sample, and A_{blank} is the absorbance of the solution other than the tested sample.

2.5.2. Reducing Power activity

The reducing power of the extracts from plant was determined by the capacity to reduce Fe^{+3} to Fe^{+2} ions assay [22]. According to this procedure, an aliquot of 400 µL of extract was mixed with an identical volume (400 µL) of both phosphate buffer (0.2 M, pH = 6.6) and potassium ferricyanide (1%). This mixture was then incubated for 20 min at 50 °C in a water bath. The reaction was terminated by adding 400 µL of trichloroacetic acid (TCA, 10%), and the mixture was centrifuged at 3000 rpm for 10 min. The supernatant (400 µL) was added to distilled water (400 µL). The diluted solution was treated with 80 µL of 0.1% ferric chloride. The colour intensity of the mixture was measured at 700 nm after 10 min of incubation. In this context, a high absorbance of the solution means a high reducing power.

2.6. Anti-inflammatory Activity

Xylene-induced ear edema in mice was used to evaluate the topical anti-inflammatory efficacy of the extracts. Mice were divided into four groups, each consisting of six animals (n = 6). Group 1 (+ control) received 0.5 mg of indomethacin topically, group 2 (- control) was treated with xylene alone, and groups 3 and 4 were treated topically with two extracts (2 mg/ear). Edema was induced by applying 30 µL of xylene to each ear, and ear thickness was measured using a digital caliper before and 2 hours after xylene application [24].

2.7. Acute Toxicity

The acute oral toxicity of the extracts was evaluated in mice following globally recognized procedures [25]. Mice were divided into three groups (n = 5) and fasted for 12 hours prior to the experiment. Mice in the first and second groups received 2 g/kg of the two extracts (in 0.5 mL of water), while mice in the third group (negative control) were given distilled water. Body weight and signs of toxicity were carefully monitored for 24 then, 48 hours, followed by a 14-day observation period.

2.8. Statistical Analysis

All determinations were performed in triplicates, and the results are expressed as the mean ± standard deviation (SD). Statistical significance was assessed using the Student's t-test with GraphPad Prism-5. Differences were considered statistically significant at $p \leq 0.05$.

3. Results and Discussions

3.1. Qualitative Phytochemical Analysis

Qualitative phytochemical tests were conducted on the decoction and methanolic extracts of the aerial parts of *Centaurium erythraea* Rafn., revealing the presence of flavonoids, coumarins, and phenolic compounds in both extracts (Table 1).

Table 1. *Phytochemical screening of Centaurium erythraea Rafn. extracts. MethE: Methanolic extract, DecE: Decoction extract*

Compounds	MethE	DecE
Saponins	-	+
Phenolic substances	+	+
Free quinones	+	+
Hydrolysable tannins	+	+
Condensed tannins	+	+
Alkaloids	+	+
Proteins	-	-
Coumarins	-	-
Flavonoids	+	+
Terpenoids	+	+
Anthraquinones	-	-

+ Presence, - Absence

These findings align with those of Afzal et al. [26], who reported the presence of terpenoids, tannins, phenolics, and sugars in the methanolic extract, while saponins were absent. Our phytochemical analysis supports these findings, confirming the presence of specific chemical families, while also noting the absence of others. This variation can be attributed to differences in several factors, including geographical, physicochemical, and biological parameters. These factors may involve the plant's harvest location, environmental conditions such as light, precipitation, topography, and soil type, as well as the season, harvest period, genetic background, extraction method used, the plant part studied, and their specific phytochemicals [19].

3.2. Quantitative Phytochemical Analysis

3.2.1. LC-MS/MS Analysis

Twenty-six phenolic compounds, including phenolic and flavonoids, which are widespread in edible plants, were analyzed in phenolic-rich the methanolic and decoction extracts (Table 2). According to the LC-MS/MS analysis results of the current study, the ethanolic and decoction extracts of *Centaurium erythraea* Rafn. had very rich phenolic content because of their high percentage of ascorbic acid, rutin, synergic acid, luteolin and other phenolic compounds. The high content of synergic acid was detected in MethE with an area of 57.52% followed by luteolin with an area of 48.90%. Contrary to DecE, in the methanolic extract of this plant, ascorbic acid was not detected. In addition, ascorbic acid was not detected in MethE (Table 2).

3.2.2. Total Polyphenols, Flavonoids and Condensed Tannins Contents

Table 3 shows the polyphenol content in the two extracts. DecE has the highest polyphenol quantity in the quantitative analysis, with 77.61 ± 2.56 µg gallic acid equivalent/mg dry weight, followed by MethE. Additionally, DecE contains a significant concentration of condensed tannins, with 81.70 ± 1.65 µg catechin equivalent (CE)/g dry weight. Both extracts also have a considerable proportion of flavonoids (Table 3).

Table 2. *LC-MS/MS data for phenolic and flavonoids compounds detected in the decoction and methanolic extracts of Centaurium erythraea Rafn. MethE: methanolic extract, DecE: Decoction extract, ND: Not determined.*

			Area %/Retention time (R_t)			
			DecE		MethE	
N°	**Compounds**	**Molecular Formula**	**%**	R_t	**%**	R_t
1	Ascorbic acid	$C_6H_8O_6$	14.08	3.078	/	/
2	Caffeic acid	$C_9H_8O_4$	2.18	3.336	$83.72*10^{-2}$	3.086
3	Gallic acid	$C_7H_6O_5$	ND	ND	01.65	1.437
4	Catechin	$C_{15}H_{14}O_6$	$00.30*10^{-2}$	1.674	$18.68*10^{-2}$	1.924
5	Rutin	$C_{27}H_{30}O_{16}$	19.76	5.696	09.59	5.702
6	Carnosic acid	$C_{20}H_{28}O_4$	$05.64*10^{-2}$	10.768	$00.53*10^{-2}$	10.882
7	Apiginin	$C_{15}H_{10}O_5$	$06.21*10^{-2}$	8.109	1.24	8.093
8	Vanilic acid	$C_8H_8O_4$	$21.27*10^{-2}$	3.373	$62.49*10^{-2}$	3.293
9	Luteolin-7-glycoside	$C_{21}H_{20}O_{11}$	$13.67*10^{-2}$	5.662	01.54	5.588
10	Salvonic acid	$C_{26}H_{22}O$	$01.12*10^{-2}$	6.689	00.33	6.904
11	Apigenin-7-O-glucoside	$C_{21}H_{20}O_{10}$	$30.65*10^{-2}$	6.355	$48.95*10^{-2}$	6.152
12	4-OH-comarin	$C_9H_6O_3$	ND	ND	$21.24*10^{-2}$	5.474
13	Chlorogenic acid	$C_{16}H_{18}O_9$	$68.89*10^{-2}$	12.834	$02.24*10^{-2}$	12.835
14	Quercetin	$C_{15}H_{10}O_7$	$24.8*10^{-2}$	7.240	$57.67*10^{-2}$	7.126
15	Myricetin	$C_{15}H_{10}O_8$	$00.53*10^{-2}$	6.104	$00.50*10^{-2}$	6.152
16	3-O- methyl quercetin	$C_{16}H_{12}O_7$	$28.96*10^{-2}$	8.094	$00.45*10^{-2}$	8.071
17	Ferulic acid	$C_{10}H_{10}O_4$	$04.95*10^{-2}$	4.839	$49.70*10^{-2}$	4.755
18	4-hydroxy cinnamic acid	$C_9H_8O_3$	09.89	4.512	1.51	4.225
19	Synergic acid	$C_9H_{10}O_5$	22.09	3.115	57.52	3.418
20	Hesperidin	$C_{28}H_{34}O_{15}$	06.84	8.061	$20.45*10^{-2}$	7.254
21	Rosmaric acid	$C_{18}H_{16}O_8$	$69.21*10^{-2}$	5.857	08.28	5.843
22	Luteolin	$C_{15}H_{10}O_6$	ND	ND	48.90	7.254
23	Galangin	$C_{15}H_{10}O_5$	$00.88*10^{-2}$	9.657	$14.51*10^{-2}$	9.452
24	Salvonic acid	$C_{26}H_{22}O$	$80.97*10^{-2}$	6.146	$00.16*10^{-2}$	6.238
25	Resveratrol	$C_{14}H_{12}O_3$	$08.68*10^{-5}$	4.743	ND	ND
26	Carnosic acid	$C_{20}H_{28}O_4$	$08.95*10^{-2}$	10.288	$31.74*10^{-2}$	11.720

Table 3. *Total polyphenol, flavonoids and tannins of Centaurium erythraea extracts. MethE: methanolic extract, DecE: Decoction extract, GAE: gallic acid, QE: quercetin, CE: catechin equivalent, respectively.*

Extracts contents/ (mg dry extract)	**MethE**	**DecE**
Total phenolic (µg GAE)	61.52 ± 5.93	77.61 ± 2.56
Total flavonoids (µg QE)	19.57 ± 0.22	19.17 ± 0.45
Condensed tannins (µg CE)	44.38 ± 1.53	81.70 ± 1.65

The total polyphenol content in the methanolic extract of *C. erythraea* reported by Bouyahya et al. [19] is 105.54 ± 0.84 mg GAE/g and 34.27 ± 1.17 mg QE/g of extract, which is higher than the polyphenol content observed in the extracts we studied. In contrast, the polyphenol concentrations in the methanolic extract reported by Tusevski et al. [27] are lower, with a value of 35.45 ± 0.041 mg GAE/g and flavonoid levels of 6.65 ± 0.06 mg QE/g dry extract, which are comparatively lower than those found in our study. On the other hand, Mihaylova et al. [28] reported lower concentrations of polyphenols (3.11 ± 0.10 mg GAE/g) and flavonoids (2.59 ± 0.05 mg QE/g) in the aqueous extract of *C. erythraea*, which is considerably less than what we found in our studied extracts. However, our two extracts demonstrated relatively high concentrations of tannins, which are generally found in the aerial parts of this plant.

3.4. *In Vitro* Antioxidant Effects

3.4.1. DPPH Radical Scavenging Assay

Plant extracts exhibit notable antioxidant activity due to their phytochemical constituents, such as flavonoids and phenolic acids [29]. In this study, the reduction of DPPH• radicals by DecE and MethE was observed at 517 nm. The free radical

scavenging activity (IC_{50}), measured using the DPPH• method, was found to be 119.00 ± 0.01 µg/mL for DecE and 317.86 ± 0.01 µg/mL for MethE, compared to the BHT as standard. DecE demonstrated strong DPPH radical scavenging activity, likely due to its higher concentration of total phenolic compounds (Figure 2).

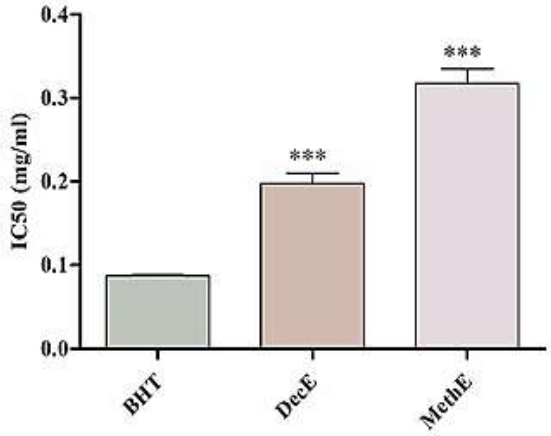

Figure 2. *Free radical scavenging effect of extracts using DPPH model. MethE: methanolic extract, DecE: Decoction extract. Results represent mean ± SD (n = 3). ***: p < 0.001 compared to the standard.*

On the other hand, Bouyahya et al. [19] revealed that the antioxidant activity of ethanolic, *n*-hexane and ethyl acetate extracts showed an IC_{50} of 382.25 ± 5.59, 376.08 ± 3.18 and 49.54 ± 2.43 µg/mL, respectively. However, we have found that 'it is less activity compared to those found. Several polyphenols, including phenolic acids, flavonoids, tannins, and other substances, are widely distributed in plants and have garnered significant attention due to their antioxidant properties and free radical scavenging abilities, which may offer potential health benefits for humans [24].

3.4.2. Reducing Power Effect

Figure 3 illustrates the antioxidant activity curves for the reducing powers of *C. erythraea* extracts. In this assay, all the extracts demonstrated the ability to donate electrons, reducing Fe^{3+} to Fe^{2+}. An increase in absorbance at 700 nm of the final reaction mixture indicates a stronger reducing ability of the compounds. In this test, DecE and MethE exhibited significant reducing power activity, with IC_{50} values of 85.32 ± 0.00 µg/mL and 86 ± 0.00 µg/mL, respectively, compared to Vitamin C as the standard (Figure 3). The antioxidant capacity of plant extracts is closely associated with the quantity and quality of phytochemicals, such as phenolics and flavonoids [29].

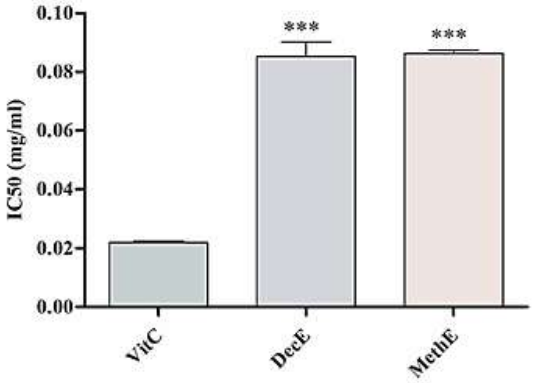

Figure 3. *Reducing power activity of extracts. Values are expressed as means ± SD (n=3). MethE: methanolic extract, DecE: Decoction extract. ns: no significant difference. ***: p < 0.001 as compared with the control.*

3.5. Anti-inflammatory Activity

3.5.1. In vivo Xylene-induced Ear Edema

In vivo topical anti-inflammatory activity of the extracts was evaluated in a mice model using the xylene-induced ear edema model. The results of our study on the anti-inflammatory efficacy of two extracts are presented in Figure 4. The edematous response was quantified by measuring the thickness of the edema with a digital caliper. Both extracts exhibited significant activity against the xylene-induced edematous response. Specifically, our findings indicated that DecE and MethE reduced the edematous response by 85.59 ± 2.08% and 81.48 ± 3.63%, respectively (Figure 4), in comparison to the indomethacin standard.

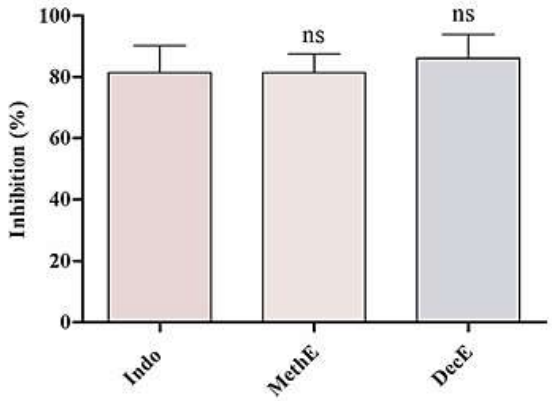

Figure 4. *Percentage inhibition of the ear edema of extracts in mice after 2 hours, at a 2 mg/ear dose. Results are conducted as the mean ± SEM (n = 6). ns: no significant difference.*

Acute inflammatory can be tested using xylene-induced ear edema. The observed changes in the The mechanism behind this process is related to neurogenous inflammation [30] involving sensory neurons that are sensitive to capsaicin [23]. In

addition, xylene ear edema model is used in steroids anti-inflammatory agents and it is less sensitive to non-steroidal ones [31]. In contrast, Mascolo et al. [32] demonstrated that the carrageenan-induced edema showed an inhibition of 19 % in the ethanolic extract witch doses not close to our results. Therefore, the carrageenan rat paw edema has shown a high edema reduction estimated 48.48 % and 75.53 % at 100 mg/kg BW and 150 mg/kg BW in CE aqueous extract [17].
This activity is related to the antioxidant activity and it is due to the presence of active secondary metabolites like polyphenols.

3.6. Acute Oral Toxicity

There was no toxicity, no alteration, and no abnormal behavior changes between the control group and the treated group after 14 days of oral administration of the decoction and methanolic extracts from *C. erythraea* at a single dose of 2 g/kg. The findings demonstrated that the administered dose's LD_{50} was greater than 2 g/kg BW. Tahraoui et al. [33] demonstrated that *C. erythraea* has an LD_{50} that is even more than 15 g/kg BW and that it has no sub-chronic harmful effect. Furthermore, Budniak et al. [34] found that at 5 g/kg, no acute toxicity was detected. These outcomes support the findings of this investigation.

4. Conclusions

In conclusion, our findings indicate that both extracts are potentially rich in phenolic substances, which are of great interest due to their biological properties. They exhibit anti-inflammatory activities, which can play a significant role in preventing various diseases. However, further research is required to investigate the mechanisms of action of this plant, along with its pharmacokinetics and toxicological profile, highlighting the importance of such studies for its potential therapeutic applications. According to these findings, this plant may be a promising new source of naturally occurring antioxidant and anti-inflammatory compounds due to their strong biological activity properties. Therefore, it could have promising potential for medical applications in the future.

Author Statements:

- **Ethical approval:** The "Algerienne Association of Sciences in Animal Experimentation" Committee authorized the experimental assays under statute No. 88-08/1988, which is associated with veterinary medical activities and the protection of animal health (No. JORA: 004/1988).
- **Conflict of interest:** The authors declare that they have no known competing financial interests in this paper
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- **Data availability statement:** The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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