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Original Article

In vitro assessment of antioxidant, anti-inflammatory, neuroprotective and antimicrobial activities of *Centaurea tougourensis* Boiss. & Reut.

[Evaluación *in vitro* de las actividades antioxidantes, antiinflamatorias, neuroprotectoras y antimicrobianas de *Centaurea tougourensis* Boiss. & Reut.]

Mohamed Sabri Bensaad[1,2], Saliha Dassamiour[2]*, Leila Hambaba[2], Chawki Bensouici[3], Hamada Haba[4]

[1]Laboratoire de Physio-Toxicologie, Pathologie Cellulaires et Moléculaires-Biomolécules (LPTPCMB), Département de Biologie des Organismes, Faculté des Sciences de la Nature et de la Vie, Université de Batna-2, Batna, Algeria.
[2]Laboratoire de Biotechnologie des Molécules Bioactives et de la Physiopathologie Cellulaire (LBMBPC), Département de Microbiologie et de Biochimie, Faculté des Sciences de la Nature et de la Vie, Université de Batna-2, Batna, Algeria.
[3]Centre de Recherche en Biotechnologie, Ali Mendjli nouvelle ville UV 03, Constantine, Algeria.
[4]Laboratoire de Chimie et Chimie de l'Environnement (LCCE), Département de Chimie, Faculté des Sciences de la Matière, Université de Batna-1, Batna, Algeria.
*E-mail: s.dassamiour@univ-batna2.dz

Abstract

Context: More than 500 *Centaurea* species compose the Asteraceae family, and most of the recent studies made on the species of this genus proved their pharmacological potential, especially to treat chronic illnesses.

Aims: To evaluate for the first time the antioxidant, anti-inflammatory, neuroprotective and anti-microbial properties of the n-butanol (n-BuOH) and ethyl acetate (EA) extracts of the aerial part of *Centaurea tougourensis*.

Methods: The antioxidant activity was determined by ABTS, galvinoxyl radical, phenanthroline, and reducing power assays, while the anti-inflammatory effects were assessed by heat-induced hemolysis and egg albumin denaturation assays. The neuroprotective activity was assessed against acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) and the anti-microbial activity by the agar disk diffusion method.

Results: Both extracts possess a great antioxidant capacity, but it was considered higher in the n-BuOH extract with respective IC_{50} values of 8.04 ± 0.21 µg/mL in ABTS assay and 4.25 ± 0.6 µg/mL in GOR assay, while the $A_{0.50}$ values were 4.46 ± 0.55 µg/mL in phenanthroline assay and 11.16 ± 0.64 µg/mL in reducing power assay. The n-BuOH extract also showed a remarkable anti-inflammatory activity with an EC_{50} of 120.81 ± 0.2 µg/mL in egg albumin denaturation assay and 154.15 ± 0.14 µg/mL in heat-induced hemolysis assay. The neuroprotective activity of the n-BuOH extract was very strong in both AChE and BChE inhibitory assays with respective IC_{50} values of 9.8 ± 0.62 µg/mL and 173.53 ± 0.04 µg/mL. EA extract was more active on microbial strains.

Conclusions: These encouraging results showed once again the pharmacological potential of *Centaurea* species.

Keywords: anti-inflammatory; antimicrobial; antioxidant; *Centaurea tougourensis*; neuroprotective.

Resumen

Contexto: Más de 500 especies de *Centaurea* componen la familia *Asteraceae* y la mayoría de los estudios de las especies de este género demostraron su potencial farmacológico, especialmente para el tratamiento de enfermedades crónicas.

Objetivos: Evaluar por primera vez las propiedades antioxidantes, antiinflamatorias, neuroprotectoras y antimicrobianas de los extractos de n-butanol (n-BuOH) y acetato de etilo (EA) de la parte aérea de *Centaurea tougourensis*.

Métodos: La actividad antioxidante se determinó mediante ensayos de ABTS, radical galvinoxilo, fenantrolina y poder reductor, mientras que los efectos antiinflamatorios se evaluaron mediante ensayos de hemólisis inducida por calor y desnaturalización de albúmina de huevo. La actividad neuroprotectora se evaluó frente a acetilcolinesterasa (AChE) y butirilcolinesterasa (BChE) y la actividad antimicrobiana mediante el método de difusión en disco de agar.

Resultados: Ambos extractos poseen un gran efecto antioxidante, pero se consideró más alto en el extracto de n-BuOH con valores de IC_{50} respectivos de 8,04 ± 0,21 µg/mL en el ensayo ABTS y 4,25 ± 0,6 µg/mL en el ensayo GOR, mientras que los valores de A0,50 fueron 4,46 ± 0,55 µg/mL en el ensayo de fenantrolina y 11,16 ± 0,64 µg/mL en el ensayo de potencia reductora. El extracto de n-BuOH mostró también una notable actividad antiinflamatoria con una CE_{50} de 120,81 ± 0,2 µg/mL en el ensayo de desnaturalización de albúmina de huevo y 154,15 ± 0,14 µg/mL en el ensayo de hemólisis inducida por calor. La neuroprotección del extracto de n-BuOH se manifestó mediante la actividad inhibidora sobre AChE y BChE, con valores de IC_{50} respectivos de 9,8 ± 0,62 µg/mL y 173,53 ± 0,04 µg mL. El extracto de EA fue más activo contra las cepas microbianas.

Conclusiones: Estos alentadores resultados demostraron una vez más el potencial farmacológico de la especie *Centaurea*.

Palabras Clave: anti-inflamatorio; antimicrobiano; antioxidante; *Centaurea tougourensis*; neuroprotector.

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INTRODUCTION

Maintaining body homeostasis is crucial to ensure optimal body's function. This includes temperature, acidity, and water level (Modell et al., 2015). This balance allows us to rapidly adapt to varying stressful environmental conditions (Torday, 2015). The regulation of homeostasis is orchestrated by nervous and endocrine systems via positive and negative feedback processes (Ramsay and Woods, 2014).

The excessive production of free radicals, including reactive oxygen (ROS) and nitrogen (RNS) species, may alter the whole-body physiology as a result of an imbalance between oxidants and antioxidants agents (Shankar and Mehendale, 2014), and this accumulation of free radicals generate oxidative stress phenomenon. Oxidative stress may also lead to DNA damage, especially that of a key organelle called mitochondria with generally results in energy loss or cell death (Gonzalez-Hunt et al., 2018). Heavy metals, UV radiations, and pollutants remain the main generator of oxidative stress, and several studies demonstrated over the years the potential of plants to reverse the oxidative process via DNA repair (Manova and Gruszka, 2015) or recombination (Schröpfer et al., 2014) mechanisms.

It is well known that it is very difficult to stop or control the propagation of microorganisms due to their microscopic size, and unfortunately, infectious diseases caused by bacteria, parasites, or viruses are increasing each day (Shen et al., 2019). The fact that these microorganisms can use animals or humans as hosts makes them more dangerous, and this symbiotic effect results in chronic infections (Godkin and Smith, 2017). Recently, the COVID-19, which is caused by a new type of coronavirus, proved that the scientific community approaches face pathogenic microorganisms should be revised to confront a comparable pandemic situation (Stevens, 2020), especially to prevent or limit the colonization of pathogenic microorganism strains.

Inflammation response is crucial for body's defense mechanism. It allows us to repair and overcome the damages caused by injury, trauma, infections, or internal dysfunctions due to autoimmune pathologies. As a result, key pro-inflammatory mediators like prostaglandins (PGs), interleukins (IL-1 and IL-6), tumor necrosis factor-α (TNF-α), nitric oxide (NO), interferon γ (IFNγ) are released in the bloodstream to face these situations and return to physiological state (Galvão et al., 2018). But long-term inflammation response can result in serious chronic illnesses like Crohn's disease or ulcerative colitis (Rubin et al., 2012).

In this 21st century, Alzheimer's and Parkinson's disease are considered the most common neurodegenerative disorders (Han et al., 2018), and several studies clearly indicated that there is a link between oxidative stress and central nervous system (CNS) injury that results in the loss of neurons and glial cells populations (Salim, 2017). The fact that this neurodegenerative process starts slowly makes it almost impossible for the scientific community to prevent or even treat diseases generated by this phenomenon.

The richness of plants in key elements like polyphenols, saponins, and tannins (Cheynier, 2012; Negri and Tabach, 2013; Seca and Pinto, 2019) may explain their remarkable pharmacological properties. Many species of the genus *Centaurea* demonstrated over the years antioxidant, immunomodulatory, anti-inflammatory, neuroprotective, antidiabetic, and anti-microbial effects (Garbacki et al., 1999; Ozçelik et al., 2009; Erel et al., 2014; Ozsoy et al., 2015). Until now, no pharmacological approach was made on *C. tougourensis,* and there are only two phytochemical studies made on this species in which researchers identified in total ten compounds. Thus, six of them were aglycone flavonoids, while the remaining four were sesquiterpene lactones (Nacer et al., 2006; 2012). The isolated compounds were respectively: 3'-O-methyl-eupatorin, jaceosidin, nepetin, eupatilin, apigenin, kaempferol, cnicin, 8a-(3,4-dihydroxy-2-methylene-butanoyloxy)-dehydromelitensin, (6R, 7R, 8S, 30R) 8a- (3, 4-dihydroxy-2- methylene-butano-

yloxy)-15-acetoxy-helianga 1 (10), 4 (5), 11 (13) trien-6-olide and finally (6R, 7R, 8S, 30R) 8a-(3, 4-dihydroxy-2-methylene-butanoyloxy)-15-oxo-helianga 1 (10), 4 (5), 11 (13) trien-6-olide. For all these reasons, we targeted this species through our study to discover all its pharmacological potential.

The objectives of the present work are to evaluate *in vitro* for the first-time antioxidant, anti-inflammatory, anti-cholinesterase, and anti-microbial activities of n-butanol and ethyl acetate extracts of an endemic Algerian species called *Centaurea tougourensis* Boiss. & Reut.

MATERIAL AND METHODS

Chemical and reagents

All solvents and standards used in this study were purchased from Sigma-Aldrich, Steinheim, Germany. This includes 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), acetylcholine (ACh), acetylcholinesterase (AChE), alpha-tocopherol (α-tocopherol), ascorbic acid, butyrylcholinesterase (BChE), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), diclofenac sodium, dimethyl sulfoxide (DMSO), ethanol, ethyl acetate (EA), ferrous cation (Fe^{2+}), ferric cation (Fe^{3+}), ferric chloride ($FeCl_3$), galantamine, galvinoxyl radical (GOR), gentamicin, methanol, penicillin, phenanthroline, potassium ferricyanide ($K_3Fe(CN)_6$, n-butanol (n-BuOH), tannic acid, trichloroacetic acid (TCA). Microbial strains were provided by the Bacteriology Laboratory of the University-Hospital Center of Batna and the Bacteriology Laboratory of Sanatorium of Batna.

Plant material

C. tougourensis was collected in spring 2019 at Belezma national park in the municipality of Fesdis (Algeria) (GPS coordinates: latitude 35.621975; longitude 6.241327) and was identified by experts in the field from the agronomic department of the Batna-1 University (Algeria), and a voucher specimen under the code (CT/2019/LPTPCMB) was deposited at the Laboratory of Improvement of the Phytosanitary Protection Techniques in Mountainous Agrosystems, Agronomy Department, Institute of Veterinary and Agricultural Sciences, University of Batna 1, Batna, Algeria.

Preparation of plant extract

The aerial parts of *C. tougourensis* were dried in a dry, ventilated place away from the sun's rays and then ground to obtain (300 g) of fine powder. Maceration was carried out three times with 3 L EtOH-H_2O (70: 30) at room temperature for 3 days. After liquid-liquid extraction with solvents (hexane, ethyl acetate, and n-butanol), were obtained 1.58% of n-butanol (n-BuOH), 1.03% of ethyl acetate (EA), and 0.42% of n-hexane as extracts yields.

Free radical scavenging assays

ABTS scavenging capacity

The scavenging capacity of *C. tougourensis* extracts against the free radical ABTS was evaluated spectrophotometrically according to Re et al. (1999). Briefly, 1 mL of ABTS cation solution was added to 500 µL of extracts or standards (BHA and BHT) dissolved in methanol at various concentrations (3.125, 6.25, 12.5, 25, 50, 100, and 200 µg/mL). The absorbances of the tested solutions were measured at 734 nm 30 min after incubation. The results were expressed as median inhibitory concentration (IC_{50}) in (µg/mL).

The ABTS scavenging activity was calculated using the following equation [1].

$$\text{ABTS scavenging (\%)} = [(A_{Control} - A_{Sample})/ A_{Control}] \times 100 \quad [1]$$

Where $A_{Control}$ is the absorbance of blank; A_{Sample} is the absorbance of positive control or sample.

Reducing power assay

To estimate the possible reducing power capacity of *C. tougourensis*, the protocol of Oyaizu (1986) was used. A volume of 10 µL of each sample solution at various concentrations (3.125-200 µg/mL) was added to 40 µL of phosphate buffer (0.2 M; pH 6.6) and 50 µL of $K_3Fe(CN)_6$ (1%). The preparation was incubated for 20 min at 50°C. A total of 50 µL of TCA (10%) and 10 µL of $FeCl_3$ (0.1%) was added to the mixture and completed with 40 µL of distilled water. The concentration of the sample giv-

ing an absorbance of 0.5 ($A_{0.5}$) was measured at 700 nm. Ascorbic acid, tannic acid, and α-tocopherol were used as standards.

Phenanthroline test

The phenanthroline assay was also investigated based on Szydlowska-Czerniaka et al. (2008) approach. A volume of 10 µL of the plant extracts or standards solutions at different concentrations (3.125-200 µg/mL) was added to 50 µL $FeCl_3$ (0.2%). Then, 30 µL of phenanthroline (0.5%) and 110 µL of methanol were added to the mixture. The obtained solution was incubated for 20 min at 30°C, and the absorbance was measured at 510 nm. BHA and BHT were used as standard, and results expressed as $A_{0.50}$ values (µg/mL).

Galvinoxyl radical (GOR) scavenging assay

To evaluate this activity, we used the protocol previously described by Shi et al. (2001). The mixture consisted of 40 µL of *C. tougourensis* extracts or standards solutions at concentrations (3.125-200 µg/mL) and 160 µL of galvinoxyl radical (0.1 mM). The samples were then incubated for 120 min, and the absorbance was measured at 428 nm. BHA and BHT were used as antioxidant standards, and results were given as IC_{50} (µg/mL).

Anti-inflammatory activity

Heat-induced hemolysis assay

This test was performed according to Gandhidasan et al. (1991) method with slight modifications. Various concentrations of n-BuOH and EA extracts of *C. tougourensis* were prepared in different concentration levels (50, 100, 200, 400, and 800 µg/mL), with diclofenac sodium as reference standard drug. A volume of 50 µL of erythrocyte suspension was added to all tubes of each series, and the tubes were then incubated in a water bath at 54°C for 20 min, while the other pair was maintained at 0°C for 20 min. The mixtures were then centrifuged at 5000 rpm for 5 min, and the absorbance of the supernatants was measured at 560 nm. The experiment was repeated three times. The median effective concentration (EC_{50}) was also calculated, and the inhibition percentage of hemolysis was calculated according to the equation [2].

$$\text{Hemolysis inhibition (\%)} = 1 - [(OD_2 - OD_1)/(OD_3 - OD_1)] \times 100 \quad [2]$$

Where OD_1: Represent the absorbance of the unheated sample; OD_2: Represent the absorbance of the heated sample; and OD_3: Represent the absorbance of the heated control.

Egg albumin denaturation assay

The possible anti-inflammatory activity of *C. tougourensis* extracts was also evaluated by the egg albumin denaturation method previously described by Mehta et al. (2017). The reaction mixture (5 mL) consisted of 200 µL of egg albumin (from fresh hen's egg) with 2.8 mL of phosphate-buffered saline (PBS, pH 6.4) and 2 mL of *C. tougourensis* at different concentrations level (50, 100, 200, 400 and 800 µg/mL). Diclofenac sodium was used as a reference. After 15 min of incubation at 37°C, the obtained solutions were heated at 70°C for 5 min, and the absorbances were then measured at 660 nm. A similar volume of distilled water was used as a control group. In addition, the EC_{50} value was calculated, and the equation [3] was used to calculate the inhibition percentage of protein denaturation.

$$\text{Inhibition (\%)} = [Vs / Vc - 1] \times 100 \quad [3]$$

Where Vs: absorbance of the test sample; Vc: absorbance of the control group.

Neuroprotective activity

In this assay, the evaluation of the acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory activities was conducted as previously described by Ellman et al. (1961). A volume of 150 µL of sodium phosphate buffer (100 mM; pH 8.0) was added to 10 µL of extract solution dissolved in ethanol at different concentrations. Then, 20 µL of AChE (5.32×10^{-3} U) or BChE (6.85×10^{-3} U) or solution was added to the mixture and incubated at 250°C for 15 min. A volume of 10 µL of DTNB (0.5 mM) and 10 µL of acetylthiocholine iodide (0.71 mM) or 10 µL of butyrylthiocholine chloride (0.2 mM) were added to the previous mixture. The absorbance was made at 412 nm, and galantamine was used as a reference drug and tested at the

same concentration range of extracts (3.125-200 µg/mL). The IC_{50} value was also calculated.

Anti-microbial test

The anti-microbial effect of n-BuOH and EA extracts of *C. tougourensis* was determined using the micro-dilution method (Carev et al., 2018). Six bacterial strains were used for this evaluation, namely, *Escherichia coli* (ATCC 25922), *pathogenic Escherichia coli*, *Pseudomonas aeruginosa* (ATCC 1117), *Staphylococcus aureus* (ATCC 25923), *Pathogenic Staphylococcus aureus,* and *Klebsiella pneumonia*. In addition, the possible anti-microbial effect of *C. tougourensis* against the yeast *Candida albicans* (ATCC 90029) was also investigated. Sterilized Wathman paper discs (6 mm) were impregnated with 10 µL of n-BuOH or EA extract solutions at different concentrations (0.00625, 0.0125, 0.025, 0.05, and 0.1, mg/mL) and filed carefully on the surface of the inoculated agar using a sterile cotton swab. Negative controls consisted of discs impregnated with dimethyl sulfoxide (DMSO). Penicillin and gentamicin were used as positive controls. The bacteria were then incubated at 37°C for 24 h, while the fungal strain was also incubated at the same temperature but for 48 h. Tests were performed in triplicate and at the end of the experiment. The minimum inhibitory concentrations (MIC) were calculated.

Statistical analysis

Data obtained from this study were expressed as mean ± SD, and the statistical analyses were performed by one-way ANOVA using GraphPad Prism version 8 (California corporation, USA). Results were considered highly significant at $p<0.001$.

RESULTS

Antioxidant capacivity

In this study, the antioxidant capacity of *C. tougourensis* was evaluated using several scavenging assays, and as shown in Table 1. It seems that the anti-radical capacity of n-BuOH extract was more efficient than ethyl acetate extract.

The results also revealed that the antioxidant properties of both extracts evolve in a dose-dependent manner to reach for the ABTS test an inhibition percentage of 90.22 ± 4.37% for n-BuOH extract while 71.74 ± 2.2% for EA extract at the highest tested concentrate (200 µg/mL). Almost the same results were found in GOR, reducing power, and phenanthroline assays. It is also interesting to see that the antioxidant activity of the n-BuOH extract of *C. tougourensis* approaches that of the standards at the highest tested concentrations with non-significant differences.

The IC_{50} and $A_{0.5}$ of the tested samples were also calculated (Table 2), in ABTS assay results were 8.04 ± 0.21 µg/mL for n-BuOH extract and 51 ± 0.15 µg/mL for EA extract, respectively but were found higher than standards values BHA (1.81 ± 0.10 µg/mL) and BHT (1.29 ± 0.30 µg/mL), respectively. In phenanthroline assay, the $A_{0.5}$ of n-BuOH extract (4.46 ± 0.55 µg/mL) was very close to the standards BHT (2.24 ± 0.17 µg/mL), and the differences were considered non-significant. On the other hand, in GOR assay, the IC_{50} of n-BuOH extract was remarkable (4.25 ± 0.6 µg/mL) and considered lower than the standard BHA (5.38 ± 0.06 µg/mL). In reducing power assay, also the same results were found when comparing the $A_{0.5}$ of n-BuOH extract (11.16 ± 0.64 µg/mL) with the standard α-tocopherol (34.93 ± 2.38 µg/mL). These results are very encouraging and demonstrate the potential of these plant extracts.

Anti-inflammatory activity

This activity was tested *in vitro* and revealed that the n-BuOH extract has an anti-inflammatory effect with a dose-effect relationship in both heat-induced hemolysis and egg albumin denaturation assays with a maximum inhibitory activity of 97.84 ± 0.006% and 96.91 ± 0.09%, respectively. But on the other hand, the n-BuOH extract showed lower inhibition of RBC hemolysis than standard diclofenac in egg albumin denaturation assay with a non-significant difference (Figs. 1-2).

Table 1. Antioxidant capacity (%) of *C. tougourensis* extracts.

Assays	Extracts	Dilution (µg/mL)						
		3.125	**6.25**	**12.5**	**25**	**50**	**100**	**200**
ABTS	n-BuOH extract	29.37 ± 1.11^{ab}	47.6 ± 0.26^{ab}	63.86 ± 5.25^{ab}	84.57 ± 0.66^{ab}	87.81 ± 0.53	89.58 ± 0.7	90.22 ± 4.37^{ns}
	EA extract	8.29 ± 2.09^{ab}	13.69 ± 1.07^{ab}	18.93 ± 0.4^{ab}	31.91 ± 1.35^{ab}	47.6 ± 1.07^{ab}	63.8 ± 2.15^{ab}	71.74 ± 2.2^{ab}
	BHA	83.42 ± 4.09	93.52 ± 0.09	93.58 ± 0.09	93.63 ± 0.16	93.63 ± 0.95	94.20 ± 0.90	95.39 ± 2.62
	BHT	59.22 ± 0.59	78.55 ± 3.43	90.36 ± 0.00	92.18 ± 1.27	93.37 ±0.86	94.87 ±0.87	96.68 ± 0.39
GOR	n-BuOH extract	41.06 ± 0.43^{b}	64.38 ± 1.08^{a}	73.59 ± 0.84^{a}	79.85 ± 1.41^{a}	82. 24 ± 0.27^{ab}	82.91 ± 0.14^{ab}	84.16 ± 0.16^{ab}
	EA extract	–	–	1.08 ± 0.36^{ab}	11.55 ± 0.39^{ab}	35.24 ± 0.52^{ab}	46.78 ± 0.67^{ab}	58.7 ± 0.4^{ab}
	BHA	39.15 ± 0.88	54.16 ± 0.27	65.02 ± 1.26	70.19 ± 0.51	70.32 ± 0.65	70.60 ± 0.10	70.94 ± 0.07
	BHT	49.23 ± 0.77	61.29 ± 0.69	68.89 ± 0.26	70.02 ± 0.50	70.49 ± 0.55	71.13 ± 0.74	71.20 ± 0.32
Reducing power	n-BuOH extract	0.22 ± 0.01^{cde}	0.35 ± 0.01^{cde}	0.54 ± 0.04^{cde}	0.73 ± 0.07^{de}	0.73 ± 0.1^{c}	0.81 ± 0.16^{ce}	0.98 ± 0.24^{e}
	EA extract	0.09 ± 0.01^{cd}	0.11 ± 0.01^{cde}	0.12 ± 0.01^{cd}	0.19 ± 0.06^{cd}	0.2 ± 0.01^{cde}	0.36 ± 0.03^{cde}	0.56 ± 0.03^{ce}
	Ascorbic acid	0.35 ± 0.05	0.46 ± 0.03	0.84 ± 0.12	0.93 ± 0.30	1.18 ± 0.34	1.37 ± 0.20	1.44 ± 0.21
	Tannic acid	0.28 ± 0.02	0.78 ± 0.06	1.02 ± 0.07	1.24 ± 0.18	0.86 ± 0.6	1.01 ± 0.21	1.02 ± 0.13
	α-Tocopherol	0.11 ± 0.00	0.16 ± 0.00	0.21 ± 0.03	0.35 ± 0.03	0.73 ± 0.03	1.37 ± 0.08	1.81 ± 0.09
Phenanthroline	n-BuOH extract	0.48 ± 0.04^{a}	0.51 ± 0.07^{ab}	0.7 ± 0.18^{b}	1.01 ± 0.11^{a}	2.08 ± 0.22^{ab}	3.21 ± 0.32^{ab}	3.84 ± 0.53
	EA extract	0.31 ± 0.05^{ab}	0.33 ± 0.01^{ab}	0.34 ± 0.01^{ab}	0.36 ± 0.02^{a}	0.39 ± 0.03^{ab}	0.45 ± 0.02^{ab}	0.76 ± 0.14^{ab}
	BHA	0.73 ± 0.02	0.93 ± 0.01	1.25 ± 0.04	2.10 ± 0.05	4.89 ± 0.06	>5	>5
	BHT	0.53 ± 0.03	1.23 ± 0.02	1.84±0.01	3.48 ± 0.03	4.84 ± 0.01	>5	>5

All values are expressed as mean ± SD (n = 3). One-way ANOVA followed by multiple Dunnet's test. Level of significance p<0.001; extracts vs. different standard substances; ^{a}p<0.001 is statistically significant with a comparison to butylated hydroxyanisole (BHA), ^{b}p<0.001 to butylated hydroxytoluene (BHT), ^{c}p<0.001 to ascorbic acid, ^{d}p<0.001 to tannic acid, ^{e}p<0.001 to α-tocopherol. ^{ns}: no significance. GOR: galvinoxyl radical scavenging assay.

Table 2. Antioxidant activities (IC_{50} and $A_{0.50}$ µg/mL) of standards and *C. tougourensis* extracts.

Extracts/standard	ABTS assay	Reducing power assay	GOR assay	Phenanthroline assay
	IC_{50} (µg/mL)	**$A_{0.50}$ (µg/mL)**	**IC_{50} (µg/mL)**	**$A_{0.50}$ (µg/mL)**
n-BuOH extract	8.04 ± 0.21	11.16 ± 0.64^{e}	4.25 ± 0.6^{ns}	4.46 ± 0.55^{a}
EA extract	51.00 ± 0.15^{ab}	138.33 ± 0.05^{cde}	154.66 ± 0.01^{ab}	139.07 ± 0.02^{ab}
BHA	1.81 ± 0.10	NT	5.38 ± 0.06	0.93 ± 0.07
BHT	1.29 ± 0.30	NT	3.32 ± 0.18	2.24 ± 0.17
Ascorbic acid	NT	6.77 ± 1.15	NT	NT
Tannic acid	NT	5.39 ± 0.91	NT	NT
α-Tocopherol	NT	34.93 ± 2.38	NT	NT

All values are expressed as mean ± SD (n = 3). One-way ANOVA followed by multiple Dunnet's test. Level of significance p< 0.001; extracts vs. different standard substances; ^{a}p< 0.001 is statistically significant with a comparison to butylated hydroxyanisole (BHA), ^{b}p< 0.001 to butylated hydroxytoluene (BHT), ^{c}p< 0.001 to ascorbic acid, ^{d}p< 0.001 to tannic acid, ^{e}p< 0.001 to α-tocopherol, NT: not tested. ^{ns}: no significance. n-BuOH: n-butanol extract; EA: ethyl acetate. GOR: galvinoxyl radical scavenging assay.

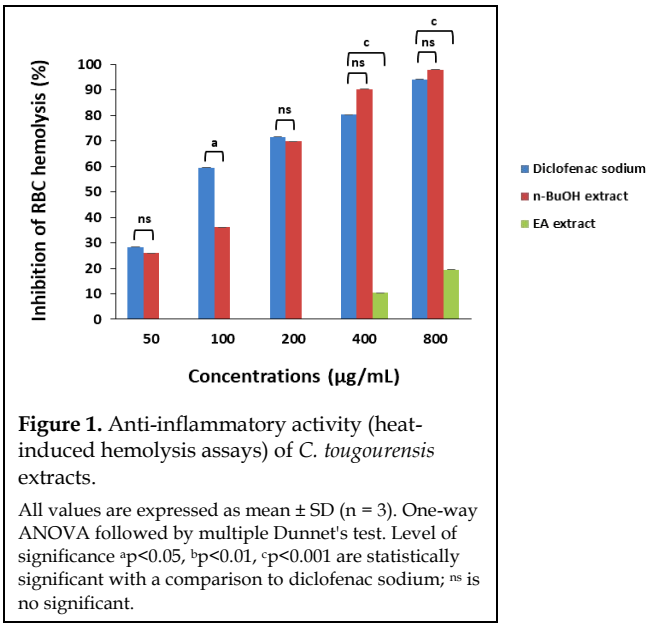

Figure 1. Anti-inflammatory activity (heat-induced hemolysis assays) of *C. tougourensis* extracts.

All values are expressed as mean ± SD (n = 3). One-way ANOVA followed by multiple Dunnet's test. Level of significance $^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.001$ are statistically significant with a comparison to diclofenac sodium; ^{ns} is no significant.

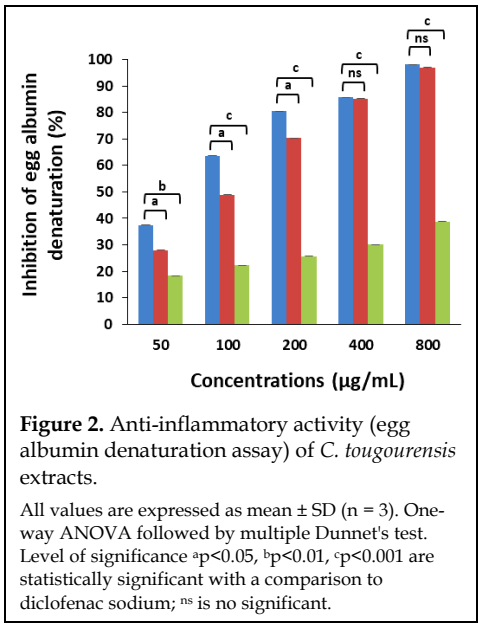

Figure 2. Anti-inflammatory activity (egg albumin denaturation assay) of *C. tougourensis* extracts.

All values are expressed as mean ± SD (n = 3). One-way ANOVA followed by multiple Dunnet's test. Level of significance $^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.001$ are statistically significant with a comparison to diclofenac sodium; ^{ns} is no significant.

Table 3. Anti-inflammatory activities (EC_{50} µg/mL) of standard and *C. tougourensis* extracts.

Extracts/standard	Albumin denaturation*	Hemolysis#
	EC_{50} (µg/mL)	EC_{50} (µg/mL)
n-BuOH extract	120.81 ± 0.2^{b}	154.15 ± 0.14^{c}
EA extract	ND	ND
Diclofenac sodium	77.77 ± 0.2	95.8 ± 0.19

All values are expressed as mean ± SD (n = 3). One-way ANOVA followed by multiple Dunnet's test. Level of significance $^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.001$ are statistically significant with a comparison to diclofenac sodium, ND: not determined. *Albumin denaturation: Egg albumin denaturation assay; #Hemolysis: Heat-induced hemolysis assay. n-BuOH: n-butanol; EA: ethyl acetate.

The EA extract did not show the same results as the n-BuOH extract; thus, a modest activity was recorded in egg albumin denaturation assay with a percentage of 38.74 ± 0.007% at the highest tested concentration while in heat-induced hemolysis assay, the activity of EA extract was almost absent.

The EC_{50} values were also recorded. In egg albumin denaturation assay, it was 120.81 ± 0.2 µg/mL for n-BuOH extract while 77.77 ± 0.2 µg/mL for diclofenac sodium and the difference was statistically significant ($p<0.01$). In heat-induced hemolysis assay; the EC_{50} values were 154.15 ± 0.14 µg/mL for n-BuOH extract and 95.8 ± 0.19 µg/mL for the standard (Table 3). The present findings clearly indicated that *C. tougourensis* could be a good candidate to replace NSAIDs drugs.

Neuroprotective activity

As presented in Figs. 3-4, the greatest inhibitory effects of *C. tougourensis* on acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) were observed at the concentration of (200 µg/mL) for both tested extracts. But the effect of *C. tougourensis* seems to be more potent on AChE with a maximum percentage inhibition of 84.71 ± 3.56% for the n-BuOH extract while the EA extract showed at the same dose a percentage of 74.11 ± 0.48%, and both presented statistically significant differences ($p<0.05$) when compared to reference standard galantamine. In BChE assay, a modest neuroprotective effect was given by the n-BuOH extract (56.12 ± 2.63%) at the concentration of 200 µg/mL with a respective IC_{50} value of 173.53 ± 0.04 µg/mL, while a non-negligible activity was ob-

served with EA extract (17.67 ± 2.28%). It was noted that in both assays, the neuroprotective activity of *C. tougourensis* extracts was strongly dependent on concentration. We also found that the IC_{50} value of n-BuOH extract (9.8 ± 0.62 µg/mL) was very close to galantamine (6.27 ± 1.15 µg/mL) in the anti-AChE assay, while the IC_{50} of EA extract was 43.27 ± 0.12 µg/mL (Table 4), which is a very promising result.

Anti-microbial activity

The possible antibacterial activity of *C. tougourensis* was also evaluated using several referenced microbial strains. As showed in Table 5, the EA extract of *C. tougourensis* was active on two microbial strains among the eight tested ones, namely: *Staphylococcus aureus* (ATCC 25923) and pathogenic *Escherichia coli*. It showed Minimum Inhibitory Concentrations (MIC) of 0.00625 mg/mL and 0.0125 mg/mL with respective inhibition zones of 8.07 ± 0.17 mm and 7.74 ± 0.22 mm, while the n-BuOH extract was only active on *Staphylococcus aureus* (ATCC 25923) with a MIC value of 0.0125 mg/mL and respective inhibition zone of 8.68 ± 0.37mm (Table 6). This allows concluding that the EA extract of *C. tougourensis* has a higher anti-microbial effect than n-BuOH extract.

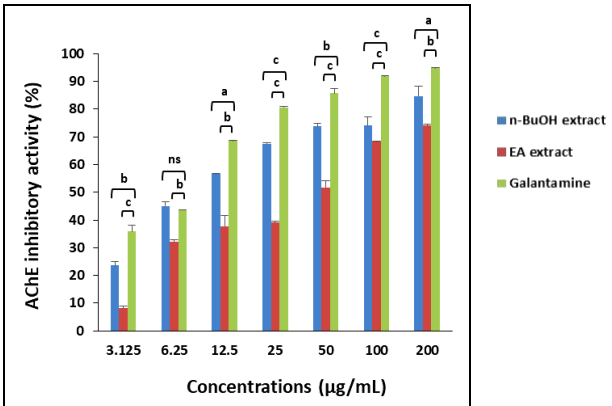

Figure 3. Neuroprotective activity (anti-acetylcholinesterase) of *C. tougourensis* extracts.

All values are expressed as mean ± SD (n = 3). One way ANOVA followed by multiple Dunnet's test. Level of significance $^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.001$ are statistically significant with a comparison to galantamine, ^{ns} is no significant.

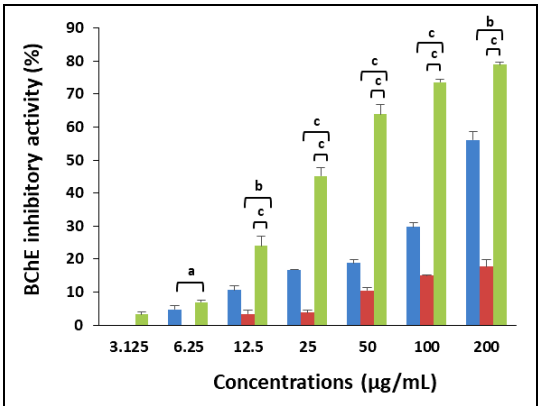

Figure 4. Neuroprotective activity (anti-butyrylcholinesterase) of *C. tougourensis* extracts.

All values are expressed as mean ± SD (n = 3). One way ANOVA followed by multiple Dunnet's test. Level of significance $^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.001$ are statistically significant with a comparison to galantamine.

Table 4. Neuroprotective activities (IC_{50} µg/mL) of standard and *C. tougourensis* extracts.

Extract/standard	AChE IA	BChE IA
	IC_{50} (µg/mL)	IC_{50} (µg/mL)
n-BuOH extract	9.8 ± 0.62^{ns}	173.53 ± 0.04^{c}
EA extract	43.27 ± 0.12^{b}	ND
Galantamine	6.27 ± 1.15	34.75 ±1.99

All values are expressed as mean ± SD (n = 3). One way ANOVA followed by multiple Dunnet's test. Level of significance $^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.001$ are statistically significant with a comparison to galantamine, ^{ns} is no significant, ND: not determined. n-BuOH: n-butanol; EA: ethyl acetate. AChE IA: acetylcholinesterase inhibitory activity; BChE IA: butyrylcholinesterase inhibitory activity.

Table 5. Sensitivity of the studied microbial strains to BuOH and EA extracts of *C. tougourensis*.

Extracts/antibiotics	Microbial strains						
	Gram-positive bacteria		Gram-negative bacteria				Yeast
	Sa	PSa	Ec	PEc	Pa	Kp	Ca
n-BuOH extract	+	–	–	–	–	–	–
EA extract	+	–	–	+	–	–	–
Penicillin	+	+	+	+	+	+	+
Gentamicin	+	+	+	+	+	+	+

Sa: *Staphylococcus aureus* (ATCC 25923); PSa: Pathogenic *Staphylococcus aureus*; Ec: *Escherichia coli* (ATCC 25922); PEc: Pathogenic *Escherichia coli*; Pa: *Pseudomonas aeruginosa* (ATCC 1117); Kp: *Klebsiella pneumonia*; Ca: *Candida albicans* (ATCC 90029). (+) Presence of inhibition zones around the discs. (-) No zones of inhibition around the discs.

Table 6. Inhibition zones diameters and Minimum Inhibitory Concentration (MIC) of *C. tougourensis* extracts on microbial strains.

Extracts/antibiotics	Dilution (mg/mL)	Inhibition zone diameter (mm)	
		Microbial strains	
		***Staphylococcus aureus* (ATCC 25923)**	***Escherichia coli* (pathogenic)**
n-BuOH extract	0.1	9.95 ± 0.14[ab]	–
	0.05	9.32 ± 0.08[ab]	–
	0.025	8.94 ± 0.23[ab]	–
	0.0125	8.68 ± 0.37[ab]	–
	0.00625	–	–
MIC (mg/mL)	/	0.0125	–
EA extract	0.1	12.04 ± 0.89[ab]	9.08 ± 0.3[ab]
	0.05	10.19 ± 0.38[ab]	8.67 ± 0.45[ab]
	0.025	9.85 ± 0.21[ab]	8.39 ± 0.28[ab]
	0.0125	9.42 ± 0.25[ab]	7.74 ± 0.22[ab]
	0.00625	8.07 ± 0.17[ab]	–
MIC (mg/mL)	/	0.00625	0.0125
Penicillin	/	32 ± 0.0	32 ± 0.0
Gentamicin	/	34 ± 0.0	36 ± 0.0

All values are expressed as mean ± SD (n = 3). One-way ANOVA followed by multiple Dunnet's test. Level of significance $p<0.001$; extracts vs. different standard substances; [a]$p<0.001$ is statistically significant with a comparison to penicillin, [b]$p<0.001$ to gentamicin.

DISCUSSION

Many diseases have been treated and eradicated due to the evolution of medicine and, more particularly, herbal medicine, which served as an important source of bioactive compounds (Proestos et al., 2020). Previous studies (Ugur et al., 2009; Escher et al., 2018) made on two *Centaurea* species; namely, *C. ensiformis* and *C. cyanus* showed significant antioxidant activities of this species with dose-dependent effect and suspected that the actual phenolic, flavonoids, and anthocyanins compounds identified in their extracts are responsible for the biological effect. Another study made by Karamać (2009) demonstrated that tannins possess the ability to retard oxidation by preventing the

oxidation of Fe^{2+} ions into its ionized form called Fe^{3+}. Dhouibi et al. (2020) underlined the antioxidant properties of *Centaurea kroumirensis* and *Centaurea sicula* and suggested that the high polyphenol contents recorded in these species could explain their great free radicals scavenging activity. But the bioactive compounds of a plant may act in a synergistic way to break the chain of free radicals (Lobo et al., 2010).

Sometimes, the innate immunity is not sufficient to deal with external stimuli, which requires the immediate use of anti-inflammatory medications (NSAIDs), but the side effect of these drugs is hazardous (Davis and Robson, 2016). Several studies showed that the richness of plants in flavonoids and terpenes (Mouffouk et al., 2020) could exert strong anti-inflammatory and antioxidant effects. A study made by Csupor et al. (2013) on eleven *Centaurea* species using both in-vitro and in-vivo assays correlated the strong anti-inflammatory activities of these species with the major flavonoids compounds identified and suggested that these bioactive compounds significantly reduced the production of COX-1 and COX-2, which are the principal pro-inflammatory mediators released during inflammation response.

Another study (Alper and Güneş, 2019) showed that *Centaurea solstitialis* could regulate the inflammatory process by modulation of key enzymes and cytokines secretion like IL1α, IL-6, and TNFα, which prevent immune system deregulation. It is well established indeed that inflammatory response and immunity are related to each other.

Based on some research results, it seems that *Centaurea* species have non-negligible antibacterial activities. A recent study carried out by Naeim et al. (2020) on various extracts of *Centaurea pumilio* demonstrated a strong anti-microbial effect of ethyl acetate extract against multidrug-resistant bacteria, but on the other hand, their n-butanol extract did not show any antibacterial effect, which is in accordance with our findings—noting that the doses used in our study are approximately the same used in the previously cited study. The ethyl acetate extract of another *Centaurea* species named *C. babylonica* showed almost the same antibacterial results against two endospore-forming bacteria (Güvensen et al., 2019), which is consistent with our result.

A recent study (Jurca et al., 2020) involving four *Streptococcus* strains demonstrated that the actual bioactive compounds from the plant, especially polyphenols and anthocyanins, might exert a significant antibacterial effect via interaction with bacterial lipopolysaccharides making them more exposed, and this lead to a significant increase of the expression of nuclear factor *kappa* B (NF-κB), and decrease the expression of the transcription factor (STAT3), which enhance the antibacterial response.

The human nervous system is the most complex and organized body system that coordinates the whole organism's activities (Galli et al., 2003). But the progressive degeneration of nerve cells could lead to serious and irreversible diseases (Forman et al., 2004), which is why finding new treatments for Alzheimer's disease became crucial.

Cholinesterase is vital for the proper function of the nervous system. Thus, the physiological level of a key neurotransmitter called acetylcholine (ACh) is maintained by this enzyme (Kutty, 1980). But in a pathological situation, this enzyme can be responsible for an abnormal level of AChE due to excessive hydrolysis, and several studies suggested that the lack of AChE could lead to memory impairments and dementia (Higley and Picciotto, 2014).

Using plants to treat neurodegenerative disorders could be a good alternative. A recent study showed that natural compounds contained in plants, especially phenolics (Phan et al., 2019), could stop the aggregation of β-amyloid (Aβ) induced by acetylcholinesterase via the inhibition of β-amyloid protein toxicity, which is one of the main cause of Alzheimer's disease since we know that the decline of the neuronal population leads to a progressive loss of cognitive functions.

A study conducted by Aktumsek et al. (2013) on three two *Centaurea* species, namely *C. polypodiifolia var. pseudobehen*, *C. pyrrhoblephara*, and *C. antal-*

yense reported a moderate inhibitory activity on acetylcholinesterase and butyrylcholinesterase, noting that phenolics, like flavonoids and saponins, were present in these plants in high contents, which is consistent with the obtained results.

The active compounds in *C. tougourensis* extract, especially flavonoids, triterpenes, mucilages, alkaloids, and saponins classes identified in our previous phytochemical screening work, may explain its significant antioxidant, anti-inflammatory, antibacterial, and anti-cholinesterase properties.

CONCLUSIONS

This study aimed to research some biological activities of *C. tougouriensis*, which have not been studied before. The study showed that *C. tougourensis* has significant antioxidant, anti-inflammatory, and anti-microbial activities as well as neuroprotective effects. However, further investigations are needed on its chemical composition in bioactive molecules to highlight the pharmacological mechanisms and the full potential of this species.

CONFLICT OF INTEREST

The authors declare no conflicts of interests.

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AUTHOR CONTRIBUTION:

Contribution	Bensaad MS	Dassamiour S	Hambaba L	Haba H
Concepts or ideas	x	x	x	x
Design	x	x	x	x
Definition of intellectual content	x	x	x	
Literature search	x	x	x	
Experimental studies	x	x	x	
Data acquisition	x			
Data analysis	x	x		
Statistical analysis	x	x		
Manuscript preparation	x	x		
Manuscript editing	x	x		
Manuscript review	x	x	x	x

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