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EXPERIMENTAL PAPER

HPLC-DAD phenolics screening and *in vitro* investigation of haemostatic, antidiabetic, antioxidant and photoprotective properties of *Centaurea tougourensis* Boiss. & Reut.

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Summary

Introduction: Traditional medicine has an important place in human history and this since antiquity. Indeed, during Egyptian and Chinese civilization era, many detailed manuscripts, describing the therapeutic effect of plants, were found which suggest that folk medicine is the basis of the actual medicine.

Objective: To investigate the phytochemical and pharmacological properties of the n-butanol (n-BuOH) and ethyl acetate (EA) extracts of the aerial part of *Centaurea tougourensis*.

Methods: The phytochemical evaluation was done based on HPLC-DAD approach. The antioxidant activity was determined by DPPH and cupric ion reducing antioxidant capacity (CUPRAC), while the hemostatic effect was performed using plasma recalcification time (PRT) method. The antidiabetic capacity was investigated by alpha-amylase inhibition assay and the photoprotective test was evaluated by the measurement of sun protection factor (SPF).

Results: 13 phenolic compounds were identified in both extracts of *C. tougourensis*. These extracts showed antioxidant, haemostatic, antidiabetic and photoprotective properties with a dose-dependent manner. Amounts of n-BuOH activities were found higher, with a respective IC_{50} value of $0.72 \pm 0.07 \mu\text{g/ml}$ in DPPH assay, an $A_{0.50}$ value lower than $3.125 \mu\text{g/ml}$ in CUPRAC assay besides a shortening rate percentage of coagulation (86.71%) in haemostatic assay, a moderate inhibition effect on alpha amylase activity with an IC_{50} value of $(711.5 \pm 0.03 \mu\text{g/ml})$ and a maximum sun protection factor of (56.035). These results were mostly found highly significant ($p < 0.001$) when compared to respective standards.

Conclusion: This study demonstrated some pharmacological effects of *C. tougourensis* which suggests that our plant could be a good candidate to treat some illnesses related to oxidative stress, bleeding or skin cancer.

Key words: *Centaurea tougourensis*, HPLC-DAD, phenolics, pharmacological properties

Słowa kluczowe: *Centaurea tougourensis*, HPLC-DAD, związki fenolowe, aktywność farmakologiczna

INTRODUCTION

Human health has always been the source of concern for the scientific community which nowadays is facing many challenges [1]. Since antiquity, humans had to face and adapt to new environmental conditions and overcome many challenges. But unfortunately, our approach to treat illnesses must improve since many conditions related to healthcare are threatening human prosperity, suggesting us to become more realistic and look for better solutions [2].

In this context, plants are a new alternative in the prevention and treatment of many diseases, especially certain types of chronic illnesses that require constant attention [3]. The scientists explained these important healing properties by the richness in natural ingredients called secondary metabolites, which are actually used in drug development process [4]. Over the years, this natural resource proved its ability to cure many human diseases. Indeed, plants have the capacity to absorb some harmful rays related to sun exposure, such as ultraviolet (UV) radiation *via* their bioactive compounds that acts as a natural filter [5], which considerably lower the risk to develop melanoma, considered as the most common skin cancer [6]. This information explains in part why phytochemicals are actually involved in health care products composition as natural sunscreen.

Plants can also significantly limit the excessive production of reactive oxygen or nitrogen species (ROS, RNS), considered as the main precursor of oxidative stress, which in result will slow down aging process [7], and lower the risk of cancer development as well as cardiovascular and neurodegenerative diseases [8, 9]. The actual bioactive agents found in plants could also mimic insulin activity, which drastically limits α -amylase function [10]. It is well known that the inhibition of α -amylase is crucial in type 2 diabetes mellitus (T2DM): prevents abnormal elevation of glycaemia [11]. The pharmaceutical industry also consider

plants as a good alternative to treat illnesses related to coagulation process [12] that may appear at external level called physical trauma [13] and/or internal level which refer to bleeding [14]. This demonstrates the pharmacological potential of plants, and may partly explain the growing interest of the scientific community to investigate this natural resource.

Among these plants there are *Centaurea*: a group of herbaceous plants, belonging to the *Asteraceae* family which distribution is mainly centred in the Mediterranean and west Asia regions [15]. *Centaurea tougourensis* is endemic to Algeria, and principally found in Aurès, Djebel Toumour, Djebel Guethiane and Guadoust regions [16]. The lack of pharmacological and phytochemical studies on *C. tougourensis* explains the interest to investigate it in depth.

Centaurea species is rich in bioactive compounds such as flavonoids, saponins, terpenes, tannins and other. That can explain their remarkable abilities to treat several illnesses related to oxidative stress, inflammation, diabetes, bleeding, cancer and neurological disorders [17-19].

This study is a continuation of previous work in which the antioxidant, anti-inflammatory, neuroprotective and antimicrobial activities of *Centaurea tougourensis* was demonstrated [20]. The present study aimed to evaluate other activities of this plant species, namely haemostatic, antioxidant and photoprotective activities of the n-butanol and ethyl acetate extracts.

MATERIALS AND METHODS

Chemicals and reagents

All solvents and standards used in this study were purchased from Sigma Aldrich (Steinheim, Germany): 2,2-diphenyl-1-picrylhydrazyl (DPPH), 3, 5-dinitrosalicylic acid solution (DNS), 4-hydroxy-

benzoic acid ($C_7H_6O_3$), acarbose ($C_{25}H_{43}NO_{18}$), acetonitrile (C_2H_3N), α -amylase, ammonium acetate ($AcNH_4$), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), caffeic acid ($C_9H_8O_4$), calcium chloride ($CaCl_2$), catechin ($C_{15}H_{14}O_6$), chlorogenic acid ($C_{16}H_{18}O_9$), citrate tubes, cinnamic acid ($C_9H_8O_2$), copper (II) chloride dihydrate ($CuCl_2 \times 2H_2O$), coumarin ($C_9H_6O_2$), distilled water, ethanol, gallic acid ($C_7H_6O_5$), methanol, neocuproine ($C_{14}H_{12}N_2$), quercetin ($C_{15}H_{10}O_7$), rutin ($C_{27}H_{30}O_{16}$), starch, sulfuric acid (H_2SO_4), tannic acid ($C_{76}H_{52}O_{46}$), trans-cinnamic acid ($C_9H_8O_2$), trans-ferulic acid ($C_{10}H_{10}O_4$), vanillin ($C_8H_8O_3$).

Plant material

C. tougourensis was collected in spring 2019 at Belezma National Park in the municipality of Fesdis (Algeria) (latitude 35.621975; longitude 6.241327) and was identified by experts in the field from agronomic department of the Batna-1 University, Algeria and a voucher specimen under the code (CT/2019/LPT-PCMB) was deposited at the Laboratory of Improvement of the Phytosanitary Protection Techniques in Mountainous Agrosystems.

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 3L EtOH- H_2O (70:30) at a room temperature for 3 days. After liquid-liquid extraction with solvents (hexane, ethyl acetate and n-butanol), 1.58% of n-butanol, 1.03% of ethyl acetate and 0.42% of n-hexane extract were obtained.

High performance liquid chromatography with diode array detection (HPLC-DAD)

The HPLC analysis of *C. tougourensis* extracts was performed by HPLC-DAD (Agilent Technology, model 2014) which is based on an advanced UV detector. A volume of 20 μ l of each extract was injected in Agilent Zorbax SB-C18 reverse phase HPLC column (150 mm x 4.6 mm x 5 μ m). The gradient elution was carried out according to Falah *et al.* [21] method with slight modification. The flow rate used for the gradient elution was 0.5 ml/min. The mobile phase consisted of acetonitrile (Solvent A) and a solution of sulfuric acid in ultra-pure water (0.2%) (Solvent

B). The UV detection was set at a wavelength equal to 254 nm and analyses were performed in triplicate at a temperature of 35°C. The standards used in this study were respectively: gallic acid, catechin, quercetin, rutin, coumarin, vanillin, *trans*-ferulic acid, cinnamic acid, *trans*-cinnamic acid, 4-hydroxybenzoic acid, tannic acid, caffeic acid and chlorogenic acid. The results were expressed as μ g standard equivalent per mass of extract (μ g x mg^{-1}) and per dry weight of plant powder (μ g x g^{-1}).

Free radical scavenging assays

DPPH scavenging activity

This activity was evaluated according to Blois [22] approach with slight modification. Briefly, an amount of 6 mg of DPPH was dissolved in 100 ml of methanol and stored at -20°C in the dark. Several dilutions of the n-BuOH and EA extracts were prepared (0.048-50 μ g/ml). For each concentration, 40 μ l of the extract was added to 160 μ l of DPPH solution and the absorbance was recorded at 517 nm. It was noted, that another test tube was used as control and prepared using the same procedure but did not contain any dose of the extract, which was replaced by methanol. BHA (butylated hydroxyanisole), BHT (butylated hydroxytoluene) were used as standards. The median inhibitory concentration (IC_{50}) value was also calculated and expressed in (μ g/ml).

In addition, the following equation was used to calculate the DPPH scavenging activity:

$$\text{DPPH scavenging effect (\%)} = \left[\frac{(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})}{\text{Abs}_{\text{control}}} \right] \times 100,$$

where $\text{Abs}_{\text{control}}$ is the absorbance of control group, and $\text{Abs}_{\text{sample}}$ is the absorbance of plant sample.

Cupric ion reducing antioxidant capacity (CUPRAC)

In order to evaluate this activity, three successive solutions were prepared; the 1st solution is composed of 1.927 g of $AcNH_4$ and 25 ml of H_2O . The 2nd solution of 42.625 mg ($CuCl_2 \times 2H_2O$) and 25 ml of H_2O and the 3rd one of 0.039 g of neocuproine (Nc) and 25 ml of ethanol. A volume of 40 μ l of each extract or standards at different concentrations (3.125–200 μ g/ml) was added to 60 μ l of the 1st solution then to 50 μ l of the 3rd solution and finally to 50 μ l of the 2nd solution in this order. In the presence

of an antioxidant, the Cu^{+2}/Nc complex is reduced to a chromogenic Cu^{+}/Nc complex. BHA and BHT were used as standards and the absorbance was measured 1 h later at 450 nm. The concentration of a sample giving an absorbance of 0.5 ($A_{0.5}$) was also calculated [23].

***In vitro* haemostatic assay**

The determination of the potential effect of *C. tougourensis* extracts on clotting time, which is also known as plasma recalcification time (PRT), was investigated according to Pandith *et al.* [24] method, both n-butanol and ethyl acetate extracts were used for this approach. Blood samples were collected from healthy adult males (25 years of age) on citrate tubes and then centrifuged at 3000 rpm for 15 min to obtain the plasma. A volume of 200 μl of different extracts concentrations (800, 400, 200, 100 and 50 $\mu\text{g}/\text{ml}$) were placed in test tubes. Another test tube was used as a control and prepared using the same procedure, but without any dose of extract which was replaced by distilled water. The tubes were kept in a water bath at 37°C for two minutes. After that, 200 μl of plasma was added to each tube and clotting reaction was initiated by addition of 200 μl of calcium chloride (CaCl_2 , 0.025 M). The coagulation time was recorded using a chronometer and triggered as soon as the plasma was added. The observation was monitored every 30 s at the beginning, then frequently until the clot formation was observed. The presence or absence of the coagula was determined by slightly tilting the tube at an angle of 45°C.

In addition, the shortening percentage of clotting time was calculated as follows:

$$S\% = \left[\frac{(T_1 - T_2)}{T_1} \right] \times 100,$$

where

S – shortening percentage of clotting time;

T1 – clotting time of control group;

T2 – clotting time of the tested samples.

α -Amylase inhibitory test

This test was performed according to Agada *et al.* [25] and acarbose was used as a standard drug. A volume of 500 μl of plant extract or acarbose was added to 500 μl of phosphate buffer solution containing α -amylase enzyme (1%). The mixture was then incubated at 37°C for 10 min. Nearly 500 μl of the starch solution (1%) were added to the preparation to initiate the reaction and incubated again at 37°C

for 15 min, then 1 ml of 3,5-dinitrosalicylic acid solution (DNS) to stop the reaction. Preparations were placed in boiling water (100°C) for 10 min and 5 ml of distilled water was then added to each one. A control was prepared using the same procedure, replacing the plant extract with distilled water. The absorbances were measured by spectrophotometer at 540 nm. The IC_{50} value was also calculated and the following formula was used to calculate the inhibition percentage of α -amylase activity:

$$\% \text{ Inhibition} = \left[\frac{(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})}{\text{Abs}_{\text{control}}} \right] \times 100.$$

Photoprotective assay

The evaluation of possible sun protection factor (SPF) of *C. tougourensis* was carried out by the previous spectrophotometric approach described by Mansur *et al.* [26]. The stock solution of n-BuOH and EA of the plant was prepared in ethanol at the concentration of (4 mg/ml). Then, the absorbances were measured in triplicate, using UV-vis spectrophotometer. Ethanol was used as blank and the readings were taken at 290–320 nm with 5 nm of intervals.

The following equation was used to calculate the SPF of the plant:

$$\text{SPF}_{\text{spectrophotometric}} = \text{CF} \times \sum_{290}^{320} \text{EE}(\lambda) \times \text{I}(\lambda) \times \text{Abs}(\lambda)$$

where

CF – correction factor (= 10);

EE – erythemic efficacy spectrum;

I – intensity spectrum of the solar simulator;

EE x I – constant values previously determined by Sayre *et al.* [27].

Statistical analysis

One-way ANOVA was performed for the statistical analyses using GraphPad Prism version 8 (California, USA). Experiments were carried out in triplicate for each sample. The results were expressed as mean $\pm$ SD (n=3) and the level of significance was set at $p < 0.05$, $p < 0.01$ and $p < 0.001$.

Statement. The authors of the publication declare that the patients/participants have been informed that their blood will be used for testing. The patients/participants provided their written informed consent to participate in this study. Patients/participants agreed to use the research results to prepare a scientific publication.

RESULTS

HPLC analysis

The HPLC analysis of n-BuOH and EA extracts of *C. tougourensis* revealed the presence of thirteen phenolic compounds based on molecular separation at three wavelengths: 280 nm, 320 nm and 350 nm (fig. 1, fig. 2). The identified compounds were principally flavonoids and phenolic acids, namely gallic acid, catechin, quercetin, rutin, coumarin, vanillin, *trans*-ferulic acid, cinnamic acid, *trans*-cinnamic acid,

4-hydroxybenzoic acid, caffeic acid and chlorogenic acid besides tannic acid as hydrolysable tannin. The n-BuOH extract contains a high amount of vanillin ($6.19 \pm 1.03 \mu\text{g}/\text{mg}$) followed by rutin ($1.89 \pm 0.19 \mu\text{g}/\text{mg}$) and catechin ($1.40 \pm 0.36 \mu\text{g}/\text{mg}$), respectively, while the highest amounts in EA extract were of chlorogenic acid ($2.34 \pm 0.46 \mu\text{g}/\text{mg}$), cinnamic acid ($1.71 \pm 0.16 \mu\text{g}/\text{mg}$) and caffeic acid ($1.01 \pm 0.11 \mu\text{g}/\text{mg}$). Noting that most of compounds were identified at 280 nm for both extracts. The contents of detected phenolic compounds in *C. tougourensis* extracts by HPLC-DAD at different wavelengths are shown in table 1.

Figure 1.

HPLC-DAD chromatograms of *C. tougourensis* n-BuOH extract at (a): 280 nm, (b): 320 nm and (c): 350 nm

Figure 2.

HPLC-DAD chromatograms of *C. tougourensis* EA extract at (a): 280 nm, (b): 320 nm and (c): 350 nm

Table 1.

Contents of detected phenolic compounds in *C. tougourensis* extracts by HPLC-DAD at different wavelengths (280, 320 and 350 nm)

Phenolic compounds	Content [$\mu\text{g}/\text{mg}$] of n-BuOH extract	Content [$\mu\text{g}/\text{mg}$] of EA extract	Content [$\mu\text{g}/\text{g}$] of plant powder
Gallic acid	–	–	–
Catechin	1.40 ± 0.36	–	22.14 ± 5.67
Quercetin	0.56 ± 0.07	–	8.95 ± 1.10

Table 1. (continued)

Phenolic compounds	Content [$\mu\text{g}/\text{mg}$] of n-BuOH extract	Content [$\mu\text{g}/\text{mg}$] of EA extract	Content [$\mu\text{g}/\text{g}$] of plant powder
Rutin	1.89 $\pm$ 0.19	–	29.99 $\pm$ 3.02
Coumarin	–	0.90 $\pm$ 0.20	9.23 $\pm$ 2.04
Vanillin	6.19 $\pm$ 1.03	0.86 $\pm$ 0.03	107.07 $\pm$ 8.33
<i>Trans</i> -ferulic acid	–	–	–
Cinnamic acid	–	1.71 $\pm$ 0.16	17.60 $\pm$ 1.62
<i>Trans</i> -cinnamic acid	0.96 $\pm$ 0.06	–	15.29 $\pm$ 0.87
4-hydroxybenzoic acid	–	0.33 $\pm$ 0.07	3.37 $\pm$ 0.73
Tannic acid	0.57 $\pm$ 0.13	0.50 $\pm$ 0.09	14.21 $\pm$ 1.47
Caffeic acid	–	1.01 $\pm$ 0.11	10.38 $\pm$ 1.14
Chlorogenic acid	–	2.34 $\pm$ 0.46	24.15 $\pm$ 4.72

Antioxidant capacity

The possible antioxidant activity of *C. tougourensis* was evaluated using DPPH and CUPRAC approaches and revealed an anti-radical capacity for both n-BuOH and EA extracts in a dose-dependent manner. However, the result obtained in n-BuOH extract test were more efficient with a maximum inhibitory activity of (77.79 $\pm$ 1.86%) in DPPH test (fig. 3) and a maximum absorbance rate of (2.66 $\pm$ 0.1) in CUPRAC assay while the results of EA extract were (58.55 $\pm$ 0.99%) and (2.41 $\pm$ 0.28) respectively for the same tested concentrations (tab. 2). It is also interesting to see that the IC₅₀ values of both extracts were lower than those of tested standards. These values were (0.72 $\pm$ 0.07 $\mu\text{g}/\text{ml}$) for the n-BuOH extract and (4.69 $\pm$ 0.04 $\mu\text{g}/\text{ml}$) for EA one, and considered highly significant ($p < 0.001$) compared to standards values which were 15.74 $\pm$ 0.47 $\mu\text{g}/\text{ml}$ for BHA and 6.55 $\pm$ 0.59 $\mu\text{g}/\text{ml}$ for BHT. Comparable results were also found for A_{0.5} values (tab. 3).

Figure 3.

DPPH scavenging activity of *C. tougourensis* extracts and standards. All values are expressed as mean $\pm$ SD (n=3). One-way ANOVA followed by multiple Dunnet's test. ^a $p < 0.05$, ^b $p < 0.01$, ^c $p < 0.001$ is statistically significant with comparison to butylated hydroxyanisole (BHA), ^d $p < 0.05$, ^e $p < 0.01$, ^f $p < 0.001$ to butylated hydroxytoluene (BHT); n-BuOH – n-butanol extract; EA – ethyl acetate

Table 2.
Results of CUPRAC assay

Extracts/standard	Concentration [$\mu\text{g}/\text{ml}$]						
	3.125	6.25	12.5	25	50	100	200
n-BuOH extract	1.85 $\pm$ 0.11 ^{cf}	1.91 $\pm$ 0.05 ^{cf}	1.96 $\pm$ 0.05 ^{bf}	2.11 $\pm$ 0.08 ^{af}	2.21 $\pm$ 0.01 ^{cf}	2.47 $\pm$ 0.02 ^{ce}	2.66 $\pm$ 0.1 ^{cd}
EA extract	1.53 $\pm$ 0.08 ^{cf}	1.57 $\pm$ 0.2 ^{cf}	1.58 $\pm$ 0.06 ^{af}	1.70 $\pm$ 0.00 ^{cf}	1.78 $\pm$ 0.03 ^{cd}	1.81 $\pm$ 0.02 ^{ce}	2.41 $\pm$ 0.28 ^c
BHT	0.19 $\pm$ 0.01	0.33 $\pm$ 0.04	0.66 $\pm$ 0.07	1.03 $\pm$ 0.07	1.48 $\pm$ 0.09	2.04 $\pm$ 0.14	2.32 $\pm$ 0.28
BHA	0.46 $\pm$ 0.00	0.78 $\pm$ 0.01	1.34 $\pm$ 0.08	2.36 $\pm$ 0.17	3.45 $\pm$ 0.02	3.76 $\pm$ 0.03	3.93 $\pm$ 0.01

All values are expressed as mean $\pm$ SD (n=3). One-way ANOVA followed by multiple Dunnet's test. Level of significance $p < 0.05$, $p < 0.01$, $p < 0.001$; extracts vs. different standard substances, ^a $p < 0.05$, ^b $p < 0.01$, ^c $p < 0.001$ is statistically significant with a comparison to butylated hydroxyanisole (BHA), ^d $p < 0.05$, ^e $p < 0.01$, ^f $p < 0.001$ to butylated hydroxytoluene (BHT). n-BuOH – n-butanol extract; EA – ethyl acetate

Table 3.Antioxidant activities (IC_{50} and $A_{0.50}$ $\mu\text{g/ml}$) of standards and *C. tougourensis* extracts

Extracts standards	DPPH assay	CUPRAC assay
	IC_{50} [$\mu\text{g/ml}$]	$A_{0.50}$ [$\mu\text{g/ml}$]
n-BuOH extract	0.72 ± 0.07^{cd}	<3.125
EA extract	4.69 ± 0.04^{ce}	<3.125
BHA	15.74 ± 0.47	3.64 ± 0.19
BHT	6.55 ± 0.59	9.62 ± 0.87

All values are expressed as mean $\pm$ SD (n=3). One-way ANOVA followed by multiple Dunnett's test. Level of significance $p < 0.05$, $p < 0.01$, $p < 0.001$; extracts vs. different standard substances, $^ap < 0.05$, $^bp < 0.01$, $^cp < 0.001$ is statistically significant with a comparison to butylated hydroxyanisole (BHA), $^dp < 0.05$, $^ep < 0.01$, $^fp < 0.001$ to butylated hydroxytoluene (BHT). n-BuOH – n-butanol extract; EA – ethyl acetate

Haemostatic test

As shown in table 4, both extracts of *C. tougourensis* shortened the clotting times in a dose-dependent manner, the best haemostatic effect was recorded for the maximum tested concentration of 4 mg/ml with a corresponding clotting time of (22.07 ± 3.72 s) for n-BuOH and (47.33 ± 7.09 s) for EA extracts which correspond to a shortening rate percentage of (86.71%) and (71.42%), respectively, and those data were considered highly significant ($p < 0.001$) when compared to control group.

Table 4.Effect of n-BuOH and EA extracts of *C. tougourensis* on coagulation time of plasma

Treatment	Concentrations [mg/ml]	Mean clotting time [s]	Shortening rate of clotting time [%]
Control	/	165.66 ± 13.05	/
n-BuOH extract	0.125	73.5 ± 4.94^c	55.63%
	0.25	61 ± 4.24^c	63.17%
	0.5	50.33 ± 1.15^c	69.61%
	1	43 ± 2.82^c	74.06%
	2	28 ± 3.13^c	83.09%
	4	22.07 ± 3.72^c	86.71%
Ethyl acetate extract	0.125	152.33 ± 12.58^{ns}	8.24%
	0.25	99.5 ± 4.94^b	39.93%
	0.5	88.5 ± 2.12^c	46.57%
	1	73 ± 11^c	55.93%
	2	53 ± 5.56^c	68%
	4	47.33 ± 7.09^c	71.42%

All values are mean $\pm$ SD, n=3. One way ANOVA followed by multiple Dunnett's test. Level of significance $^ap < 0.05$, $^bp < 0.01$, $^cp < 0.001$ compared with control group. ns – no significance

Antidiabetic assay

In this test, both extracts of *C. tougourensis* showed a moderate inhibitory effect of α -amylase activity, but the activity of n-BuOH extract seems to be slightly higher than EA extract's one. The maximum inhibitory activity was observed at the tested concentration of 1000 $\mu\text{g/ml}$ with respective inhibition percentages of (61.36%) for n-BuOH and (52.27%) for EA extracts while acarbose value was (94.61 ± 1.14 %) (fig. 4). IC_{50} values were also calculated and were (711.5 ± 0.03 $\mu\text{g/ml}$) for n-BuOH and (962 ± 0.02 $\mu\text{g/ml}$) for EA extracts respectively against (284.33 ± 0.06 $\mu\text{g/ml}$) for acarbose (tab. 5). Differences between *C. tougourensis* extracts and the standards were considered to be highly significant ($p < 0.001$) for all tested concentrations.

Figure 4.

α -Amylase inhibitory activity of *C. tougourensis* extracts. All values are expressed as mean $\pm$ SD (n=3). One way ANOVA followed by multiple Dunnett's test. Level of significance $^ap < 0.05$, $^bp < 0.01$, $^cp < 0.001$ is statistically significant with comparison to acarbose

Table 5.Determination of IC_{50} values of standard and *C. tougourensis* extracts in α -amylase inhibitory assay

Extracts/standard	IC_{50} [$\mu\text{g/ml}$]
n-BuOH extract	711.5 ± 0.03^c
EA extract	962 ± 0.02^c
Acarbose	284.33 ± 0.06

All values are expressed as mean $\pm$ SD (n=3). One way ANOVA followed by multiple Dunnett's test. Level of significance $^ap < 0.05$, $^bp < 0.01$, $^cp < 0.001$ is statistically significant with a comparison to respective standards. EC_{50} represent the median effective concentration

Table 6.
Sun protection factor (SPF) calculation from *C. tougourensis* extracts

Wavelength λ [nm]	<i>C. tougourensis</i> extracts				
	EE (λ)x I(λ) (normalized)	n-BuOH extract		EA extract	
		Absorbances	SPF	Absorbances	SPF
290	0.0150	5.966±0.015	0.894	0.897±0.001	0.134
295	0.0817	5.921±0.011	4.837	0.893±0.002	0.729
300	0.2874	5.896±0.005	16.945	0.871±0.002	2.503
305	0.3278	5.814±0.015	19.058	0.837±0.002	2.743
310	0.1864	5.32±0.015	9.916	0.801±0.001	1.493
315	0.0837	4.428±0.03	3.706	0.771±0.000	0.645
320	0.0180	3.776±0.017	0.679	0.754±0.002	0.135
Total	1	-	56.035	-	8.382

Photoprotective assay

The possible photoprotective activity of *C. tougourensis* was also evaluated based on sun protection factor (SPF) and, as indicated in table 6, the n-BuOH extract showed a maximum SPF of 56.035. On the other hand, the EA extract showed a maximum SPF of 8.382. When referring to the European commission guidelines of sun protection factor (tab. 7), it is clear that n-BuOH extract of *C. tougourensis* has a high SPF while EA extract has a weak SPF.

Table 7.

European commission recommendation on sun products categories

Indicated category	Indicated protection factor	Sun protection factor measured
Low protection	6	6–9.9
	10	10–14.9
	15	15–19.9
Average protection	20	20–24.9
	25	25–29.9
High protection	30	30–49.9
	50	50–59.9
Very high protection	50+	≤60

DISCUSSION

The phytochemical investigation on *Centaurea tougourensis* revealed that it contained considerable amounts of several flavonoids and phenolic acids, which may contributed to the pharmacological properties of this plant species. These compounds may act in a synergistic way as antioxidant agents

to reduce DPPH (2,2-diphenyl-1-picrylhydrazyl) into a non-radical form called (2,2-diphenyl-1-picrylhydrazine) *via* hydrogen atom donating capacity. On the other hand, CUPRAC results suggest that Cu^{+2} were reduced to Cu^{+} in the presence of neocuproine. Rutin and quercetin are probably one of the most potent antioxidants agents and several studies [28–30] using both mice and rats as model reported that these flavonoids may considerably reduce oxidative stress and lipid peroxidation in several tissues including brain, kidney but also in liver by protecting and enhancing the expression of key genes involved in antioxidant processes like glutathione S transferase α ($\text{GST}\alpha$), paraoxonase-1 (PON-1) and glutamate-cysteine ligase (GCL) which in result, will considerably decrease the plasma level of malondialdehyde (MDA) and glutathione (GSH) considered as the principal markers of oxidative stress [31]. Phenolic acids play also an important role in antioxidant reactions; gallic acid, cinnamic acid and chlorogenic acid are the best examples to illustrate it. These two compounds can increase superoxide dismutase, glutathione and catalase levels, which will considerably reinforce antioxidant defense mechanisms [32, 33]. On the other hand, caffeic acid is also an important antioxidant agent due to its unique capacity to reduce α -toxin production by 95%, which is really important since α -toxin is a pro-oxidant and a virulence factor that may alter the polarity and diffusion processes of epithelial membranes leading to an irreversible deregulation of cellular redox balance [34]. Several studies [35–37] showed that *Centaurea* species possess non-negligible antioxidant properties due to their richness in various phenolic components which strongly contribute to the suppression of reactive oxygen species (ROS) or reactive nitrogen

species (RNS) by inhibiting the enzymes involved in their production or down-regulating the expression of their respective genes. The antioxidant effect of polyphenols may also improve cardiac function by significantly reducing ROS and RNS production which considerably reduces cardiomyocytes damage to prevent the possible necrosis or apoptosis of these cells [38]. When these stressful agents are excessively produced, they may represent a real threat for whole body function and their harmful effects are observed in both organelles and cell levels. A previous work on *C. tougourensis* showed its antioxidant potential using ABTS, galvinoxyl radical, phenanthroline, and reducing power assays [20], which is consistent with the result of this study.

Another study using rats showed that polyphenols may prevent skeletal muscle ageing *via* an enhancement of PGC-1 β expression [39], leading to an important increase of anti-oxidative defense mechanisms.

A study of Kose *et al.* [40] on rats as experimental model proved that the bioactive compounds present in plant extracts, especially flavonoids, and phenolic acids can significantly shorten bleeding process by accelerating blood arrival at injury sites *via* vasoconstriction which greatly enhances clotting mechanism *via* a cascade made of platelets [41] and other key pro-coagulation mediators like Factor III (thromboplastin). A study made by de Jesus *et al.* [42] showed that tannic acid can activate platelets and enhance their adhesion process in injured tissues especially in intestine which will considerably accelerate coagulation process to stop bleeding and limit the appearance of ulcers, which is in accordance with the findings of this study. It was also reported that catechin can regulate platelets activation process especially in patients who suffer from peripheral artery disease [43] suggesting that this compound has an influence on haemostatic phenomenon.

Flavonoids may prevent microvascular lesions related to myocardial infarction and myocardial disorders [44] by enhancing haematopoiesis process *via* an elevation of cytosolic Ca²⁺ levels [45] resulting from a molecular cascade; the G protein activates phospholipase C (PLC) which in turn leads to the synthesis of inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DG) [46]. This phenomenon significantly increases the platelet aggregation and other blood elements to accelerate the healing process in lesion area [47].

Recent studies underlined possible pro-coagulant effect of flavonoids, steroids and terpenes using both *in-vitro* and *in-vivo* approaches [48, 49] and showed

that these classes of bioactive compounds can oppose the effect of anti-coagulant drugs such as warfarin [50] and this despite the fact that flavonoids and warfarin does not share the same binding sites as shown previously by the docking approach of Rimac *et al.* [51]. The presence of these molecules was confirmed in *C. tougourensis*.

Type 2 diabetes is one of the most dangerous illnesses of current decade, which may be the result of several factors like mitochondrial or pancreatic β -cell dysfunction, insulin resistance, and impaired glucose tolerance [52].

In several studies made in sector of biotechnology it was reported that plants may effectively prevent or treat type 2 diabetes due to their richness in flavonoids and phenolic acids which may oppose the oxidative stress process in particular the production of reactive oxygen species (ROS) [53]. Among these phenolic compounds, a particular lactone named coumarin has a strong antidiabetic activity. A study made by Laddha and Kulkarni [54] showed that diabetic patient who received medication containing coumarin had a considerable attenuation of cardiovascular and cerebrovascular diseases normally generated by diabetes complications. It was also reported that coumarin may protect salivary and pancreatic glands from oxidative stress damage; since these glands are responsible of the production of α -amylase [55]. Coumarin may also limit the excessive production of alpha amylase and α -glucosidase by activating AMP-activated protein kinase (AMPK) signalling pathway [56]. It is well known that there is a strong link between oxidative stress and alpha amylase activity. In fact, a high liberation of free radicals will automatically deregulate alpha amylase activity and it was proved that free radicals accumulation will induce an over-expressing of HAP1 gene in a *S. cerevisiae* strain which in turn induce an overproduction of recombinant α -amylase [57]. In long term, this phenomenon generate several complications related to diabetes [58]. It was also proved in diabetic rats model that a reduction of oxidative stress process will significantly increase insulin sensitivity [59], and this may increase the activity of the transporter GLUT4, which has the principle function favouring glucose uptake to reduce glycaemia to physiological rate [60]. A study carried out by Bandyopadhyay *et al.* [61] demonstrated that the increase in GLUT4 could be due to an important increase of transport transduction pathway in which series of proteins will act *via* a cascade; these proteins are phosphatidylinositol-3 kinase (PI3K), protein kinase C (PKC), and

peroxisome proliferator-activated receptors (PPAR), respectively. Recently, a study made on eight *Centaurea* species showed that they can significantly decrease alpha amylase activity and associated that to the richness of these plants in phenolics, flavonoids and tannins [62] which is in perfect accordance with our previous finding.

The antioxidant and anticancer properties of vanillin are well-known, but this compound has also non negligible anti-hyperglycaemic effect. Lu *et al.* [63] showed that this compound can regulate the serum level of glucose and insulin in diabetic rats and even conserve the integrity of pancreas tissues. Another study carried out by Iannuzzi *et al.* [64] proved that this compound could prevent diabetes complications by blocking glycation of insulin, precisely *via* a non-covalent binding process between vanillin and positively charged arginine 22 (Arg22) of insulin. This process allows insulin to recover its biological effect and reduce blood glucose level to physiological state. Noting that, a comparable hypoglycaemic effect was observed in diabetic rats treated using 4-hydroxybenzoic acid. In addition, researchers noted an important increase in liver glycogen content [65].

Sunlight is vital for all living organisms, for example, it helps regulate circadian rhythm and offers the capacity to make vitamin D which is an important nutrient that helps bones fixing as well as absorbing calcium and phosphorus to enhance the mineralization process [66]. But, on the other hand, sun also emits other categories of rays that can be harmful like broad-spectrum UV radiation also called carcinogen sun [67]. In recent studies [68, 69] it was proved, using formulations approach, that sunscreen made of plants is effective against UV radiation. These researchers noted that the secondary metabolites present in plants, especially lignin and alkaloids, can absorb UV-B rays [70] but flavonoids have the best photoprotection in the UV-B range with a non-negligible UV-A protection. Among these flavonoids, quercetin and catechin proved their ability to prevent collagen fragmentation, premature skin cancer and skin photoaging processes [71-73] by significantly reducing and neutralizing free radical and oxygen-mediated damage in cells and also in extracellular matrix [74]. A recent study showed that tannic acid may effectively protect L929 fibroblasts cell from UVB-irradiation by inhibiting the expression of a key enzyme called matrix metalloproteinase-1 (MMP-1) [75]. In the same study a non-negligible photoprotective effect exerted by gallic acid was reported, which agrees with

the results of our study. In another research work the photoprotective effect of caffeic acid on human diploid skin fibroblasts (KF1) against UV-B and UV-C rays and no sign of cytotoxicity was detected [76] was underlined. Quercetin and rutin proved their ability to stabilize the chemical composition and molecular structure of sunscreen constituents *via* mutual co-regulation effects [77], and even enhancing the photoprotection of sunscreen constituents with a synergistic way [78]. *Trans-ferulic* acid offers also a good protection against UV radiation and reached an SPF value of 37% [79], this is why these phyto-compounds are part of the composition of many cosmetic sunscreens such as Sun Bum and alba Botanica [80].

In fact, in 2006 the European Commission published a guideline on sun products categories [81] and according to indicated protection factors we can suggest that *C. tougourensis* may be a good candidate to enter in the composition of natural sunscreen.

Otherwise, human skin contains a key pigment called melanin which gives colour to skin, eyes and hair. This pigment acts as a natural sunscreen to limit UV penetration [82]. A recent study made by Liu-Smith *et al.* [83] showed that flavonoids compounds can actually enhance melanin synthesis process and even treat melanoma *via* activation of the transcriptional factor MITF and other key enzymes, namely dopachrome tautomerase DCT2 and tyrosinase-related protein 1 (TYRP-1), thus lead to a remarkable inhibition of the proliferation, migration, and invasion of melanoma cells [84].

CONCLUSIONS

In the present study, we investigated the antioxidant, antidiabetic, haemostatic and photoprotective effects of *C. tougourensis*, and the various results obtained from this study supported our previous work and suggest that 13 phenolic compounds identified in this species could possibly treat some disorders; indeed, tannic acid and catechin could effectively treat illnesses related to haemophilia or vascular bleeding, while rutin, quercetin, coumarin, vanillin and phenolic acids may treat some chronic illnesses related to oxidative stress like cardiovascular diseases, cancer and diabetes. Several pathways have already been proposed to explain their beneficial properties. The highest photoprotective effect recorded in the n-BuOH extract also gives a lot of hope to treat some kind of skin cancers, noting that catechin, gallic acid and caffeic acid are well-known

photoprotective agents, but further investigations are needed to clearly understand the full potential of this species.

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