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Full Length Research Paper

Phytochemical analysis, antioxidant and antimicrobial activities of leaves and flowers ethyl acetate and n-butanol fractions from an Algerian endemic plant *Calycotome spinosa* (L.) Link

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Calycotome spinosa* (L.) Link is one of the important plants in traditional medicine especially in Algeria. The present work deals with the phytochemical screening, the flavonoids extraction from leaves and flowers of this plant, the quantification of total polyphenols and flavonoids, as well as the evaluation of antioxidant and antimicrobial properties of ethyl acetate and n-butanol fractions. While, the rest, interacted with its phenolics identification. The results of preliminary phytochemical screening showed that *C. spinosa* contained various metabolites: Polyphenols, flavonoids, alkaloids, saponins etc. In addition, the findings of polyphenols determination using Folin Ciocalteu was high in all the fractions: 107.75±0.41 and 64.24±1.81 mg gallic acid equivalents/g extract for leaves ethyl acetate and n-butanol respectively and 81.45±0.6 and 96.06±2.72 mg gallic acid equivalents/g extract for flowers ethyl acetate and n-butanol successively. Conversely, the flavonoids content in the four fractions by $AlCl_3$ was less important than polyphenols. Moreover, the obtained fractions were evaluated for their antioxidant capacity using DPPH assay. Besides, the obtained result revealed that Ethyl acetate and n-butanol fractions of leaves exerted very high antioxidant activities with IC_{50} equal to 45.25±1.8 and 52.80±2.05 µg/mL, respectively compared with flowers. Furthermore, the antimicrobial activity of those fractions against many microbial strains have also been investigated using the disc diffusion method; the leaves ethyl acetate fraction showed the highest activity against *Acinetobacter baumannii* with a zone inhibition diameter equal to 22±0.06 mm. Additionally, high-performance liquid chromatography/diode-array detector (HPLC/DAD) analysis performed with *C. spinosa* fractions revealed the richness of plant in many phenolic compounds.

Key words: *Calycotome spinosa* (L.) Link, medicinal plant, phenolics and flavonoids content, antioxidant activity, antimicrobial potential, high-performance liquid chromatography/diode-array detector (HPLC/DAD).

INTRODUCTION

Medicinal plants are the effective source of secondary metabolites which are used in traditional as well as modern medicines (Patil et al., 2014) and they have played an important role in the drug development

(Edeoga et al., 2005). In Africa, more than 80% of the populations use these plants to provide health care (WHO, 2002; Tonye and Mayet, 2007). For many centuries, in Algeria as in all countries of the Maghreb, medicinal and aromatic plants are used mainly in rural areas (Reguieg, 2011).

Within the framework of the bio-diversity study of certain vegetable resources in Algeria, the aromatic and medicinal plants of the Algerian Northeast which are little valued this day were very interested in the past (Larit et al., 2012), as the plant belongs to *Calycotome* genus.

Calycotome spinosa (L.) Link belongs to the Papillionaceae (Fabaceae) family; it is a spiny shrub, trifoliate with yellow flowers during the spring season, widespread in the Mediterranean undergrowth forest and prefers siliceous well watered soils (Quezel and Santa, 1963). Its vernacular name is El Guendoul. According to our bibliographic search, no profound phytochemical study of this plant has been reported. The aerial parts of this genus, Calycotome, are traditionally used as an antitumoral agent and efficient for the treatment of furuncle, cutaneous abscess and chilblain in the Sicilian folk medicine (Djeddi et al., 2015). Additionally, the special thing about *C. spinosa* is that its foliage is very rich in crude protein (33.7% DM), making this legume an excellent supplement protein for low quality forage and undergrowth fibrous products. Unfortunately, this species is also excessively rich in phenols and total tannins (Mebirouk-Boudechiche et al., 2015). Flavonoids are a broad class of plant pigments that are ubiquitously present in fruit and vegetable derived foods (Robards and Antolovich, 1997; Pietta, 2000).

As a part of a systematic research study on the constituents, antioxidant activity and antimicrobial potential of Algerian natural plants, the phytochemical and biological properties of *Calycotome villosa* (Chikhi et al., 2014; Djeddi et al., 2015) and *Retama raetam* (Djeddi et al., 2013) have already been reported.

The present investigation was undertaken to report the phytochemical screening and the extraction of flavonoïds with its quantification, to evaluate the antioxidant activity and also to check the antimicrobial potential of leaves and flowers fractions from *C. spinosa* against different microbial strains, as well as to identify its phenolic compounds.

MATERIALS AND METHODS

Plant material

Leaves and flowers of *C. spinosa* (L.) Link were collected in April 2014 from natural population in Constantine located in the North-East of Algeria (Chattaba forest in Ain Smara). This forest is located 15 km south-west of Constantine and culminating at 689 m above sea level. It is characterized by a Mediterranean climate with hot summer (Figure 1). The plant was identified by Mrs. Nadra Khalfallah (Department of Biology and Vegetable Ecology, Faculty of Natural and Life Sciences, Mentouri University, Constantine, Algeria).

Systematic and botanical study of species

The systematic classification of the studied species *C. spinosa* (L.) Link is now presented as the following (GRIN, 2009): Kingdom: Plants (Plantae); Subkingdom: Vascular Plants (Tracheobionta); Superdivision: Spermaphytes (Spermatophyta); Division: Angiosperms; Class: Edudicotyledons (Eudicots); Subclass: True Rosidae I; Order: Fabales; Family: Fabaceae (Papilionaceae/Leguminosae); Subfamily: Faboideae Rudd (Papilionoideae Juss); Genus: Calicotome Link; Species: *C. spinosa* (L.) Link. The local vernacular name of this plant is 'El-Gandoul' without distinction between different species.

Calicotome (or Calycotome), from Greek *Calyx* (calyx) and *Temno* (cut), alludes to the calyx which breaks circularly and seems clipped after flowering. Calicotomes are thorny and broom shrubs, with slender and discarded stems, forming bushes that can reach 2 m in height. The branches are green, then brown with age, and end with thorns (Thomas, 2004). This plant is also characterized by spiny branches, divariate, strongly striated, glabrescent; leaves blackening by desiccation, leaflets subsessile, obovate, obtuse, glabrous above, with hairs applied below; stipules very small; solitary or fasciculated flowers 2 to 4; pedicels 2 to 3 times longer than the calyx, bearing at the top a bi-trifid bract ordinarily longer than broad; acute careen; clover 30 to 40 mm by 6 to 8, glabrous, shiny and black when ripe, upper suture only slightly winged, with straight margin; 3 to 8 seeds (Quezel and Santa, 1963). It is found in its native form in some areas to the south of Spain and north Africa, particularly Algeria, Tunisia, Morocco, and Libya.

This plant has a thermophilic trend and a heliophilous behavior, the soil consists of various siliceous rocks alterites, with limited water reserves. It is located in stations, often characterized by a water-balance more or less deficit (Rameau et al., 2008). The plant is highly flammable and contributes the spread of fires. Its roots often carry nodules containing bacteria allowing the fixation of atmospheric nitrogen (Damerdji and Djeddi, 2006). The trifoliate leaves and the yellow flowers are characteristic of Fabaceae family. Cultivated as an ornamental plant, bees harvest very sweet, scanty nectar at the base of the stamens tubes. It prefers siliceous soils. It is very common in Algeria (Damerdji, 2008-2009; Damerdji, 2012).
This forage shrub is among the most consumed by goats in extensive system. It is characterized by its high palatability, its resistance to climatic conditions, its abundance and its annual availability (Mebirouk-Boudechiche et al., 2015).

Tested microorganisms

The following clinical bacterial strains from the “University Hospital Ibn Badiss”, Algeria were used in this test: *Staphylococcus aureus* (ATCC-25923) and *Bacillus sibtilis* (ATCC- 6633) (Gram positive), *Escherichia coli* (ATCC-25922), *Pseudomonas aerugenosa* (ATCC-27853), *Salmonella abony* (NCTC 6017), *Klebseilla pneumoniae* and *Acinetobacter baumannii* (Gram negative). Furthermore, one pathogenic yeast: *Candida albicans* (ATCC 10231), was also tested.

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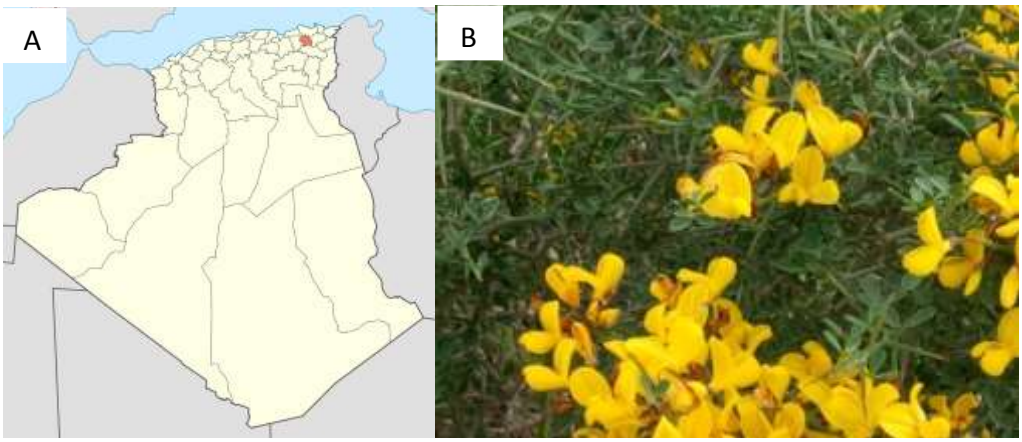

Figure 1. (A) The studied area; (B) *Calycotome spinosa* (L.) Link plant (April, 2014).

The bacteria were sub-cultured on nutrient agar slants, incubated at 37°C for 24 h and stored at 4°C in the refrigerator to maintain the stock culture; some of these bacteria are involved in various skin infections (Valia and Valia, 2008).

Phytochemical screening

Preliminary screening of primary and secondary metabolites such as sugar, proteins, polyphenols, flavonoids, alkaloids, saponins, and volatile oils were carried out according to the common phytochemical methods described by Harborne (1973), Trease and Evans (1983), Sofowara (1993), and Raaman (2006).

The results have been classified according to: highly positive: +++; fairly positive: ++; weakly positive: +; negative test: -.

Fehling test (carbohydrates)

The extract (1 ml) was mixed with 1 ml Fehling solutions (A + B) and heated until boiling. The appearance of a brick red precipitate indicates the presence of carbohydrates (sugar).

Biuret test (proteins)

One milliliter of $CuSo_4$ (1%) and NaOH (5N) was added into 1 ml of extract (0.05 g/10 ml). The appearance of a pink color indicates the presence of proteins.

Polyphenols test

The reaction with ferric chloride ($FeCl_3$) allowed the polyphenols characterization. A drop of 2% of ferric chloride solution was added to 2 ml of extract. The appearance of a dark blue-green coloration indicates the presence of polyphenols.

Flavonoids test

Flavonoids are highlighted by adding 4 ml of sodium hydroxy alcoholic solution ($FeCl_3$) to the extract. The appearance of a yellow color indicates the presence of flavonoids.

Alkaloids test

A powder of 10 g was added into 50 ml of 10% H_2SO_4. After 24 h of soaking at room temperature, the macerated is filtered and washed with water to obtain 50 ml of filtrate. 5 drops of Mayer reagent was added into 1 ml of the collected filtrate and was allowed to wait 15 min. The presence of alkaloids is confirmed by the appearance of a yellow precipitate.

Saponins test (Foam test)

Test solution was mixed with water and shaken and observed for the formation of froth, which is stable for 15 min for a positive result.

Essential oils test

The essential oil extraction was performed by hydro-distillation in a Clevenger apparatus. Three distillations were carried out by boiling 200 g of fresh plant material with 1 L of water in 2 L flask surmounted by a column of 60 cm in length connected to a refrigerant. The yield of essential oil was determined relatively to the dried matter.

Preparation of plant extracts (flavonoïds extraction)

In this study, the freshly cut leaves and flowers of *C. spinosa*, each part alone, were air dried at room temperature in a dark place. They were stored in the dark until analysis. Dried powdered (100 g) for each part were extracted with a methanol/distilled water system (8:2, 500 ml), this maceration was repeated three times after each 24 h at room temperature.

The obtained crude extracts after evaporation were dissolved in water (300 ml) and filtrated through filter paper (Whatman No. 1), then extracted successively for three times with equal volume of petroleum ether, chloroform $CHCl_3$, ethyl acetate EtOAc and finally with n-butanol BuOH using a decanted ampoule (Bekkara et al., 1998).

The final extracts were evaporated to dryness in vacuum by rotary evaporator at 45°C. The obtained yields of leaves and flowers fractions were as follows: 455 and 333 mg (Petroleum ether), 218 and 222 mg ($CHCl_3$), 547.5 and 444 mg (EtOAc), and 307.9 and 233 mg (BuOH fraction) in that order.

Determination of total phenolic content

The total phenolics content of the four fractions was quantified according to the method described by Singleton et al. (1999) using Folin-Ciocalteu reagent and gallic acid as a reference standard. Indeed, 0.2 ml of each sample was tested in triplicate and 1 ml of Folin-Ciocalteu reagent (10%) was added to each tube. The tubes were maintained at room temperature for 5 min, afterward, 0.8 ml of sodium carbonate Na_2CO_3 (7.5%) was added and mixed well. Then, the samples were incubated for 60 min at room temperature (25°C) in obscurity. The absorbance was measured at 760 nm with a UV/Visible spectrophotometer (Shimadzu, UV-1280). Results were expressed as milligram gallic acid equivalents (GAE)/1 g extract. The standard curve was prepared with gallic acid in six different concentrations (6.25, 12.5, 25, 50, 100, and 200 µg/ml).

Determination of flavonoids content

The total flavonoids content of leaves and flowers ethyl acetate and n-butanol fractions from *C. spinosa* was measured spectrophotometrically using the aluminium chloride colorimetric method (Chang et al., 2002; Ramful et al., 2011). In fact, 1 ml of each sample was mixed with 1 ml of 2% aluminium chloride ($AlCl_3$) solution. The samples were incubated for 30 min at room temperature in obscurity. By using a spectrophotometer, absorbance was measured at 430 nm. The samples were analyzed in triplicate. Results were expressed as milligram quercetin equivalents/1 g extract. The calibration curve was prepared by quercetin standard solution at different concentrations: 0.02, 0.04, 0.06, 0.08, 0.1, 0.12, 0.14, 0.16, 0.18, and 0.2 mg/ml.

Radical scavenging activity (DPPH method)

From the obtained fractions, different concentrations were prepared in methanol: 0.01, 0.02, 0.04, 0.06, 0.08, and 0.1 mg/ml. The antioxidant activity of leaves and flowers ethyl acetate and n-butanol fractions from *C. spinosa* was carried out using a modified DPPH free radical-scavenging activity (2, 2-diphenyl-1picrylhydrazyl) (Lebeau et al., 2000; Molyneux, 2004).

According to this method, 1 ml of methanol solution of DPPH (24 µg/ml concentration) was added to 500 µl methanol solution of fractions of the various concentrations and allowed to stay in the dark for 30 min. Shorter times have also been reported by some authors, such as 5 min (Lebeau et al., 2000) or 10 min (Schwarz et al., 2001), but in our experiments, the time of 30 min proved to be the optimum (time needed for stable signals). After this time, the absorbance was measured at 517 nm in a spectrophotometer against a control consisting of methanol (0. 5 mL) and DPPH (1 mL). Each test was repeated three times. The result was expressed as micrograms of extract inhibiting 50% of the control using the following formula:

$$\text{Inhibition (\%)} = [(A_0 - A_1)/A_0] \times 100$$

where A_0 is the absorbance of the control (sample without extracts) and A_1 is the absorbance of samples with extracts.

The value of the IC_{50} was determined graphically using the plot obtained from different concentrations of each fraction. A low IC_{50} value indicates a strong antioxidant activity.

Antimicrobial activity

According to the standard M2-A8 from Clinical Laboratory Standards Institute (CLSI) 2003) for bacteria and National Committee for Clinical Laboratory Standards (NCCLS) (2004), document M44-A for yeasts, the agar disc diffusion method was used for the evaluation of antimicrobial activity of the different tested fractions (Bauer et al., 1966; National Committee for Clinical Laboratory Standards (NCCLS), 1997).

All bacteria species were cultured overnight at 37°C in Mueller Hinton medium (Bio-Rad). The inoculums were prepared using the bacteria and the yeast from 24 h culture on Mueller Hinton medium (Bio-Rad) for bacteria and on Sabouraud dextrose agar for yeasts, suspensions were made in a sterile saline solution (0.9%). The turbidity of the suspensions was adjusted to obtain a final concentration to match that of a 0.5 McFarland standard (1×10^8 colony forming units (CFU)/mL). Suspensions of the tested micro-organisms (0.1 ml of 10^7-10^8 cells/ml) were spread over the surface of Petri plates using a distilled swab. Filter paper discs (Whatman No. 1; 6 mm in diameter) were impregnated with 10 µl of the sample and placed on the inoculated agar plates. The minimum inhibitory concentration (MIC) of ethyl acetate and n-butanol fractions of *C. spinosa* leaves and flowers were studied using MH agar in square Petri dishes seeded by multiple inoculators as described in a previous work of Abedini et al. (2014). The four fractions were tested at five final concentrations (25, 50, 100, 150 and 200 mg/mL) against the eight micro-organisms. The agar plates were incubated 24 h at 37°C for bacteria and 48 h at 30°C for yeasts. The activity was then visually estimated by the presence or absence of colonies. Positive antimicrobial controls were also used for bacteria (chloromphenicol 15 µg/disc) and yeasts (fluconazol 10 µg/disc). The inhibition zones diameters (IZD) have been measured in millimeters (Jirovetz et al., 2003). All experiments were performed in triplicates and the results were given in mean ± standard deviation (SD).

MIC values were recorded as the lowest concentrations of compounds enabling growth inhibition. DMSO was checked for absence of antimicrobial activity.

Analysis of phenolic compounds by HPLC-DAD chromatography

The phenolic acids content of leaves and flowers ethyl acetate and n-butanol fractions from *C. spinosa* was measured using the HPLC analysis system (Sycam, Gilching, Germany) according to methods described by Jin et al. (2011). A sunfire C18 column (25 cm×4.6 mm, Waters Co., Milford, MA, USA) with a C18 guard column (2 cm×4.6 mm) was used at 25°C. The injection volume was 10 µL. The separated phenolic acids were detected at 280 nm with a photodiode array detector (PDA, S3210; Sycam). The mobile phase consisted of 0.1% formic acid in 10% acetonitrile (solvent A) and 0.1% formic acid in 90% acetonitrile (solvent B). Flow rate was kept at 1 mL/min for a total running time of 45 min and the gradient program was as follows: 100% A at 0 to 2 min, 100% A to 90% A at 2 to 6 min, 90% A to 37% A at 6 to 31 min, 37% A to 50% A at 31 to 41 min, 50% A to 100% A at 41 to 45 min, and 100% A at 45 to 50 min.

Statistical study

All tests were performed in triplicate. Results are presented as mean± SD of three independent determinations.

Chemicals

All chemicals [methanol, petroleum ether, chloroform, ethyl acetate, n-butanol, Folin-Ciocalteu reagent, sodium carbonate (Na_2CO_3), gallic acid, aluminum chloride ($AlCl_3$), quercetin, the free radical 2,2'-diphenyl-1-picryl-hydrazyl (DPPH), butylated hydroxytoluene (BHT), dimethylsulfoxyde (DMSO), and chloramphenicol] were

Table 1. Prelimanary phytochemical screening of *C. spinosa* Leaves and flowers.

Phytochemical test (class of compounds)	Leaves	Flowers
Ferric chloride test (phenolic compounds)	+++	++
Alkaline reagent test (flavonoids)	++	+++
Mayer's test (alkaloids)	+++	+++
Tannins	+	+
Foam test (saponins)	++	+
Coumarins	++	++
Volatile oil test (Essential oil)	-	-
Fehling's test (sugar)	++	++

Highly positive: +++; fairly positive: ++; weakly positive: +; Negative test: -

purchased at Sigma-Aldrich and all other ingredients used were of analytical grade.

RESULTS AND DISCUSSION

Phytochemical screening of *C. spinosa* (L.) Link

Thousands of diverse natural products are produced by plants and many of these are involved in plant defense. The phytochemical diversity of antimicrobial compounds include terpenoids, saponins, phenolics and phenyl propanoids, pterocarpans, stilbenes, alkaloids, glucosinolates, hydrogen cyanide, indole and also elemental sulphur, the sole inorganic compound (Cooper et al., 1996).

In this study, the phytochemical analysis of the *C. spinosa* leaves and flowers extracts (Table 1) showed the presence of different groups of secondary metabolites such as polyphenols, flavonoids, alkaloids, tannins, coumarins and saponins which are of medicinal importance. When the chemical profile of the studied plant is compared to the previously studied species, it appears similar. Aberkane et al. (2013) reported the presence of 04 flavonoids (Glucopyranosyl chrysin type) in *C. spinosa*. Furthermore, El Antri et al. (2004) showed that *C. villosa* subsp. *intermedia* collected from Morocco contains two flavonoids belonging to the same type: chrysin-7-O (ß-D glycopyranoside) and chrysin-7-O-ß-D-[(6´´-acetyl) glycopyranoside]. Then, more resent study revealed that one alkaloid as well as a paraben derivative was extracted from the same species (El Khamlichi et al., 2014).

The obtained physical and spectroscopic data are in concordance with literature values (Cheng et al., 2001; Perveen et al., 2009). Although there are many types of paraben derivatives by organic synthesis (Crombie et al., 1979; Hirota et al., 1981), methyl-4-hydroxybenzoate 2 was isolated for the first time from *C. villosa subsp. intermedia*. In addition to that, Djeddi et al. (2015) showed the presence of alkaloids, flavonoids, sterols and triterpenes in dichloromethane and methanol *C. villosa* extracts collected from Edough Mountain (Annaba, Algeria).

Total phenolics content of leaves and flowers ethyl acetate and n-butanol fractions from *C. spinosa* L. (Link)

The calibration curve was determined from all readings of gallic acid calibration standard absorbance. The correlation coefficient for standard curve exceeded 0.99 for gallic acid. Under the assay conditions, a linear relationship between the concentration of standard and the absorbance at 760 nm was obtained. Phenolic compounds such as flavonoids, phenolic acids, and tannins possess diverse biological activities, such as anti-inflamatory, anti-atherosclerotic and anti-carcinogenic activities that may be related to their antioxidant activity (Chung et al., 1998).

Thus, the total phenolics content of the four fractions of *C. spinosa* leaves and flowers was also evaluated, using the Folin-Ciocalteu method. The variation of phenolics content was quite large (Table 2). Leaves ethyl acetate fraction was found to have the highest phenolic content with 107.75±0.41 mg GAE/g, followed by flowers and leaves n-butanol fractions (96.06±2.72 and 81.45±0.60 mg GAE/g, respectively). The phenolics content of flowers ethyl acetate fraction showed also a significant content but less than the previous ones with 64.24±1.81 mg GAE/g.

The total phenolics content of leaves and flowers ethyl acetate and n-butanol fractions from *C. spinosa* analyzed with this method are shown in Table 2.

The recent study on hydromethanolic crude extracts of 20 Algerian medicinal plants reported that the amount of total phenolics, measured by Folin-Ciocalteu method, varied widely in herb materials and ranged from 3.96 to 259.65 mg GAE/g extract. The highest total phenolic content was detected in *Salix alba* cortex with 259.65 mg GAE/g extract, followed by *C. spinosa* leaves (228.42±8.86 mg GAEs/g extract),while *Ajuga iva* aerial part was the lowest one (3.96 mg GAE/g extract) (Krimat

Table 2. Total phenolic content of leaves and flowers ethyl acetate and n-butanol fractions from *C. spinosa* in 1 g extract expressed in mg GAEs.

Fraction	Total phenolic content (mg GAEs/gram extract)	
	Leaves	Flowers
Ethyl acetate	107.75±0.41	64.24±1.81
N-Butanol	81.45±0.60	96.06±2.72

The results are the mean of triplicate estimation ± standard error.

Table 3. Total flavonoids content of leaves and flowers ethyl acetate and n-butanol fractions from *C. spinosa* in 1gram extract expressed in mg QE.

Fraction	Total flavonoids content (mg QE/1 g extract)	
	Leaves	Flowers
Ethyl acetate	20.87 ±0.13	16.30 ±0.05
N-Butanol	17.03 ±0.06	8.19 ±0.44

The results are the mean of triplicate estimation ± standard error.

et al., 2014).

It is well known that the amount of phenolic compounds vary with respect to families and varieties (Sini et al., 2010; Belmekki and Bendimerad, 2012). Furthermore, a moderate correlation between the total phenolics content and antiradical properties tested by DPPH assay was also observed (Krimat et al., 2014). According to some authors, the antioxidant capacity was not exclusively dependent on phenolics content but it may be due to other phytoconstituents or combine effect of them (Wong et al., 2006; Ho et al., 2012). Phenolic compounds have different antioxidant activity depending on their chemical structure (Tatiya et al., 2011).

Flavonoids content of leaves and flowers ethyl acetate and n-butanol fractions from *C. spinosa* (L.) Link

The calibration curve was determined from all readings of quercetin calibration standard absorbance.

Under the described assay conditions, a linear relationship between the concentration of quercetin and the absorbance at 430 nm was obtained. The correlation coefficient for standard curve exceeded 0.977 for quercetin. The total flavonoids content of leaves and flowers ethyl acetate and n-butanol fractions from *C. spinosa* in quercetin equivalents are represented in Table 3.

The highest value was obtained in leaves ethyl acetate fraction (20.87±0.13 mg QE/1 g extract) followed by leaves n-butanol fraction (17.03±0.06 QE/1 g extract). This was significantly followed by the important flavonoids content in flowers ethyl acetate fraction (16.30±0.05 mg QE/1 g extract). Although, the lowest total flavonoids content was observed in flowers n-butanol fraction with 8.19±0.44 mg QE/1 g extract.

Through the study of Krimat et al. (2014) on hydromethanolic crude extracts of 20 Algerian medicinal plants, the range for total flavonoids content was from 1.13 to 26.84 mg QE/g extract. *Rhamnus alaternus* leaves showed the highest flavonoids content while *S. alba* cortex showed the lowest one with 26.84 and 1.13 mg QE/g extract, respectively. Lower flavonoids content (4.87±0.12 mg QE/g extract) of *C. spinosa* leaves was also observed.

The relationship between the antioxidant activity and the phenolic contents, flavonoids and condensed tannin of *Retama monosperma* was evaluated by Belmokhtar et al. (2014). It was found that the antioxidant activities of the different fractions (chloroform, ethyl acetate, butanol and methanol) of the hydromethanolic extract of the different parts of this plant using DPPH method represented a significant high correlation between flavonoids content and antioxidant activities (r=0.91).

In vitro antioxidant effects of leaves and flowers ethyl acetate and n-butanol fractions from *C. spinosa* L. (Link) (DPPH method)

The free radical-scavenging activity of leaves and flowers ethyl acetate and n-butanol fractions from *C. spinosa* was determined by measuring the decrease of absorbance of DPPH free radical at 517 nm in the presence of various concentrations of extracts. The initial absorbance of DPPH decreases by increasing extracts concentration.

The antioxidant activity was determined by calculating the IC_{50}, as expressed in µg/mL. As shown in Figure 2, the IC_{50} values were 45.25±1.8 and 52.80±2.05 for leaves

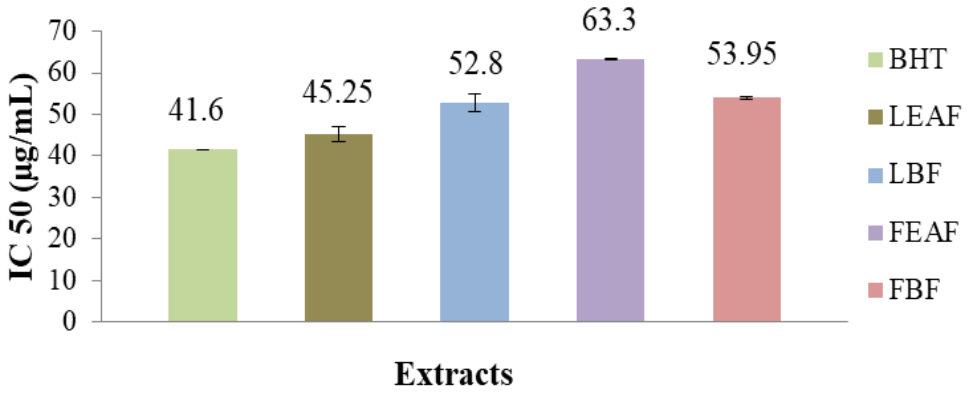

Figure 2. IC_{50} of BHT leaves and flowers ethyl acetate and n-butanol fractions from *C. spinosa*: BHT (Butylated hydroxytoluene), LEAF (leaves ethyl acetate fraction), LBF (leaves n-butanol fraction), FEAF (flowers ethyl acetate fraction), FBF (flowers n-butanol fraction).

ethyl acetate and n-butanol fractions from *C. spinosa* and 63.3±0.12 and 53.95±0.19 for flowers ethyl acetate and n-butanol fractions, respectively. These results showed that the leaves fractions are generally more active than the flowers fractions. However, a low activity of the leaf fractions of *C. spinosa* was noted compared to the activity of BHT as standard (41.06±0.01 µg/mL).

Conversely, the ethyl acetate fraction of leaves demonstrated superior activity compared with their n-butanol fraction. The results exhibited a correlation between the polyphenol contents of leaves and flowers fractions and their antioxidant activities. It was found that leaves and flowers ethyl acetate and n-butanol fractions with high free radical scavenging activity correlate well relatively to the great important content of phenolic compounds found in the corresponding fractions (107.75 ±0.41, 64.24 ±1.81 and 81.45 ±0.60, 96.06 ±2.72 mg GAE/g extract, respectively) (Table 2).

Many studies have shown a correlation between the total polyphenol contents of plants and their antioxidant abilities (Karou et al., 2005; Lamien-Meda et al., 2008; El Hajaji et al., 2010).

According to Chikhi et al. (2014), the two extracts of *C. villosa* leaves (essential oil and ethanol) showed a good antioxidant/free radical scavenging activity using DPPH method. The weakest radical scavenging capacity was exhibited by the essential oil (60%), whereas the strongest activity was exhibited by the ethanol extract (96%) at a concentration of 200 µg/ml when compared with the effect of ascorbic acid at this concentration (98.61%).

Furthermore, the hydromethanolic crude extract of *C. spinosa* leaves was tested for antioxidant activity using the DPPH assay. The results were expressed as EC_{50}, which is defined as the concentration of substrate at 50% inhibition (Krimat et al., 2014). The value of EC_{50} was important with 29. 20±0.80 µg/mL compared with the DPPH radical scavenging effect of ascorbic acid which was higher (EC_{50}=4.1 µg/mL) than all the plant species studied, except *Pistacia lentiscus* which showed no significant difference with ascorbic acid (P>0.05).

In vitro antimicrobial effects of leaves and flowers ethyl acetate and n-butanol fractions from *C. spinosa* (L.) Link

The *in vitro* antimicrobial activity was carried out on the four fractions of *C. spinosa* (leaves and flowers ethyl acetate and n-butanol) (Table 4). The dried extracts were dissolved in DMSO to obtain the required concentrations which were evaluated for their antimicrobial activities against the tested microorganisms. The result of inhibition diameters (expressed in mm) and that of micro-dilution (expressed in mg/mL) of the *C. spinosa* leaves and flowers fractions against eight microbial strains is shown in Tables 4 and 5.

The diameters of inhibition ranged from 7±0.41 to 22±0.06 mm for the leaves fractions and from 7±0.76 to 13±0.12 mm for the flowers fractions. The MIC that was obtained ranged from ≤25 to 200 mg/mL for the leaves fractions and from 50 to 200 mg/mL for the flowers fractions. These values showed that the leaves fractions are more active than that from the flowers fractions. The sensitivity of the different strains was classified by the diameter of the inhibition (Ponce et al., 2003).

The most tested strains were somewhat sensitive to the tested fractions. Chloramphenicol had a high activity on all the tested bacterial strains compared to the activity of plant extracts. The extremely sensitive bacterial strain to the tested fractions was *A. baumannii* while the least sensitive was *E. coli*. The antibacterial activity obtained with fractions of *C. spinosa* leaves could justify their use in traditional medicine such as bacterial infections.

Additionally, these results demonstrated that leaves ethyl acetate fraction were the most active of all tested

Table 4. Antimicrobial activity of *Calycotome spinosa* aerial part (leaves and flowers) fractions and determination of strain sensitivity.

Microorganism		Inhibition zone (mm)						
		Ethyl acetate (2 mg/disc)		n-Butanol (2 mg/disc)		Standards (+)Control		(-)Control
		Leaves	Flowers	Leaves	Flowers	Chloramphenicol (15 µg/disc)	Fluconazol (10 µg/disc)	DMSO
Gram +	*S. aureus*	11±0.78	13±1.08	10±1	9±0.58	40±0.07	NT	ND
	B. sibtilus	13±0.65	11±0.75	8±0.07	9±0.15	33±0.13	NT	ND
Gram -	*E. coli*	ND	ND	8±0.00	ND	28±0.34	NT	ND
	P. aeruginosa	12±0.93	7±0.76	9±1.87	7±0.41	32±0.22	NT	ND
	K. pneumonia	12±0.55	ND	10±0.88	ND	30±0.17	NT	ND
	A. baumannii	22±0.06	13±0.31	12±1.99	13±0.12	44±0.11	NT	ND
	S. abony	16±1.53	13±0.61	10±0.09	8±0.34	35±0.44	NT	ND
Yeast	*C. albicans*	ND	ND	ND	ND	NT	35.2±0.24	ND

ND: Not determined; NT: Not tested.

Table 5. Minimum inhibitory concentration (MIC) of leaves and flowers ethyl acetate and n-butanol fractions from *C. spinosa*.

Fraction	MIC (mg/ml)							
	S. aureus	*B. sibtilus*	*E. coli*	*P. aeruginosa*	*K. pneumonia*	*A. baumannii*	*S. abony*	*C. albicans*
LEAF	150	**50**	ND	50	50	**≤ 25**	**25**	ND
LBF	200	200	200	150	150	50	50	ND
FEAF	50	50	ND	200	ND	50	150	ND
FBF	150	150	ND	200	ND	50	200	ND

ND: Not determined.

fractions, particularly against *A. baumannii* and *S. abony* (22±0.06 and 16±1.53 mm, respectively), but it showed less interesting activity against *B. sibtilus*, *P. aeruginosa*, *K. pneumoniae* and *S. aureus* (13±0.65, 12±0.93, 12±0.55, 11±0.78 and 11±0.78 mm, successively).

Several studies on polyphenol point out that these metabolites have an antibacterial activity (Zhentian et al., 1999; Meng et al., 2001; Berahou et al., 2007). For leaves n-butanol fraction, a less important activity was observed against the different bacterial strains: *A. baumannii, S. aureus, S. abony, K. pneumoniae, P. aeruginosa, B. sibtilus* and *E. coli* with the following inhibition zones diameters (12±1.99, 10±1, 10±0.09, 10±0.88, 8±0.07, and 8 mm in that order).

On the other hand, the different flowers fractions (ethyl acetate and n-butanol) revealed an important activity against *A. baumannii* (13±0.31 and 13±0.12 mm, successively), however, a less important antimicrobial potential against *P. aeruginosa* (7±0.76 and 7±0.41, respectively) was observed and no activity of flowers n-butanol fraction was shown against *K. pneumoniae, E. coli* and *C. albicans.*

E. coli and *S. aureus* are recognized as food contaminants (Al-Zoreky and Nakahara, 2003). Thus, extracts from *C. spinosa* could be used as food additives or preservatives.

A previous study of Djeddi et al. (2015) on *C. villosa* plant revealed that dichloromethane (non-polar) crude extract presented a strong antimicrobial activity against *Klebseilla pneumoniae* (20.5±2.7 mm) as well as Acinetobacter spp. (15.7±1.3), a moderate antimicrobial potential against *E. coli* (12.9±0.9 mm), *P. aeruginosa* (13.1±2.3 mm), *S. marcescens* (10.2±0.3 mm) and no effect against *P. mirabilis.*

Furthermore, Loy et al. (2001) have shown that the essential oil and methanol crude extract of *C. villosa* leaves gathered in Italy were potentially very toxic and very active against several gram (±) bacteria, especially *S. aureus* ATCC 25923 (20 and 10 mm, respectively), *Bacillus lentus* B 60 (10 and 11 mm in that order), *E. coli* ATCC 25922 (15 and 10 mm), and *K. pneumoniae* 52 (12 and 10 mm).

According to Chikhi et al. (2014), the *in vitro* antimicrobial activities of essential oil and ethanol extracts of *C. villosa* showed an important activity. A high activity was observed by essential oil with antimicrobial

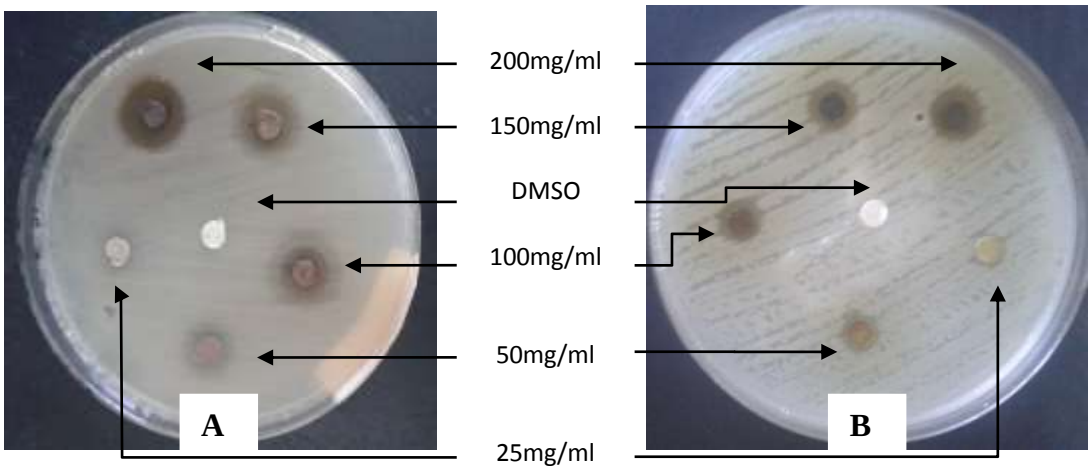

Figure 3. Leaves fractions from *C. spinosa* against *B. sibtilus* and *S. abony*; A: ethyl acetate fraction against *B. sibtilus; B:* n-butanol fraction against S. abony.

inhibition zones of 14 and 13 mm against *S. aureus* and *E. faecalis,* respectively and low activity against *K. pneumoniae* and *Salmonella typhimurium* with an antimicrobial inhibition zone of 11 mm. The ethanol extract had an average activity against two poisonous bacteria: *S. aureus* and *S. typhimurium*, with inhibition zone of 10 mm. However, *C. albicans* strain did not show any inhibition.

Additionally, the antimicrobial screening of hydromethanolic crude extracts of 20 Algerian plant species against four bacteria species (*Bacillus* species, *S. aureus*, *E. coli*, and *P. aeruginosa*) and one yeast (*C. albicans*) by Krimat et al. (2014) revealed that all the tested extracts had an antimicrobial activity showing different selectivity for each microorganism. *C. spinosa* extract was found to be active against *Bacillus* spp., *S. aureus* and *C. albicans* with different inhibition zones 7.0, 10 and 7.0 mm, respectively, while no inhibitory effect on *E. coli* and *P. aeruginosa* was observed for *C. spinosa* extract.

In general, the Gram positive bacteria were found to have more susceptibility compared to Gram negative bacteria. This is in line with earlier studies which attribute the observed differences to the variation in chemical composition and structure of cell wall of both types of microorganisms (Pirbalouti et al., 2010; Nalubega et al., 2011; Madureira et al., 2012; Sulaiman et al., 2013). Two examples of antimicrobial effect of *C. spinosa* are shown in Figure 3.

Phenolic acids composition

Phenolic compounds are known to have antioxidant properties with beneficial health effects and the composition of individual phenolic acids is correlated with antioxidant activity (Salminen et al., 2001). Therefore, it is important to investigate the composition of phenolic compounds in *C. spinosa* leaves and flowers fractions as potential antioxidants. The quantitative spectrum of phenolic acids in ethyl acetate and n-butanol fractions from leaves and flowers was determined using the HPLC system at 280 nm (Figure 4).

Ethyl acetate and n-butanol fractions from *C. spinosa* leaves and flowers, each part alone, were investigated for the presence of phenolic acids.

Eight different types of phenolic compounds (8 phenolic acids) were detected. Ascorbic, gallic, vanilinic, salicylic and coumaric acids were detected from all the four fractions; however; cafeic acid was only detected in leaves butanol fraction (LBF). On the other hand, chlorogenic acid was merely detected from the two butanol fractions (LBF and FBF) and methoxycinnamic acid was also single noticed in flowers fractions (FEAF and FBF). Previous works have demonstrated the preventive effects of chlorogenic acid against lipid peroxidation (Ohnishi et al., 1993) and also in the strongest DPPH radical scavenging activity in different *in vitro* assays compared with other hydroxycinnamic acids (Chen and Ho, 1997).

The most abundant phenolic acids were ascorbic acid (9.642%) and vanilinic acid (6.014%) in FBF, as well as methoxycinnamic acid (5.218%) in FEAF. In this study, it was possible to detect the cafeic acid in the leaves of *C. spinosa* only in butanol fraction (0.052%) and it was absent in the other fractions. It is known that these phenols (ascorbic, vanilinic, methoxycinnamic and cafeic acids) are among the best antioxidants.

In comparison with the ascorbic acid and vanilinic acid, chlorogenic acid was found in much smaller amounts in FBF (0.089%). The presence of ascorbic and vanilinic acids in high levels can be closely related to the lowest values of IC_{50} obtained for butanol fraction in the DPPH assay. It has been confirmed that ascorbic acid

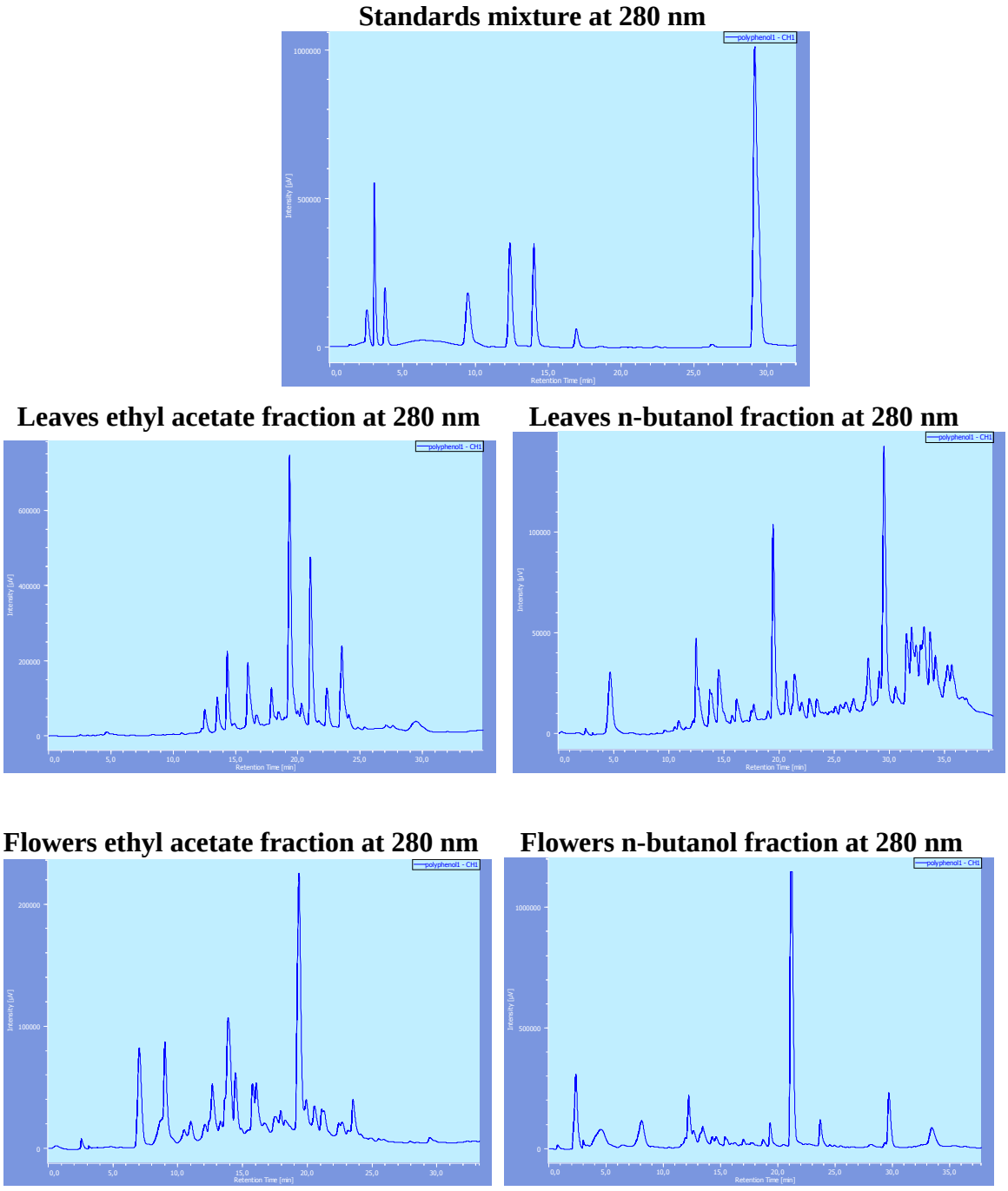

Figure 4. HPLC chromatograms of phenolic acids (standards mixture), leaves and flowers ethyl acetate and n-butanol fractions from *Calycotome spinosa* at 280 nm. Phenolic acids (1-8): 1 (ascorbic acid), 2 (gallic acid), 3 (cafeic acid), 4 (chlorogenic acid), 5 (vanillinic acid), 6 (methoxycinnamic acid), 7 (salicylic acid), 8 (coumaric acid).

possessed a higher ability for scavenging DPPH free radicals than ABTS+ free radicals in contrast to gallic and coumaric acids (Badanai et al., 2015).

The mechanism by which the phenolic acids exert their antioxidant activity is probably due to their chemical structures (Chen and Ho, 1997).

Although chlorogenic acid was found in lower concentrations in the butanol fractions of *C. spinosa* leaves and flowers, this compound is found in most plant species (Hynes and O'Coinceanainn, 2004) and a variety of studies have demonstrated the beneficial effects of chlorogenic acid on different pathophysiological effects, such as antihypertensive and antihyperglycemic effects, prevention of the development of human colon cancer

and inhibition of proliferation of tumor cells of different lines and anti-inflammatory action (Chen et al., 2009; Marrassini et al., 2010). The phenolic acids found in this study for *C. spinosa* are known to have many biological activities, which can thus be correlated with the use of this plant.

Conclusion

In frame of this work, the phytochemical screening, the phenolics and flavonoids quantification, the radical scavenging activity and antimicrobial potential of leaves and flowers fractions from *C. spinosa* were evaluated. In light of these experiments, the preliminary screening showed interesting results and indicated the antimicrobial potential of *C. spinosa*. So, it could be concluded that the different fractions (Ethyl acetate and n-butanol) have an important content of polyphenols. In a further set of experiment, it was shown that leaves ethyl acetate fraction has a very high scavenging activity (IC_{50} equal to 45.25±1.8 µg/mL) and relatively good phenolics content (107.75±0.41 mg GAE/g extract). The different fractions showed also a good antibacterial activity against *A. baumannii*; they can possibly be used as antimicrobial agents in new drugs for the therapy of infectious diseases caused by pathogens. As well, HPLC/DAD analysis performed with *C. spinosa* revealed the presence of eight phenolic compounds and the butanol fractions showed larger number of phenolics. These results confirmed that *C. spinosa* aerial part, usually employed in traditional medicine of Algeria can be regarded as a source of very efficient antioxidant compounds, and moreover this activity could explain their therapeutic and preventive usefulness.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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