SUPPLEMENTARY MATERIAL

Antioxidant and antibacterial activities and polyphenolic constituents of *Helianthemum sessiliflorum* Pers.

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In this study the various extracts of aerial parts of *Helianthemum sessiliflorum* Pers. were examined *in vitro* for possible source of antioxidants and for antibacterial activity. The antioxidant activity was performed by DPPH radical scavenging method which showed that ethyl acetate extract possessed the best antioxidant potential ($IC_{50} = 32.75 \pm 2.07 \mu\text{g/ml}$). The significant linear correlation was realized between the values of the total phenolic/flavonoid content and antioxidant activity of plant extracts. The ethyl acetate and *n*-butanol extracts showed moderate antibacterial activity. In addition, the phytochemical study of *n*-butanol extract afforded nine known phenolic compounds (**1-9**). This is the first report of six of them (**1, 3, 5-8**) in Cistaceae family. The structural identification of the isolated compounds was achieved using several spectroscopic methods.

Keywords: *Helianthemum sessiliflorum*, Cistaceae, polyphenol, antioxidant activity, antibacterial activity, NMR

Experimental

General experimental procedures

The separation and purification of *n*-butanol extract were realised using column chromatography (SiO₂: 320–400 mesh, Merck, Polyamide: SC-6 and Sephadex LH-20 (25–100 µM). The TLC and preparative TLC used in this study were Silica gel (Kieselgel 60 F₂₅₄, Merck) and RP-18 reversed-phase, detection at 254 and 366 nm. The structures of isolated compounds were established using several spectroscopic methods including NMR experiments (Bruker-Avance-600 Spectrometer; δ in ppm rel. to Me₄Si as internal standard, *J* in Hz), UV spectra (Beckman DU-600 spectrophotometer; λ_{max} in nm), optical rotation measurement (Perkin-Elmer 241 polarimeter) and MS spectra were obtained by (ESI-MS ion trap Bruker Esquire, EI-MS Bruker Micromass Q-TOF). Spectrophotometer used in biological study was a Perkin-Elmer (Lambda 950).

Preparation of plant extracts

The aerial parts of *H. sessiliflorum* were collected on May 2011 in Biskra (south of Algeria) and identified by Prof. Bachir Oudjhih of Agronomic Institute of Batna-1 University (Algeria) where a voucher specimen number (664/LCCE) was deposited. Air-dried and powdered aerial parts of *H. sessiliflorum* (1 kg) were extracted three times with 70% ethanol (10 L each) for 24 h at room temperature. The ethanol extract was suspended in water and then successively partitioned with cyclohexane (3 × 150 ml), ethyl acetate (3 × 150 ml) and *n*-butanol (3 × 150 ml).

Phytochemistry study of n-butanol extract

The *n*-butanol extract (8.89 g) was chromatographed on a Polyamide column eluted with H₂O/MeOH (100-0 → 0-100) to give five main fractions (F1 → F5). Fraction F1 (40 mg) was chromatographed on SiO₂ column (CHCl₃/MeOH: 100-0 → 0-100) to give several free sugars. Fraction F3 (100 mg) was fractionated on Polyamide column with Toluene/MeOH (97-3, 95-5, 90-10, 85-15, 80-20, 75-25, 70-30, 60-40, 50-50) as system of elution. The fraction F3-3 (50 mg) was subjected to Polyamide column with the same eluent system than Sephadex column (CHCl₃/MeOH: 70-30) to lead compound **1** (8 mg). Fraction F4 (292 mg) was also submitted to CC Polyamide (Toluene/MeOH : 100-0 → 0-100) and afforded five fractions. The first fraction F4-1 (30 mg) gave after purification on CC Polyamide (Toluene/MeOH: 100-0, 99-1, 97-3, 95-5, 90-10, 85-15,

80-20, 70-30, 60-40, 50-50, 40-60) compounds **2** (10 mg) and **3** (13 mg). The second fraction F4-2 (55 mg) provided compounds **4** (12.5 mg) and **5** (9 mg) after separation on Polyamide CC (Toluene/MeOH : 100-0 → 0-100), then on Sephadex CC using MeOH as eluent. The last fraction F4-5 (20 mg) was subjected to prep. TLC (SiO₂) using CHCl₃/MeOH/H₂O (7-3-0.5) to obtain compound **6** (5 mg). Purification of fraction F5 (550 mg) over Polyamide CC with Toluene/MeOH (100-0 → 0-100) allowed the isolation of compound **7** (12 mg). Fraction F2 (50 mg) was submitted to CC Polyamide with solvent system Toluene/MeOH (100-0 → 50-50), then CC Sephadex with MeOH as eluent and followed by prep. TLC (RP-18) eluted with MeOH/H₂O (4-6) to give 8 mg of compounds **8** and **9**.

Total phenol content (TPC)

The concentration of phenolic compounds in plant extracts was determined using Folin-Ciocalteu method (Singleton et al. 1999). 0.5 ml of methanolic solution of various extracts were mixed with Folin Ciocalteu reagent (2.5 ml, 1:10 diluted with distilled water) for 5 min and aqueous Na₂CO₃ (2.5 ml, 7.5 %) were then added. The mixture was allowed to stand for 45 min in thermostat at 45 °C and the phenols were determined by colorimetric method at 765 nm. The standard curve was prepared using different concentrations of gallic acid in methanol (20-200 µg/ml) and calibration line was constructed (r = 0.988). Total phenolic content was expressed in terms of micrograms of gallic acid equivalents per milligrams of dry extract.

Total flavonoid content (TFC)

Total flavonoid content in *H. sessiliflorum* extracts was measured by the aluminium chloride spectrophotometric assay (Zhishen et al. 1999). The sample contained 2 ml of methanol solution of the extract in the concentration of 2 mg/ml and 2 ml of 4% AlCl₃ solution dissolved in methanol. The samples were incubated for an hour at room temperature. The standard curve was prepared using the standard solution of quercetin in methanol (0.5-5 mg) and the absorbance was determined using spectrophotometer at 415 nm for constructing a calibration line (r = 0.989). Total flavonoid content of the different extracts was expressed in micrograms of quercetin equivalents per milligrams of extract.

Evaluation of antioxidant activity

The ability of the plant extracts to scavenge *in vitro* 1,1-diphenyl-2-picryl-hydrazyl (DPPH)-free radicals was assessed by the standard method, adopted with suitable modifications (Kumarasamy et al. 2007). The solution of extracts was prepared in methanol to achieve the concentration of 2 mg/ml. Diluted solutions were mixed with 2 ml of methanolic solution of DPPH in concentration of 2 mg/ml. These solution mixtures were kept in dark for 30 min at room temperature and optical density was measured at 517 nm. The same procedure was repeated for the standard solution of quercetin. Percentage of inhibition was calculated using equation:

$$\% \text{ inhibition} = [1 - (\text{Abs sample} / \text{Abs control})] \times 100$$

Linear graph of concentration versus percentage inhibition was prepared and IC₅₀ values (micromolar concentration required to inhibit DPPH radical formation by 50%) were calculated. The antioxidant activity of each sample was expressed in terms of IC₅₀.

Evaluation of antibacterial activity

In vitro antibacterial activity of different extracts was investigated by the disk diffusion method (Bauer et al. 1996) using eight bacterial species belonging to four different groups: bacterial banal (*Streptococcus faecalis* ATCC 14504 and *Escherichia coli* ATCC 25922), opportunistic pathogenic bacteria (*Salmonella typhimurium* ATCC 14028 and *Aeromonas hydrophyla* ATCC 7966), infectious pathogenic bacteria (*Salmonella gallinarum* ATCC 9184 and *Salmonella cholera*) and exotoxic pathogenic bacteria (*Staphylococcus aureus* ATCC 25923 and *Bacillus cereus* ATCC 14579) (Tortora et al. 2004) collected from antibacterial laboratory of hospital public institution of Djelfa, Algeria. Different concentrations of plant extracts (50, 100, 200, 300, 400, 500, 600, 700 mg/ml) were prepared using DMSO as solvent. The sterile filter paper discs (6 mm diameter) were saturated by 20 µl of different concentrations of each extract and then were placed on lawn cultures prepared. Similarly, each plate carried a blank disk by adding DMSO alone to serve as a negative control, and antibiotic disk (6 mm diameter) was also used as positive control. The Petri dishes were subsequently incubated at 37 °C for 24 h and the inhibition zone around each disc was measured in mm.

Statistical analysis

Data are expressed as mean $\pm$ standard derivation from three separate observations. For antioxidant assays, one way ANOVA test ($p < 0.05$) was used to analyze the differences among IC₅₀ of various extracts. The correlation and linear regression analysis were performed using Microsoft Office Excel 2007.

References

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8 and 9

Figure S1. Structures of isolated compounds **1-9**

Table S1. Total phenolic and flavonoid contents in the plant extracts

Extract	TPC (μg of GAE/mg of extract)	TFC (μg of QE/mg of extract)
Water	7.35 ± 0.42	5.50 ± 0.19
<i>n</i> -butanol	40.02 ± 2.81	35.48 ± 0.36
Ethyl acetate	42.51 ± 1.01	46.70 ± 0.22
Cyclohexane	1.31 ± 0.84	0.23 ± 0.62

Each value is expressed as mean $\pm$ standard error (n=3)

Table S2. The IC₅₀ values of plant extracts of DPPH radical scavenging assay

Extract	IC ₅₀ values ($\mu\text{g/ml}$)
Water	427.51 ± 3.14
<i>n</i> -butanol	94.03 ± 1.52
Ethyl acetate	23.75 ± 2.07
Cyclohexane	637.66 ± 9.01

Each value is expressed as mean $\pm$ standard error (n=3)

Table S3. Correlations between the IC₅₀ values of DPPH assay, phenolic and flavonoid contents of plant extracts

Extract	Correlation (R^2) TPC and DPPH	Correlation (R^2) TFC and DPPH
Water	0.4031	0.3517
<i>n</i> -butanol	0.3172	0.3834
Ethyl acetate	0.9627	0.9598
Cyclohexane	0.1002	0.1253

Figure S2. Antibacterial activity of plant extracts against some clinical bacterial pathogens

Compound 1: isolaricireinol 9'-O- β -D-glucopyranoside (Wen et al. 2012)

Figure S3. Mass spectrum ESI-MS of compound 1

Figure S4. ^{13}C -NMR spectrum of compound 1 (150 MHz, CD_3OD)

Figure S5. ^1H -NMR spectrum of compound 1 (600 MHz, CD_3OD)

Compound 2: Tiliroside (Gomes et al. 2011)

Figure S6. Mass spectrum ESI-MS of compound 2

Figure S7. ¹³C-NMR spectrum of compound 2 (150 MHz, CD₃OD)

Figure S8. ¹H-NMR spectrum of compound 2 (600 MHz, CD₃OD)

Compound 3: Nicotiflorin (Han et al. 2004)

Figure S9. Mass spectrum ESI-MS of compound 3

Figure S10. ^{13}C -NMR spectrum of compound 3 (150 MHz, CD_3OD)

Figure S11. ^1H -NMR spectrum of compound 3 (600 MHz, CD_3OD)

Compound 4: Kaempferol 3-*O*-vicianoside (Sohretoglu et al. 2011).

Figure S12. Mass spectrum ESI-MS of compound 4

Figure S13. ^{13}C -NMR spectrum of compound 4 (150 MHz, CD_3OD)

Figure S14. ^1H -NMR spectrum of compound 4 (600 MHz, CD_3OD)

Compound 5: Rutin (Riehle et al. 2013).

Figure S15. Mass spectrum ESI-MS of compound 5

Figure S16. ¹³C-NMR spectrum of compound 5 (150 MHz, CD₃OD)

Figure S17. ¹H-NMR spectrum of compound 5 (600 MHz, CD₃OD)

Compound 6: Neoisorutin (Krenn et al. 2003).

Figure S18. Mass spectrum ESI-MS of compound 6

Figure S19. ¹³C-NMR spectrum of compound 6 (150 MHz, CD₃OD)

Figure S20. ¹H-NMR spectrum of compound 6 (600 MHz, CD₃OD)

Compound 7: Vicenin-2 (Xie et al. 2003).

Figure S21. Mass spectrum ESI-MS of compound 7

Figure S22. ^{13}C -NMR spectrum of compound **7** (150 MHz, CD_3OD)

Figure S23. ^1H -NMR spectrum of compound 7 (600 MHz, CD_3OD)

Compounds 8 and 9: Hovetrichosid C (Yoshikawa et al. 1998).

Figure S24. Mass spectrum ESI-MS of compounds 8 and 9

Figure S25. ^1H -NMR spectrum of compounds 8 and 9 (600 MHz, CD_3OD)