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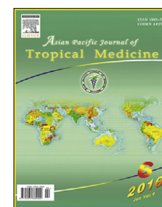

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Chemical constituents, cytotoxic, antifungal and antimicrobial properties of *Centaurea diluta* Ait. subsp. *algeriensis* (Coss. & Dur.) Maire

Hanène Zater[1,2,3], Joëlle Huet[4], Véronique Fontaine[5], Samir Benayache[1], Caroline Stévigny[3], Pierre Duez[3,6*], Fadila Benayache[1*]

[1]*Unité de recherche: Valorisation des Ressources Naturelles, Molécules Bioactives et Analyses Physicochimiques et Biologiques (VARENBIOMOL), Faculté des Sciences Exactes, Université Frères Mentouri Constantine 1, 25000 Constantine, Algeria*

[2]*Université Ziane Achour, Cité du 5 Juillet, Route Moudjbara BP: 3117, 17000 Djelfa, Algeria*

[3]*Laboratoire de Pharmacognosie, de Bromatologie et de Nutrition Humaine, Université Libre de Bruxelles (ULB), 1050 Bruxelles, Belgium*

[4]*Laboratoire de Biopolymère et Nanomatériaux Supramoléculaire, Université Libre de Bruxelles (ULB), 1050 Bruxelles, Belgium*

[5]*Unité de Microbiologie Pharmaceutique et Hygiène, Faculté de Pharmacie, Université Libre de Bruxelles (ULB), 1050 Bruxelles, Belgium*

[6]*Service de Chimie Thérapeutique et de Pharmacognosie, Université de Mons (UMONS), 7000 Mons, Belgium*

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ABSTRACT

Objective: To investigate the chemical composition of a moderately polar extract ($CHCl_3$ soluble part of the MeOH–H_2O extract) obtained from the aerial parts (leaves and flowers) of *Centaurea diluta* Ait. subsp. *algeriensis* (Coss. & Dur.) Maire, a species endemic to Algeria and Morocco on which no reports are available to date. To evaluate *in vitro* the cytotoxic, antifungal and antimicrobial activities of this extract and the cytotoxic and antimicrobial activities of its isolated secondary metabolites.

Methods: The cytotoxic effects of the extract were investigated on 3 human cancer cell lines i.e. the A549 non-small-cell lung carcinoma (NSCLC), the MCF7 breast adenocarcinoma and the U373 glioblastoma using a MTT colorimetric assay. Biological data allowed to guide the fractionation of the extract by separation and purification on silica gel 60 (CC and TLC). The isolated compounds which were characterized by spectral analysis, mainly HR-ESIMS, HR-EIMS, UV and NMR experiments (^1H, ^{13}C, COSY, ROESY, HSQC and HMBC) and comparison of their spectroscopic data with those reported in the literature, were evaluated for cytotoxic activities on six cancer cell lines (A549, MCF7, U373, Hs683 human glioma, PC3 human prostate and B16-F10 murine melanoma). The direct and indirect antibacterial and antifungal activities were determined using microdilution methods for the raw extract and TLC-bioautography and microdilution methods against standard and clinical strains for the isolated compounds.

Results: The raw extract reduced cell viability with IC_{50}s of 27, 25 and 21 μg/mL on A549, MCF7 and U373, respectively. Five secondary metabolites: two phenolic compounds (vanillin **1**, paridol **3**), a lignan [(−)-arctigenin **2**] and two flavonoid aglycones (eupatilin **4** and jaceosidin **5**), were then isolated from this extract. Moderate cytotoxic effects were observed for (−)-arctigenin **2** (IC_{50}s: 28 and 33 μM on Hs683 and B16-F10, respectively), eupatilin **4** (IC_{50}s: 33 and 47 μM on B16-F10 and PC3, respectively) and jaceosidin **5** (IC_{50}s: 32 and 40 μM on PC3 and B16-F10, respectively).

Conclusions: All the isolated compounds were described for the first time from this species. Although inactive against 7 tested microorganisms (fungi, bacteria and yeast, human or plant pathogens), the raw extract was able to potentiate the effect of beta-lactam

*Corresponding authors. Pierre Duez, Laboratoire de Pharmacognosie, de Bromatologie et de Nutrition Humaine, Université Libre de Bruxelles (ULB), 1050 Bruxelles, Belgium; Fadila Benayache, Unité de recherche: Valorisation des Ressources Naturelles, Molécules Bioactives et Analyses Physicochimiques et Biologiques (VARENBIOMOL), Faculté des Sciences Exactes, Université Frères Mentouri Constantine 1, 25000 Constantine, Algeria.

Tel/Fax: +213 31 81 11 03

E-mails: pierre.duez@umons.ac.be, fbenayache@yahoo.fr

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antibiotics on methicillin-resistant *Staphylococcus aureus* (MRSA), reducing the minimal inhibitory concentrations (MICs) by a factor of 2-32-fold. No synergy was found between the extract and streptomycin. From the five isolated compounds only jaseosidin **5** showed a moderate antimicrobial activity.

1. Introduction

The genus *Centaurea* (tribe Cynareae, family Asteraceae) is one of the most widely distributed plant genera in the world. *Centaurea* includes more than 500 species, 45 of which grow spontaneously in Algeria, with 7 species localized in the Sahara [1,2]. Although, to our best knowledge, no traditional uses or pharmacological studies are reported so far for the species *Centaurea diluta* (*C. diluta*), many other *Centaurea* species are well known in traditherapy. For example, in Turkey, dried flowers of *Centaurea cyanus* are used in infusion to relieve diarrhea, gain energy, increase appetite, and to relieve chest tightness; *Centaurea calcitrapa* is used (infusion) as a febrifuge; *Centaurea jacea* is used to reduce fever, to start menstruation, to relieve constipation and increase appetite [3,4]. In Tunisia, *Centaurea furfuracea*, an endemic species from the desert regions of the North of Africa [5], is used as astringent and diuretic [6], while, in Algeria, the roots of *Centaurea incana* are used in the area of Aurès for the treatment of liver diseases [7] and *Centaurea pullata* is used in the preparation of a local traditional dish called “El Hammama” [8]. Various studies have shown medicinal properties of *Centaurea* species, mainly as analgesic [9], cytotoxic [10], antibacterial [11] and antifungal [12].

Centaurea typically present high structural diversity in major bioactive compounds, including triterpenes, flavonoids, lignans and sesquiterpene lactones [13–21]. In specimens of *C. diluta*, cultivated in the botanical garden of Technical University of Braunschweig, Germany, polyacetylenic compounds have been reported [22–24]. In the essential oil of *C. diluta* Aiton aerial parts, collected from Sicily, Italy [25], the most abundant compounds were fatty acids and derivatives, notably hexadecanoic acid (21.3%) and (Z,Z)-9,12-octadecadienoic acid methyl ester (12.2%), followed by hydrocarbons (15.3%), terpenoids being present in low amounts (2.8%).

Given the interest of *Centaurea* pharmacology and phytochemistry, the present paper concentrates on a relatively unknown subspecies, *C. diluta* Ait. subsp. *algeriensis* (Coss. & Durieu) Maire [26], endemic to Algeria and Morocco [2].

2. Material and methods

2.1. Chemicals, reagents and general

Solvents were analytical grade. Trypsin 0.5% in EDTA, RPMI1640 red phenol and fetal bovine serum (FBS) were purchased from Gibco® Invitrogen (Merelbeke, Belgium). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was obtained from Sigma Aldrich® (Bornem, Belgium). Dimethyl sulfoxide (DMSO) was obtained from Merck® (Overijse, Belgium). RNAse-free water was from Braun® (Machelen, Belgium). The Penicillin V was purchased from Certa SA ACA Pharma NV, the ampicillin, amoxicillin and oxacillin were purchased from Sigma–Aldrich.

The absorbance of the reaction mixture of MTT test was measured by spectrophotometer microplate reader Model 680XR, Bio-Rad®, Nazareth Eke (Belgium). The cells were counted by Cells Culture Counter, Beckman (Analis®, Suarlée, Belgium). The following apparatus were also used: optical microscope PCM-type Axiovert S100 (Zeiss, Nederlands) and laminar flow hood class II (IKS®, Leerdam, Nederlands).

Melting points were determined on a SMP10 Büchi B-540 Stuart Biocote apparatus and are uncorrected. Plant material powdering: Mill: Culatti, CZ13 model, Reference DCFH48. TLC: pre-coated aluminum foil silica gel 60 F_{254} & TLC silica gel 60 F_{254} Plastic roll 500 × 20 cm (Merck KGaA, Germany), visualized using UV lamp (CAMAG 254 nm & 366 nm) and by detection with a spraying reagent (vanillin-sulfuric at 10% and/or anisaldehyde) followed by heating at 100 °C for 3–5 min. Column chromatography (CC): silica gel 60 (Merck KGaA, Germany, 230–400 mesh ASTM). Routine preparative thin-layer chromatography (PLC): silica gel plates (20 × 20 cm Silica gel 60 PF_{254}, Merck), Optical rotation: Perkin–Elmer 241 polarimeter at λ_{Na} 589 nm.

UV spectra were recorded using a Thermo Electron Corporation evolution 300 spectrophotometer. ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker Avance 300, 400 MHz and Varian 600 MHz; 2D-NMR experiments (COSY, HSQC, HMBC, NOESY and ROESY) were performed on Bruker Avance 400 MHz or Varian 600 MHz spectrometers. Spectra of compounds **1**, **2** and **3** were recorded in $CDCl_3$, compound **4** in DMSO-d_6 and compound **5** in CD_3OD. A Shigemi tube was used for compound **2**.

High resolution mass spectra in positive mode were recorded by direct infusion using a 6520 series quadrupole time-of-flight (Q-TOF) mass spectrometer (Agilent, Palo Alto, CA, USA) fitted with an electrospray ionization (ESI) source in positive mode. The error between the observed and calculated masses is expressed in ppm; below 5 ppm, the compounds were considered to correspond to predicted formula.

2.2. Plant material

The aerial parts of *C. diluta* Ait. subsp. *algeriensis* (Coss. & Dur.) were collected in the flowering stage in the area of Djelfa (1 038 m, 34° 53′ 39.6″ N, 3° 3′ 56.3″ E) in June 2012. The plant was authenticated by Professor Mohamed Kaabache, specialist in the identification of Algerian *Centaurea* species (Ferhat Abbas University, Setif, Algeria). A voucher specimen has been deposited in the National Herbarium of Belgium (National Botanical Garden of Meise) under the number BR0000013666187.

2.3. Extraction and isolation

Air-dried aerial parts (leaves and flowers, 1.5 kg) of *C. diluta* Ait. subsp. *algeriensis* (Coss. & Dur.) were powdered (slight grinding with controlled temperature, up to 35 °C) and macerated at room temperature with MeOH–H_2O (77:23, v/v) (25 L) for 48 h, four times. The filtrates were combined, concentrated under reduced pressure, diluted in H_2O (600 mL) under magnetic stirring and maintained at 4 °C for one night to precipitate a maximum of chlorophylls. After filtration, the resulting solution was successively extracted with solvents with increasing polarities (petroleum ether, chloroform, ethyl acetate and *n*-butanol) [27,28]. The present study focused on the chloroform soluble part which was dried with anhydrous Na_2SO_4, filtered and concentrated under vacuum at room temperature to yield the $CHCl_3$ extract (4.0 g, yield: 0.27%, w/w). The chloroform extract was fractionated by column chromatography (120 g of silica gel; CH_2Cl_2/EtOAc/MeOH step gradients) to yield 23 fractions (F_1–F_{23}), combined according to their TLC profiles.

Fraction F_3 (26.2 mg) (CH_2Cl_2/EtOAc 98:2) was subjected to preparative TLC on silica gel; eluting with petroleum ether/EtOAc/acetone (6:3:1) yielded vanillin **1** as white crystals (3.5 mg) [29,30].

Fractions F_4 (12.2 mg) (CH_2Cl_2/EtOAc 98:2), F_5 (13.0 mg) (CH_2Cl_2/EtOAc 98:2) and F_6 (28.5 mg) (CH_2Cl_2/EtOAc 95:5) were combined and rechromatographed by CC (600 mg of silica gel; cyclohexane/EtOAc/acetone step gradients) to yield 21 subfractions (F'_1–F'_{21}) according to TLC profiles. Subtraction F'_3 (cyclohexane/EtOAc/acetone 6:6:2) yielded (−)-arctigenin **2** (3.2 mg) [17,31] and subtraction F'_4 (cyclohexane/EtOAc/acetone 5:2.5:2.5) gave paridol **3** (4.5 mg) [32,33].

Fraction F_8 (CH_2Cl_2/EtOAc 95:5) (44.4 mg) yielded up on concentration a yellowish compound which was washed with MeOH to obtain eupatilin **4** (7.5 mg) as needles [11,34]. Fraction F_{10} (CH_2Cl_2/EtOAc 87.5:12.5) (19.60 mg) was chromatographed on preparative plates of silica gel eluted with CH_2Cl_2/EtOAc (4:1) to give jaceosidin **5** as a yellowish powder (5.0 mg) [35,36].

2.4. Cell cultures

The human cancer cell lines included the A549 (Deutsche Sammlung von Mikroorganismen und Zellkulturen, DSMZ code ACC107), NSCLC carcinoma, the U373 (European Collection of Cell Culture, ECACC 08061901) glioblastoma, the PC3 prostate carcinoma (DSMZ code ACC465), the Hs683 glioma (American Type Culture Collection, ATCC code HTB-138) and the MCF7 (DSMZ code ACC115) breast adenocarcinoma. The murine tumor cell line included the B16-F10 (American Type Culture Collection ATCC code CRL-6475) melanoma.

2.4.1. Viability assay

The cytotoxic properties of the raw chloroform extract and isolated compounds were assessed, using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [37,38]. Briefly, this test is based on the capability of living cells to reduce the yellow MTT to a blue formazan compound, a reaction mediated by the mitochondrial succinate dehydrogenase. Cells were seeded (cells per well, A549: 1 500; B16F10: 1 000; Hs683: 1 500; MCF7: 2 800; PC3: 3 000; U373: 1 800) and allowed to adhere for 24 h before adding test compounds (100 µL; final concentrations from 10^{-4} M to 10^{-8} M). In the same condition, for the chloroform extract, cells were seeded (cells per well, A549: 1 200; MCF7: 2 500; U373: 1 800; 100 µL; final concentrations from 100 µg/mL to 10^{-2} µg/mL). The cells in medium alone without drug were considered as a negative control. After 72 h contact, the culture medium was replaced by a 0.5 mg/mL MTT solution in RPMI medium without phenol red (100 µL/well). After 3 h incubation, the formazan crystals were centrifuged and dissolved in 100 µL/well of DMSO. The absorbance of each well was then measured at 570 nm and 690 nm (reference) wavelength. The IC_{50} values were calculated as follows:

$$IC_{50} = [(X_2 - X_1) \times (50 - Y_1)/(Y_2 - Y_1)] + X_1, \text{ where.}$$

X_1 and X_2: are the higher and lower concentrations that border the concentration that reduces the global cell growth by the value closest to 50%.

Y_1 and Y_2: are the mean percentages of viable cells at the X_1 and X_2 concentrations.

2.5. Antimicrobial and antifungal assays

2.5.1. Microorganisms

The microorganisms used in the antimicrobial tests were: (1) Gram-positive bacteria: *Staphylococcus aureus* ATCC 6538 (*S. aureus* ATCC 6538), *Staphylococcus aureus* C98506 (*S. aureus* C98506), *Staphylococcus aureus* C100459 (*S. aureus* C100459) and *Staphylococcus aureus* ATCC 33591 (*S. aureus* ATCC 33591); (2) Gram-negative bacteria: *Escherichia coli* ATCC 25922 (*E. coli* ATCC 25922) and a plant pathogen: *Pseudomonas syringae* DC 3000; (3) plant pathogen fungi: *Fusarium oxysporum*, *Fusarium oxysporum* sporulent, *Cladosporium cucumerinum*, *Botrytis cinerea*, *Colletotrichum lagenarium* and *Pythium aphanidermatum*; and (4) a plant pathogen yeast: *Rhodotorula aurantiaca*. The ATCC strains were obtained from the American Type Culture Collection; strains C98506 and C100459 were clinical isolates, a generous gift from the Centre Hospitalier Universitaire of Charleroi, Belgium (Mr. Lerson). Strains C98506, C100459 and ATCC 33591 are methicillin-resistant *Staphylococcus aureus* (MRSA). The different plant pathogens were provided by the Centre Wallon de Biologie Industrielle, Bio-Industrie Unité Gembloux Agro-Bio Tech, Université de Liège, 5030 Gembloux, Belgique (Dr. Ongena).

2.5.2. Direct and indirect antimicrobial effects

Direct and indirect antibacterial effects were evaluated by a broth microdilution method [39].

The raw extract and isolated compounds, dissolved in DMSO, were further diluted in Mueller Hinton broth (MHB), the final DMSO concentration being maximum 4%. These solutions were transferred into 96-wells plates and serially diluted using MHB. The bacterial inoculum prepared from an overnight culture, diluted in 0.85% NaCl to achieve 0.5 Mc Farland (10^8 cells/mL), was further diluted 1/100 to be inoculated in the 96-wells plates (100 µL/well). The plates were incubated at 37 °C for 24 h, added with an aqueous solution of MTT (0.8 mg/mL) and reincubated for 4 h. The minimum inhibitory

concentrations (MIC) were the lowest concentrations that completely inhibited the growth of microorganisms, detected by unaided eyes using the MTT staining.

2.5.3. Direct and indirect antibacterial bioautography

TLC was performed for the extract and the purified compounds on precoated silica gel 60 F_{254} glass plates (Merck, Darmstadt, Germany). Plates were thoroughly dried at room temperature. One mL of 0.5 Mc Farland microorganism suspension was added to 9 mL MH agar (10^7 CFU/mL) at 37 °C and poured on the TLC plates. After solidification, the plates were incubated overnight at 37 °C. The bioautography was subsequently visualized by spraying MTT (0.8 mg/mL) followed by an additional incubation at 37 °C for 4 h [40].

To study indirect antibacterial activity against MRSA, a sub-inhibitory concentration of penicillin V (1 μg/mL) was incorporated in the mixture of MHB and agar; products with no direct antibacterial activity were selected, chromatographed, and bioautographed with this medium as described above.

3. Results

3.1. Structural elucidation of compounds **1–5**

The structures of the isolated compounds were established by spectral analysis, mainly UV–Vis, HRESI-MS, ^{1}H-, ^{13}C-, and 2D-NMR (COSY, ROESY, HSQC and HMBC) as well as by comparing their spectroscopic data with those reported in the literature.

Vanillin **1**: White crystals; MP = 82 °C; UV (MeOH) λ_{max}(nm): 230, 279, 309; HRESI-QTOF-MS (positive mode) *m/z*: 153.0545 $[M+H]^+$ (calculated for $C_8H_9O_3$: 153.0546), 175.0372 $[M+Na]^+$ (calculated for $C_8H_8O_3Na$: 175.0366), 191.0216 $[M+K]^+$ (calculated for $C_8H_8O_3K$: 191.0105), measured exact mass: 152.0471 (calculated for $C_8H_8O_3$: 152.0473), molecular formula $C_8H_8O_3$;^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm, *J*/Hz): 9.83 (1H, s, H-7), 7.44 (1H, d, *J* = 1.8 Hz, H-2), 7.41 (1H, dd, *J* = 9.0, 1.8 Hz, H-6), 7.03, (1H, d, *J* = 9.0 Hz, H-5), 6.62 (1H, brs, 4-OH), 3.98 (3H, s, OCH_3-3); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 190.86 (C, C-7), 151.62 (C, C-4), 147.05 (C, C-3), 130.33 (C, C-1), 127.45 (CH, C-6), 114.04 (CH, C-5), 108.75 (CH, C-2), 56.40 (CH_3, OCH_3-3).

(−)-Arctigenin **2**: White powder; MP = 103 °C; $[\alpha]_D^{20} = -17.27°$ (EtOH, *c*, 0.145); HRESI-QTOF-MS (positive mode) *m/z*: 373.1655 $[M+H]^+$ (calculated for $C_{21}H_{25}O_6$: 373.1646), 395.1478 $[M+Na]^+$ (calculated for $C_{21}H_{24}O_6Na$: 395.1467), measured exact mass: 372.1569 (calculated for $C_{21}H_{24}O_6$: 372.1577). These data led to the molecular formula $C_{21}H_{24}O_6$; HRESI-QTOF-MS/MS: *m/z*:355.1556 $[M+H–H_2O]^+$ ($C_{21}H_{23}O_5$) which confirm the presence of a hydroxyl group; ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm, *J*/Hz): 6.80 (1H, d, *J* = 7.9 Hz, H-5′), 6.72 (1H, d, *J* = 8.1 Hz, H-5), 6.62 (1H, d, *J* = 1.7 Hz, H-2′), 6.59 (1H, dd, *J* = 7.9, 1.7 Hz, H-6′), 6.53 (1H, dd, *J* = 8.1, 1.7 Hz, H-6), 6.44 (1H, d, *J* = 1.7 Hz, H-2), 5.50 (1H, brs, 4′-OH), 4.12 (1H, dd, *J* = 9.0, 7.4 Hz, H-9α), 3.87 (1H, dd, *J* = 9.0, 7.6 Hz, H-9β), 3.83 (3H, s, OCH_3-3′), 3.80 (3H, s, OCH_3-4), 3.79 (3H, s, OCH_3-3), 2.92 (1H, dd, *J* = 14.1, 5.3 Hz, H-7′a), 2.89 (1H, dd, *J* = 14.1, 7.1 Hz, H-7′b), 2.61(1H, dd, *J* = 14.7, 7.4 Hz, H-7a), 2.54 (1H, m, H-8′), 2.52 (1H, m*, H-7b), 2.47 (1H, m, H-8), *: partially overlapped by the signal of H-8′; ^{13}C NMR (150 MHz, $CDCl_3$) δ (ppm): 178.96 (C, C-9′), 149.24 (C, C-3), 148.05 (C, C-3′), 146.91 (C, C-4), 144.76 (C, C-4′), 130.65 (C, C-1), 129.72 (C, C-1′), 122.32 (CH, C-6′), 120.80 (CH, C-6), 114.31 (CH, C-5′), 111.97 (CH, C-2′), 111.71 (CH, C-2), 111.48 (CH, C-5), 71.53 (CH_2, C-9), 56.12 (CH_3, OCH_3-4), 56.07 (CH_3, OCH_3-3′), 56.02 (CH_3, OCH_3-3), 46.82 (CH, C-8′), 41.14 (CH, C-8), 38.42 (CH_2, C-7), 34.74 (CH_2, C-7′). Our results which were confirmed by the analysis of the ROESY spectrum experiment complete the spectroscopic data previously reported for this molecule [31,41].

Paridol **3**: White powder; MP = 128 °C; HRESI-QTOF-MS (positive mode) *m/z*: 153.0544 $[M+H]^+$ (calculated for $C_8H_9O_3$: 153.0546), 175.0369 $[M+Na]^+$ (calculated for $C_8H_8O_3Na$: 175.0369), 343.0543 $[2M+K]^+$ (calculated for $C_{16}H_{16}O_6K$: 343.0546), measured exact mass: 152.0472, (calculated for $C_8H_8O_3$: 152.0473), molecular formula $C_8H_8O_3$.

HRESI-QTOF-MS/MS of $[M+H]^+$: 153.0547 $[M+H]^+$, 135.0239 $[M+H–H_2O]^+$, 121.0289 $[M+H–CH_3OH]^+$, these two last ions confirmed the presence of the hydroxyl and methoxyl groups in the molecule; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm, *J*/Hz): 7.95 (2H, d, *J* = 8.9 Hz, H-2 & H-6), 6.86 (2H, d, *J* = 8.9 Hz, H-3 & H-5), 5.98 (1H, brs, 4-OH), 3.88 (3H, s, OCH_3-7); ^{13}C NMR (75 MHz, $CDCl_3$) δ 167.08 (C, C-7), 159.90 (C, C-4), 132.06 (CH, C-2 & C-6), 122.93 (C, C-1), 115.33 (CH, C-3 & C-5), 52.08 (CH_3, OCH_3-7).

Eupatilin **4**: Yellow crystals; MP = 236 °C; UV (MeOH) λ_{max}(nm): 276, 340; +NaOH: 276, 320, 360 (with hypochromic effect); +$AlCl_3$: 282, 368; +$AlCl_3$ + HCl: 283, 361; +NaOAc: 276, 366; +NaOAc + H_3BO_3: 276, 357; HRESI-QTOF-MS (positive mode) *m/z*: 345.0968 $[M+H]^+$ (calculated for $C_{18}H_{17}O_7$: 345.0969), 367.0788 $[M+Na]^+$ (calculated for $C_{18}H_{16}O_7Na$: 367.0788), 383.0537 $[M+K]^+$(calculated for $C_{18}H_{16}O_7$ K: 383.0528), 689.1736 $[2M+H]^+$ (calculated for $C_{36}H_{33}O_{14}$: 689.1865), 712.1717 $[2M+Na]^+$ (calculated for $C_{36}H_{32}O_{14}Na$: 712.1718), 727.1077 $[2M+K]^+$ (calculated for $C_{36}H_{32}O_{14}$ K: 712.1424), measured exact mass: 344.0888 (calculated for $C_{18}H_{16}O_7$: 344.0896), molecular formula $C_{18}H_{16}O_7$; ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm, *J*/Hz): 13.04 (1H, s, OH-5), 7.68 (1H, dd, *J* = 8.5, 2.0 Hz, H-6′), 7.56 (1H, d, *J* = 2.0 Hz, H-2′), 7.13 (1H, d, *J* = 8.5 Hz, H-5′), 6.97 (1H, s, H-3), 6.64, (1H, s, H-8), 3.88(3H, s, 3′-OCH_3), 3.85, (3H, s, 4′-OCH_3) 3.75, (3H, s, 6-OCH_3); ^{13}C NMR (100 MHz, DMSO-d_6); δ (ppm): 182.01 (C, C-4), 163.26 (C, C-2), 157.12 (C, C-7), 152.83 (C, C-5), 151.96 (C, C-9), 149.06 (C, C-4′), 148.84 (C, C-3′), 131.11 (C, C-6), 122.83 (C, C-1′), 120.10 (CH, C-6′), 111.55 (CH, C-5′), 109.26 (CH, C-2′), 104.22 (C, C-10), 103.36 (CH, C-3), 94.33 (CH, C-8), 59.97 (CH_3, OCH_3-6), 55.88 (CH_3, OCH_3-4′), 55.76 (CH_3, OCH_3-3′).

Jaceosidin **5**: Yellowish powder; MP = 237 °C, UV (MeOH) λ_{max}: 276, 346; +NaOH: 276, 314, 360 (with hyperchromic effect); +$AlCl_3$: 282, 368; +$AlCl_3$ + HCl: 283, 361; +NaOAc: 278, 366; +NaOAc + H_3BO_3: 276, 357; HRESI-QTOF-MS (positive mode) *m/z*: 331.0811 (calculated for $C_{17}H_{15}O_7$: 331.0812), 353.0625 $[M+Na]^+$ (calculated for $C_{17}H_{14}O_7Na$: 353.0632), 661.1542 $[2M+H]^+$ (calculated for $C_{34}H_{29}O_{14}$: 661.1552), 683.1485 $[2M+Na]^+$ (calculated for $C_{34}H_{28}O_{14}Na$: 683.1371), 701.0942 $[2M+K]^+$ (calculated for $C_{34}H_{28}O_{14}K$: 701.1137), 991.2454 $[3M+H]^+$(calculated for $C_{51}H_{43}O_{21}$: 991.2291), measured exact mass: 330.0744, (calculated for $C_{17}H_{14}O_7$: 330.0740), molecular formula $C_{17}H_{14}O_7$; ^{1}H NMR

(400 MHz, CH_3OH-d_4) δ (ppm, *J*/Hz): 7.52 (1H, dd, *J* = 8.5, 2.0 Hz, H-6′), 7.50 (1H, d, *J* = 1.9 Hz, H-2′), 7.48 (1H, d, *J* = 8.5 Hz, H-5′), 6.94 (1H, s, H-8), 6.64 (1H, s, H-3), 3.96 (3H, s, OCH_3-3′), 3.88 (3H, s, OCH_3-6).^{13}C NMR (75 MHz, CH_3OH-d_4) δ (ppm): 184.21 (C, C-4), 166.36 (C, C-2), 158.92 (C, C-7), 154.83 (C, C-5), 154.70 (C, C-9), 151.70 (C, C-4′), 149.44 (C, C-3′), 132.92 (C, C-6), 123.90 (C, C-1′), 121.64 (C, C-6′), 116.91 (CH, C-5′), 110.34 (CH, C-2′), 105.58 (C, C-10), 103.59 (CH, C-3), 95.65 (CH, C-8), 61.04 (CH_3, OCH_3-6), 56.50 (CH_3, OCH_3-3′).

3.2. Biological activities

3.2.1. Cytotoxic effects

The $CHCl_3$ extract showed cell growth inhibitory activity against all 3 tested cell lines in the μg/mL range (Figure 1). These results are in agreement with previous data from an Algerian *Centaurea* species; the raw chloroformic extract of *Centaurea musimomum* (*musimonum*) Maire showed on KB cells, cytotoxic activity with growth inhibition of 89% at 10 μg/mL and 26% at 1 μg/mL [10].

The evaluation of the isolated compounds **1** to **5** indicated moderate growth inhibitory/cytotoxic activities for eupatilin **4** (33–85 μM), jaceosidin **5** (32–49 μM), and (−)-arctigenin **2** (28–82 μM) (Figure 1).

Our results showed that the chloroformic extract displayed more significant cytotoxic effects on cancer cells A549, MCF7 and U373 than the isolated pure compounds. This could be attributed to the synergetic interactions, more especially as this extract contains flavonoids for which it is thought that they may have a role to play in increasing the biological activity of other compounds by synergistic or other mechanisms [42].

3.2.2. Antifungal and antimicrobial activities

Although inactive against 7 tested microorganisms (fungi, bacteria and yeast, human or plant pathogens, Table 1), the raw extract was able to potentiate the effect of beta-lactam antibiotics on methicillin-resistant *S. aureus* (MRSA), reducing the minimal inhibitory concentrations (MICs) by a factor of 2-32-fold (Table 2). In a direct antibacterial TLC-bioautography assay, compound **5** (jaceosidin), showed the highest activity (Tables 3 and 4). This was further investigated in a direct antibacterial assay, but the activity was relatively quite low on Gram positive and negative bacteria (MIC of 200 μg/mL on MRSA C98506, MRSA C100459, MRSA ATCC 33591, MSSA ATCC 6538, *E. coli* ATCC 25922).

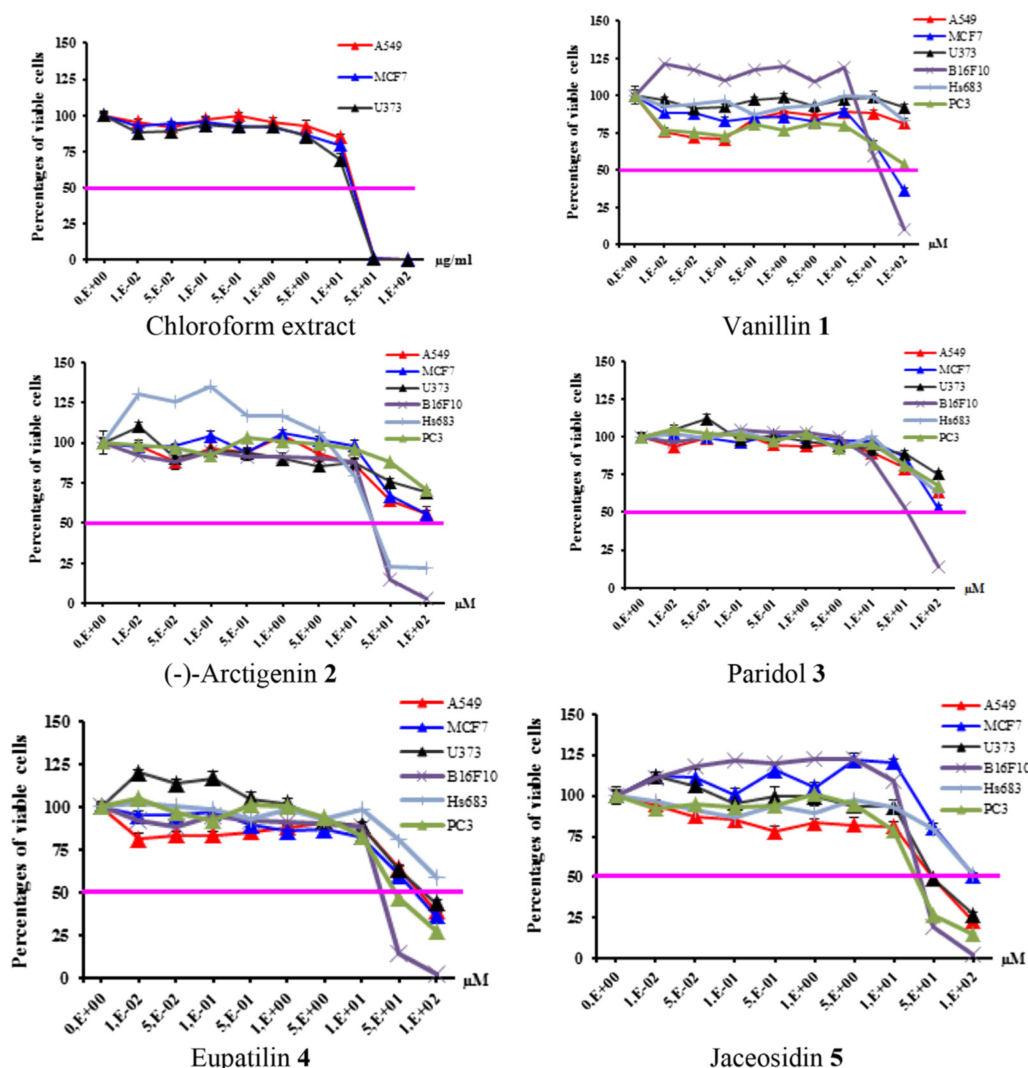

Figure 1. Cytotoxic effects (IC_{50}) of the chloroform extract and the isolated compounds on different tumor cell lines.

Table 1

MIC of the chloroform extract (μg/mL).

Microorganisms tested		MIC μg/mL
Staphylococcus aureus ATCC 6538	Gram+	2 000
Escherichia coli ATCC 25922	Gram−	2 000
Pseudomonas syringae DC 3000	Gram−	[a]
Fusarium oxysporum	Fungus	[a]
Fusarium oxysporum sporulent	Fungus	[a]
Cladosporium cucumerinum	Fungus	[a]
Botrytis cinerea	Fungus	[a]
Colletotricum lagenarium	Fungus	[a]
Rhodotorula aurantiaca	Fungus	[a]

[a] No effect at 250 μg/mL, the highest concentration tested for plant pathogens.

Table 2

Impact of the chloroform extract (200 μg/mL) on the susceptibility of the MRSA towards various beta-lactam antibiotics.

Tested mixture	MIC of the antibiotic (μg/mL)	
	MRSA C98506	MRSA C100459
Penicillin V alone (with DMSO 4%)	16	8
Penicillin V + chloroform extract (200 μg/mL)	8	2
Ampicillin alone (with DMSO 4%)	16	4
Ampicillin + chloroform extract (200 μg/mL)	8	4
Amoxicillin alone (with DMSO 4%)	16	8
Amoxicillin + chloroform extract (200 μg/mL)	4	8
Oxacillin alone (with DMSO 4%)	16	4
Oxacillin + chloroform extract (200 μg/mL)	0.5	2

4. Discussion

4.1. Phytochemical investigation

We report in this work the isolation, purification and structural elucidation of chemical components of the chloroform soluble part of the MeOH–H_2O (77%) extract obtained from the aerial parts (leaves and flowers) of *C. diluta* Ait. subsp. *algeriensis* (Coss. & Durieu) Maire (Asteraceae). No report is available so far on the phytochemistry of this species endemic to Algeria and Morocco. The present phytochemical investigation allowed the isolation of a lignan [(−)-arctigenin], flavonoids (eupatilin and jaceosidin) and phenols (vanillin and paridol). These results are in agreement with major studies reported on different *Centaurea* species [14,43–48].

4.2. Biological activities

4.2.1. Cytotoxic effects

The raw extract and the isolated compounds were evaluated for cytotoxic activity. Moderate cytotoxic effects were observed for three compounds, (−)-arctigenin **2**, eupatilin **4** and jaceosidin **5**, with IC_{50}s in the range 25–50 μg/mL. These data are in agreement with previous studies. Indeed, arctigenin (unspecified stereoisomer) as tumor specific agent that showed cytotoxicity to lung cancer (A549), liver cancer (HepG2) and stomach cancer (KATO III) cells, but not cytotoxic to several normal cell lines [49]. Arctigenin specifically inhibited the proliferation of cancer cells, which might consequently lead to the induction of apoptosis and is cytotoxic for human hepatocellular carcinoma cell lines, the IC_{50} values after 12 h, 24 h and 48 h of treatment were respectively 38.29, 1.99 and 0.24 μM [50], the highest activity was demonstrated with IC_{50} values of 0.73 μM (HeLa), 3.47 μM (MCF7) and 4.47 μM (A431) [46]. Eupatilin reduces aortic smooth muscle cell proliferation and migration by inhibiting PI3K, MKK3/6, and MKK4 activities (IC_{50}, in Hec1A and KLE cells was 82.2 and 85.5 μM) [51,52] and jaceosidin can induce G2/M cell

Table 3

Antibacterial activity of the purified compounds (**1**–**5**) measured by a direct. TLC-bioautography.

Compounds		Inhibition zone in mm MRSA C98546		Inhibition zone in mm MRSA C100459	
		Test 1	Test 2	Test 1	Test 2
Vanillin	**1**	4	4	0	0
(−)-Arctigenin	**2**	1.5	1.5	0	0
Paridol	**3**	2	2	0	0
Eupatilin	**4**	5	5	6	6
Jaceosidin	**5**	19	19	21	21

Table 4

Antibacterial activity of eupatilin **4** and jaceosidin **5**, measured by a direct. TLC-bioautography with different amounts spotted.

Compounds		Inhibition zone in mm MRSA C98546		Inhibition zone in mm MRSA C100459	
		Test 1	Test 2	Test 1	Test 2
Eupatilin	**4** (15 μg)	7	8	22	19
Eupatilin	**4** (25 μg)	6	8	17	14
Eupatilin	**4** (50 μg)	8	9	20	19
Jaceosidin	**5** (15 μg)	15	15	>21	>21
Jaceosidin	**5** (25 μg)	15	17	>21	>21
Jaceosidin	**5** (50 μg)	>21	>21	>21	>21

cycle arrest by inactivating cdc25C-cdc2 *via* ATM-Chk1/2 activation [53].

4.2.2. Antifungal and antimicrobial activities

The raw extract and the isolated secondary metabolites were evaluated for antimicrobial activity. Although the raw extract didn't show any antimicrobial effect on various bacteria or fungi, it could potentiate the effect of beta-lactam antibiotics on methicillin-resistant *S. aureus* (MRSA), reducing the minimal inhibitory concentrations (MICs) by a factor of 2-32-fold. Jaceosidin **5** showed a moderate antimicrobial activity (MIC of 200 μg/mL on MRSA C98506, MRSA C100459, MRSA ATCC 33591, MSSA ATCC 6538, *E. coli* ATCC 25922). This is in agreement with previous results [54]. Jaceosidin **5** had the greatest potency (MICs 16–32 μg/mL) against most *S. aureus* isolates [55].

The identification of five compounds, vanillin, (−)-arctigenin, paridol, eupatilin and jaceosidin, from the aerial parts (leaves and flowers) of *C. diluta* Ait. subsp. *algeriensis* (Coss. & Dur.) M. (Asteraceae) emphasized the possible relevance of this plant for Algerian traditional medicine and it is surprising that no report has been published so far on eventual ethnomedical uses of this species. This may be due to a low distribution of this species or to an eventual toxicity that could have discouraged its use in traditherapy; this warrants investigation. A promising effect on bacterial resistance needs to be further investigated to identify the compound(s) able to reverse bacterial beta-lactam resistance.

Conflict of interest statement

We declare that we have no conflict of interest.

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