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Original Article

Chemical characterization, antioxidant, tyrosinase and elastase inhibitory activities of *Colutea arborescens* aerial parts guided by chemical and biological assays

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ABSTRACT

This study presents the bio-guided chemical investigation of 80% methanol extract of aerial parts of bladder tree or *Colutea arborescens*. Liquid-liquid partitioning in solvents of increasing polarity combined with biological screening showed that the EtOAc and *n*-BuOH soluble fractions were the most active parts of the extract. These fractions were chemically profiled by a ^{13}C NMR-based dereplication method, resulting in the identification of fifteen compounds. The dereplication process was completed by purification of minor compounds of the *n*-BuOH fraction. Fourteen known compounds (**1**–**14**) were isolated, including two tri-glycosylated flavonoids containing an apiofuranose unit (**12** and **14**). Their structures were elucidated by spectroscopic methods including 1D and 2D NMR and high-resolution electrospray ionization mass spectrometry. The antioxidant activity of fractions and isolated compounds were evaluated using 2,2,1-diphenyl-1-picrylhydrazyl and hydroxyl radicals scavenging and by cupric ion reducing antioxidant capacity assays. In parallel, their inhibitory properties against mushroom tyrosinase and human neutrophil elastase enzymes were assessed. Quercetin-type flavonoids showed the best antioxidant activities and a tri-glycosylated quercetin exhibited both antioxidant and anti-elastase activities. Phenylethyl-glucoside exhibited a significant tyrosinase inhibitory activity.

KEYWORDS: *Colutea arborescens*, flavonoid, glycoside, dereplication, antioxidant, tyrosinase, elastase.

INTRODUCTION

Skin aging is a complex biological process influenced by oxidative stress and leading to matrix remodeling associated with hyperpigmentation phenomena. Prevention of these dynamic processes is a major issue for the dermo-cosmetics sector and substantial research efforts are being made to discover new protective ingredients [1-3]. Plants contain a wide range of secondary metabolites, which are commonly used as active molecules in pharmaceuticals or herbal cosmetics [4-8]. A number of plant extracts as well as purified natural compounds have been explored for the preparation of novel cosmeceutical formulations, with specific objectives such as sun protection, anti-aging, anti-wrinkling, antioxidant. Most of these plants belong to the families Asteraceae, Lamiaceae, Fabaceae, Poaceae, Malvaceae and Rosaceae [9].

Fabaceae, also known as Leguminosae, is an economically important family of flowering plants.

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A few plants from this family have cosmeceutical applications, in skin care or depigmentation formulations [9]. The papilionaceous corolla and leaves of Fabaceae species are a rich source of flavonoids, phenolic compounds, and alkaloids, which could be used to slow down skin aging [7, 10]. The genus *Colutea* (Fabaceae family, Faboideae subfamily, Galegeae tribe) comprises about thirty species, mostly occurring in the Mediterranean regions and in Asia [11, 12]. The typical papilionaceous flowers are grouped into axillaries at the head of twigs, most often yellow or red. Chemical investigations of the genus *Colutea* have revealed the presence of flavonoids [13], phenols [14], chalcones [15], sugars [16], triterpenes and alkaloids [17]. *Colutea* species are traditionally used for their ornamental and laxative properties, but their dermo-cosmetic activities, including antioxidant properties, whitening effect and anti-elastase activity have not yet been studied.

In line with our investigation for local plants with anti-aging properties [18, 19] we focused on *Colutea arborescens* L., a perennial non-climbing shrub growing in calcareous soils in southern and central Europe, as well as in south and eastern France [20]. *C. arborescens*, or common bladder senna, is a vigorous shrub of up to 3 m, with pale green pinnate leaves, 3-5 pairs of obovate or oblong leaflets and short axillary racemes of pea-like yellow flowers 2 cm across, followed by 8 cm long bladder-like fruits [20]. Leaves are used as purgative like senna. Some isoflavones have been isolated from seeds and pods of *C. arborescens* [21], as well as isoflavanes from root bark [22, 23], and a methoxyflavonol from leaves and flowers [24]. To our knowledge, the antioxidant, anti-tyrosinase and anti-elastase potency of *C. arborescens* aerial parts and their relation to chemical composition have not been reported.

Thus, the aim of the present study is to investigate the antioxidant, tyrosinase and elastase inhibitory activities of the crude MeOH extract, fractions and isolated compounds obtained from the aerial parts of *C. arborescens*. A ^{13}C NMR-based dereplication methodology combined to a bio-guided fractionation and purification procedure were used for metabolite identification.

MATERIAL AND METHODS

General experimental procedures

1D and 2D-NMR measurements were recorded at 298 K in CH_3OH-d_4 or DMSO-d_6 on a Bruker Avance AVIII-600 spectrometer (Karlsruhe, Germany) equipped with a 5 mm TCI cryoprobe. 2D-NMR experiments were performed using standard Bruker microprograms (TopSpin 3.2 software). HR-ESI-MS experiments were realized using a Micromass Q-TOF micro instrument (Manchester, UK), in the positive-ion mode in the range *m*/*z* 100-2000, with a mass resolution of 20 000 and an acceleration voltage of 0.7 kV.

Semi-preparative high-performance liquid chromatography (HPLC) was realized on a Dionex apparatus equipped with an ASI-100 automated sample injector, a STH 585 column oven, a P580 pump, a diode array detector UVD 340S, the Chromeleon® software version 6.8 and a prepacked C_{18} column (Interchim, 250 × 10 mm, 5 µ). The mobile phase was composed of H_2O with TFA (0.0025%) and CH_3CN with a flow rate of 5 mL/min and the chromatograms were monitored at 205, 254, 300 and 360 nm. Column chromatography (CC) was carried out on HP-20 resin (Sigma Aldrich).

Thin layer chromatography (TLC) was carried out on silica gel 60 F_{254} pre-coated aluminum plates (0.2 mm, Merck). The spots were visualized under UV light (254 and 366 nm) and then sprayed with 50% H_2SO_4 followed by heating.

A FLUOstar Omega spectrophotometer (BMG LABTECH) was used for measuring the absorbance of antioxidant and anti-tyrosinase assays. An Infinite F200 PRO spectrofluorimeter (Tecan, Lyon, France) was used to measure the fluorescence of anti-elastase assay.

Plant material

Aerial parts of *Colutea arborescens* L. (Fabaceae) were collected in Cormontreuil (Northeastern of France: 49°2167′N, 4°05′E) in May 2016, authenticated by Dr. Abdulmagid Alabdul Magid and dried at room temperature. A Voucher specimen was deposited at the Herbarium of the Botanic Laboratory-Faculty of Pharmacy, University of Reims Champagne-Ardenne, under the sheet reference (MA-CA-2016-05).

Extraction and isolation

The dried and powdered *C. arborescens* aerial parts (300 g) were macerated in MeOH/H_2O (4/1, v/v, 3 x 3 L, 24 h) at room temperature. The macerate was concentrated to about 1 L at 40 °C under vacuum. An aliquot of the aqueous solution (100 mL) was evaporated to dryness to obtain the hydromethanolic extract (HME) (4.1 g). The aqueous solution (1 L) of HME was extracted successively with dichloromethane (3 x 750 mL), ethyl acetate (3 x 750 mL) and *n*-butanol (3 x 750 mL), then dried under reduced pressure to yield DCMF (7.4 g), EAF (1.6 g), and *n*-BF (16.3 g) fractions, respectively and a water-soluble part (10.4 g).

The *n*-BF fraction (16 g) was dissolved in 1 L H_2O and then subjected to a Diaion HP-20 macroporous resin column (5.5 x 26 cm), eluting sequentially with a mixture of MeOH-H_2O (0%, 25%, 50%, 75% and 100% MeOH, 1.8 L each) to provide five fractions *n*-BFA – *n*-BFE, respectively.

Centrifugal partition chromatography

Centrifugal partition chromatography (CPC) experiments were carried out using a lab-scale Fast Centrifugal Partition Extractor FCPE300® column (Rousselet Robatel Kromaton, Annonay, France). The liquid phases were pumped by a KNAUER Preparative 1800 V7115 pump (Berlin, Germany). The column was coupled on-line with a UVD 170 S detector set at 210, 254, 280 and 366 nm (Dionex, Sunnivale, CA, USA). Fractions of 20 mL were collected by a Pharmacia Superfrac collector (Uppsala, Sweden). The solvent system was *n*-heptane/EtOAc/methanol/water (1/1/1/1, v/v/v/v) for EAF and EtOAc/CH_3CN/water (3/3/4, v/v/v) for *n*-BFC. The column rotation speed was set at 1200 rpm and the flow rate at 20 mL/min. EAF and *n*-BFC were subjected independently to CPC.

EAF (1.6 g injected) was dissolved in 25 mL of a mixture of both lower phase (20 mL) and upper phase (5 mL). The upper phase of the biphasic solvent system was pumped for 140 min in the ascending mode. Then the column was extruded by pumping the organic phase in the descending mode still at 20 mL/min. Fractions of 20 mL were collected over the whole experiment. All fractions were analyzed by TLC and HPLC and then pooled, giving fractions $EAF_{1\text{-}13}$.

n-BFC (700 mg injected) was dissolved in 8 mL of a mixture of both lower phase (6.4 mL) and upper phase (1.6 mL). The CPC method was identical to EAF, except for the pumping time of the upper phase which was 110 min. All fractions were analyzed by TLC and HPLC and then pooled, giving fractions $n\text{-}BFC_{1\text{-}17}$.

NMR analyses and dereplication

As a first step in this developed ^{13}C NMR-based dereplication method [25], structures and names of metabolites already described in the genus *Colutea* (n = 13) were collected from the reports available in the literature. The predicted ^{13}C NMR chemical shifts of each one was then stored in a local database already comprising 3218 structures of natural compounds (NMR Workbook Suite 2012, ACD/Labs, Ontario, Canada). In the second step, all CPC fractions were analyzed by ^{13}C NMR. NMR parameters used were described previously in the literature [18, 19]. The last step consisted of the binning of all ^{13}C NMR signals followed by hierarchical clustering analysis (HCA) and the visualization of the resulting dataset as a heat map [18, 19]. Each cluster of chemical shifts values isolated by HCA was then used as a target for a structure search within the database for compound identification. Additional 1D and 2D NMR spectra (^1H NMR, HSQC, HMBC, and ^1H-^1H-COSY) were recorded and analyzed to confirm the structures of the identified compounds.

HPLC purification of *n*-BFC

Fraction $n\text{-}BFC_5$ (53 mg) was subjected to semi-preparative HPLC using the gradient system (15-18% CH_3CN, 25 min; 18-20% CH_3CN, 10 min; 20% CH_3CN, 5 min) to yield compounds **3** (t_R = 17.4 min, 4.4 mg), **4** (t_R = 21.5 min, 1.7 mg), **5** (t_R = 24.4 min, 1.2 mg) and **6** (t_R=37.6 min, 7 mg). Fraction $n\text{-}BFC_6$ (45 mg) was purified by semi-preparative HPLC with gradient system (15-18% CH_3CN, 25 min; 18-20% CH_3CN, 10 min; 20% CH_3CN, 5 min) to yield compounds **7** (t_R = 12.4 min, 6.5 mg), **8** (t_R = 15.1 min, 2.1 mg), **9** (t_R = 15.1 min, 2.1 mg), **5** (t_R = 24.4 min, 1.8 mg), **10** (t_R = 26.5 min, 5 mg) and **11** (t_R = 37.6 min, 2.6 mg). Fraction $n\text{-}BFC_{15}$ (61 mg) was subjected to semi-preparative HPLC using the gradient system (5-20% CH_3CN, 8 min; 20% CH_3CN, 10 min) to

yield compound **12** (t_R = 10.7 min, 10.2 mg). Fraction *n*-BFC_{17} (27 mg) was purified by semi-preparative HPLC with gradient system (5-20% CH_3CN, 8 min; 20% CH_3CN, 10 min) to yield compounds **13** (t_R = 10.6 min, 2.7 mg), **12** (t_R = 10.7 min, 5.2 mg), **1** (t_R = 11.2 min, 3.2 mg), **2** (t_R = 11.2 min, 3.2 mg) and **14** (t_R = 11.7 min, 1.6 mg).

DPPH radical scavenging activity

Extracts, fractions and compounds **3-5**, **7**, **12** and **14** were tested for their DPPH radical scavenging activity. The free radical scavenging capacity was determined by using the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical [26]. Briefly, 5 µL of different concentrations of the samples (dissolved in H_2O/DMSO 9/1, v/v) were added to 95 µL of a DPPH solution (158 µM, dissolved in EtOH/H_2O (1/1, v/v)) freshly prepared. The reaction proceeded for 30 min at 37 °C on a 96-well microplate and the absorbance was then read at 515 nm. The DPPH inhibition percentage was calculated as followed: % inhibition $[(Ab_{control} - Ab_{sample})/Ab_{control}] \times 100$. A DPPH solution in EtOH 50% was used as a control. The curve of the % scavenging activity against the concentration of sample was prepared by MSExcel-based program to obtain the IC_{50}. Samples were prepared at concentrations of 200, 100, 50, 25, 12.5 and 6.25 µg/mL. Ascorbic acid and quercetin were used as positive controls. All the tests were conducted in triplicate for each concentration examined.

Hydroxyl radical scavenging activity

Extracts, fractions, and compounds **5**, **7** and **11** were tested for their hydroxyl radical scavenging activity. Hydroxyl radical scavenger ability was determined following the procedure described in the literature [18]. Hydroxyl radical was generated before the assay from Fenton reaction between 1.5 mM $FeSO_4$ and 6 mM H_2O_2, (10:7, v/v) at 37 °C for 30 min and detected by their ability to hydroxylate salicylate. The reaction mixture (300 µL) contained 100 µL $FeSO_4$ (1.5 mM), 70 µL H_2O_2 (6 mM, freshly prepared), 30 µL sodium salicylate (20 mM) and 100 µL of varying concentrations of samples (1330, 665, 332.5, 166.25, 83.12 and 41.56 µg/mL) dissolved in H_2O/DMSO (9/1, v/v). After incubation for 1 h at 37 °C, the absorbance of the hydroxylated salicylate complex was measured at 562 nm. Ascorbic acid and quercetin were used as positive controls. The scavenging activity of hydroxyl radical effect was calculated as follows: $[1 - (A_1 - A_2)/A_0] \times 100$, where A_0 is absorbance of the control (without sample), A_1 is absorbance in the presence of the sample and A_2 is absorbance without sodium salicylate. All the tests were conducted in triplicate and IC_{50} was determined by interpolation of concentration % inhibition curve obtained by MSExcel based program.

Power cupric ion reducing (CUPRAC)

Extracts, fractions, and compounds **5**, **6** and **10-12** were tested for their power cupric ion reducing. The cupric ion reducing activity (CUPRAC) was determined following the procedure described in the literature [18]. Samples were prepared at concentrations of 572, 286, 143, 71.5, 35.75, 17.87, 8.94, 4.47, 2.23, 1.12 and 0.56 µg/mL and dissolved in H_2O/DMSO (9/1, v/v). 45 µL of each concentration was added to premixed reaction mixture containing $CuCl_2$ (90 µL, 10 mM), freshly prepared neocuproine (90 µL, 7.5 mM, dissolved in distilled water and ethanol in proportion 8/2, v/v) and NH_4Ac buffer (90 µL, 1M, pH 7.0). Similarly, a blank was prepared by adding sample solution (45 µL) to premixed reaction mixture (270 µL) without $CuCl_2$. The reaction proceeded for 30 min at room temperature on a 96-well microplate and the absorbance was then read at 450 nm. Ascorbic acid, quercetin and trolox were used as positive controls. The power cupric ion reducing was calculated as follows: $[1 - A_0 / (A_1 - A_2)] \times 100$, where A_0 is absorbance of the control (without sample), A_1 is absorbance in the presence of the sample and A_2 is absorbance of the blank. All the tests were conducted in triplicate and IC_{50} were determined by interpolation of concentration % inhibition curve obtained by MSExcel based program.

Tyrosinase enzyme assay

Extracts, fractions, and compounds **6**, **7** and **12** were tested for their ability to inhibit tyrosinase. The tyrosinase inhibitory activity was determined according to the method described previously [27] with a few modifications. Briefly, L-DOPA was used as the substrate in this experiment. Samples were prepared at concentrations of 1330, 665, 332.5, 166.25, 83.12, 41.56 and 20.78 µg/mL and

dissolved in phosphate buffer solution (PBS, 20 mM, pH 6.8) and DMSO in proportion 9/1 (v/v). 100 µL of each concentration was added to a 96-well microplate and then 100 µL of 135 U/mL fungal tyrosinase in PBS was added. After pre-incubation at room temperature for 10 min in the dark, 100 µL of L-DOPA (0.5 mM in PBS) was added. The reaction mixture was incubated for another 5 min at room temperature. The amount of dopachrome in the mixture was determined by the measurement of the absorbance of each well at 475 nm. Kojic acid was used as a positive control. The inhibitory percentage of tyrosinase was calculated according to the following equation: % inhibition = $\{[(A - B) - (C - D)]/(A - B)\} \times 100$, where A is absorbance without sample, B is absorbance without sample and tyrosinase, C is absorbance with sample, and D is absorbance with sample and without tyrosinase. All the tests were conducted in triplicate. The IC_{50} values were determined by interpolation of concentration % inhibition curve obtained by MSExcel based program.

Elastase enzyme assay

Extracts, fractions, and compounds **5**, **7** and **11-13** were tested for their ability to inhibit elastase. Elastase inhibition measurement was carried out using Human Leukocyte Elastase (HLE) (Merck Biosciences). Tests were performed in pre-coated 96-well microplates with 1% Serum Albumin Bovine. HLE (0.8 µM) was incubated for 1 h at 27 °C in Tris buffer (50 mM Tris-HCl pH 7.5 containing 500 mM NaCl) containing 0.1 to 1000 µg/mL of tested sample. Sample solvent was used as a control. The assay was initiated by adding HLE fluorogenic substrate MeOSuc-Ala-Ala-Pro-Val-AMC (λ_{exc} = 380 nm/ λ_{em} = 460 nm) at a final concentration of 80 µM. The rate of each substrate cleavage was measured in triplicate for each concentration examined, using an Infinite F200 PRO spectrofluorimeter (Tecan, Lyon, France) with one measure per minute for 60 min. HLE activity was calculated according to the following equation: % HLE activity = ($Slope_{sample}$ x 100)/ $Slope_{control}$, where $slope_{sample}$ and $slope_{control}$ are the slope of the fluorescence values as a function of time. Non-linear regression analysis with Graphpad software (La Jolla, USA) allowed us to calculate the IC_{50}.

RESULTS AND DISCUSSION

Preliminary bio-guided evaluation of crude extract and fractions of *C. arborescens* aerial parts

The water-soluble fraction of a 80% hydromethanolic extract (HME) of *C. arborescens* aerial parts was partitioned successively with solvents of increasing polarity, resulting in a dichloromethane fraction (DCMF), ethyl acetate fraction (EAF) and *n*-butanol fraction (*n*-BF). The free radical scavenging activities of HME and the resulting fractions were determined by the DPPH and hydroxyl radical assays and the cupric reducing capacity was evaluated by the CUPRAC assay. Their ability to inhibit mushroom tyrosinase and human neutrophil elastase activity was also tested. The results are shown in Table 1. For EAF and *n*-BF, a substantial cupric ion reducing capacity was observed (IC_{50} 2.6 µg/mL and 5.6 µg/mL, respectively), as well as moderate DPPH (IC_{50} 107.0 µg/mL and 138.2 µg/mL, respectively) and hydroxyl radical scavenging activities (IC_{50} 123.3 µg/mL and 332.5 µg/mL, respectively). A moderate tyrosinase inhibitory activity was also observed for EAF and *n*-BF (IC_{50} 138.0 µg/mL and 516.5 µg/mL, respectively). Concerning the elastase inhibitory activity, all extracts showed less than 9% inhibition at 10 µg/mL. The EAF and *n*-BF showing the most interesting biological activities were chemically investigated through a bioassay-guided isolation strategy to tentatively determine the active constituents.

Chemical profiling of the EtOAc fraction (EAF)

The major compounds of EAF were identified using a dereplication method combining centrifugal partition chromatography (CPC), NMR analyses, clustering of NMR peak emergence profiles and data base search, without purification of individual components [25]. The CPC fractionation of EAF was performed with the biphasic solvent system *n*-heptane/EtOAc/methanol/water (1/1/1/1, v/v/v/v) which was selected to recover moderately polar compounds, to afford thirteen CPC sub-fractions (EAF_{1-13}). After ^{13}C NMR analyses of EAF_{1-13}, all spectra of the fraction series were processed and submitted to Hierarchical Clustering Analysis (HCA) for the recognition of similarities between emergence profiles of ^{13}C NMR peaks throughout

Table 1. Antioxidant, tyrosinase and elastase inhibitory activities of crude extracts, fractions and compounds isolated from *C. arborescens*.

	DPPH radical scavenging activity IC_{50} (µg/mL)	**OH radical scavenging activity IC_{50} (µg/mL)**	**Power cupric ion reducing (CUPRAC) IC_{50} (µg/mL)**	**Mushroom tyrosinase inhibition IC_{50} (µg/mL)**	**Human neutrophil elastase inhibition IC_{50} (µg/mL)**
CAM	(34%)[a]	222.7 ± 5.8	9.9 ± 0.9	961.7 ± 5.2	(9%)[c]
DCMF	167.2 ± 4.0	(30%)[b]	18.8 ± 1.8	127.2 ± 1.6	n.d.
EAF	107.0 ± 6.1	123.3 ± 11.6	2.6 ± 0.1	138.0 ± 3.9	(< 5%)[c]
***n*-BF**	138.2 ± 1.6	332.5 ± 0	5.6 ± 0.5	516.5 ± 12.4	(9%)[c]
***n*-BFA**	(30%)[a]	n.d.	n.d.	1171.7 ± 11.6	n.d.
***n*-BFB**	133.3 ± 4.3	113.2 ± 0.8	2.7 ± 0	176.3 ± 11.0	(29%)[c]
***n*-BFC**	93.3 ± 1.5	121.2 ± 3.7	2.2 ± 0	133.3 ± 2.8	(16%)[c]
***n*-BFD**	(50%)[a]	890.8 ± 3.8	11.9 ± 0.4	422.5 ± 10.9	(7%)[c]
***n*-BFE**	(38%)[a]	651.7 ± 2.9	16.7 ± 0.6	(45%)[b]	(< 5%)[c]
***n*-BFC_2**	22.7 ± 1.4	278.3 ± 2.1	52.9 ± 0.1	(26%)[b]	92.7 ± 1.1
***n*-BFC_5**	15.8 ± 1.1	459.7 ± 5.7	48.4 ± 2.7	51.2 ± 11.7	136.9 ± 1.1
***n*-BFC_6**	22.5 ± 1.5	(47%)[b]	52.8 ± 0	355.7 ± 1.5	355.4 ± 1.1
***n*-BFC_{10}**	13.9 ± 0.5	(40%)[b]	45.7 ± 0.1	(17%)[b]	189.0 ± 1.2
***n*-BFC_{11}**	(45%)[a]	(33%)[b]	59.2 ± 0.7	(39%)[b]	246.8 ± 1.2
***n*-BFC_{14}**	60.5 ± 1.3	(48%)[b]	37.0 ± 1.7	(43%)[b]	156.5 ± 1.2
***n*-BFC_{15}**	37.5 ± 2.0	175.3 ± 2.5	44.4 ± 0	(10%)[b]	59.0 ± 1.1
***n*-BFC_{17}**	(44%)[a]	(49%)[b]	59.2 ± 0.7	(25%)[b]	n.d.
Quercetin[d]	5.4 ± 0.2	52.2 ± 4.8	13.6 ± 1.3	15.1 ± 0[1]	6.0 ± 0[2]
Ascorbic acid[d]	2.3 ± 0.4	229.2 ± 2.3	13.3 ± 0.5		
Kojic acid[d]				8.5 ± 0.5	
Trolox[d]			5.4 ± 0.3		
	IC_{50} (µM)	**IC_{50} (µM)**	**IC_{50} (µM)**	**IC_{50} (µM)**	**IC_{50} (µM)**
3	103.5 ± 3.5	n.d.	n.d.	n.d.	n.d.
4	1136.3 ± 19.8	n.d.	n.d.	2381.2 ± 0[42]	n.d.
5	(12%)[a]	4178.7 ± 232.9	(33 %)[d]	63.3 ± 2.5[49]	1779.8 ± 3.9
6	11.2 ± 0.4[50]	n.d.	37.7 ± 1.1	(< 5%)[e]	> 100[63]
7	(51%)[a]	1709.3 ± 913.9	n.d.	(< 5%)[e]	693.7 ± 4.4
10	28.8 ± 0.8[60]	145.8 ± 4.9[60]	13.9 ± 0.8	(< 5%)[f, 51]	u.i.[64]
11	(< 50%)[b, 61]	751.9 ± 43.7	54.7 ± 0.8	(< 5%)[f, 55]	86.1 ± 1.9
12	45.9 ± 0.5	n.d.	38.8 ± 1.1	(10%)[e]	101.3 ± 1.5

Table 1 continued..

13	(< 5%)[c, [62]]	n.d.	n.d.	n.d.	1386.5 ± 2.9
14	1852.1 ± 0[56]	314.0 ± 16.2[4]	n.d.	n.d.	n.d.
15	n.d.	26.2 ± 4.8[4]	n.d.	n.d.	n.d.
16	(27%)[a]	n.d.	n.d.	n.d.	n.d.
Quercetin[g]	17.9 ± 0.7	172.7 ± 15.9	45.0 ± 4.3	50.0 ± 0[51]	20.0 ± 0[7]
Ascorbic acid[g]	13.1 ± 2.3	1301.5 ± 13.1	75.5 ± 2.8		
Kojic acid[g]				59.8 ± 3.5	
Trolox[g]			21.6 ± 1.2		

[a] % Inhibition at 200 µg/mL, [b] % inhibition at 44.6 µg/mL, [c] % inhibition at 8.1 µg/mL, [d] % inhibition at 1330 µg/mL, [e] % inhibition at 572 µg/mL, [f] % inhibition at 1000 µg/mL, [g] % inhibition at 10 µg/mL, [h] used as positive control, n.d. not done, u.i. undetectable inhibition.

the fractionation process. In this way, ^{13}C NMR signals belonging to the same compounds were grouped to build "chemical shift clusters" represented in the heat map given in Figure 1. As a result, 7 major chemical shift clusters corresponding to the major metabolites of the EAF (Figure 1), colored in yellow, were revealed by the heat map.

With the help of an in-house database containing predicted chemical shift values of natural metabolites, the correlated chemical shifts of cluster 1 in fractions EAF_{2-3} were assigned to benzoic acid. The identification of benzoic acid was easily confirmed by checking HSQC, HMBC and COSY data of fraction EAF_2 and by comparison with literature data [28]. By means of the same database search strategy, clusters 2 to 6 were identified as: *p*-hydroxybenzoic acid [29] (cluster 2; fractions EAF_{4-7}), *Z*/*E*-*p*-coumaric acid [30] (clusters 3 and 3'; fractions EAF_{4-5}), *Z*-3-octenedioic acid (cluster 4; fractions EAF_{7-8}), oleic acid [31] (cluster 5; fractions EAF_{2-4}), and a mixture of pinitol [32] and rhamnocitrin-3-*O*-β-D-glucopyranoside [33, 34] (cluster 6; fractions EAF_{9-10}).

Chemical profiling of *n*-BuOH fraction (*n*-BF)

The *n*-BF was composed of a complex mixture of more polar metabolites as compared to the EAF. Therefore, five fractions (*n*-BFA – *n*-BFE) were firstly produced from the *n*-BF on a Diaion HP-20 column to simplify its chemical composition and then evaluated for their biological activities (Table 1). The results showed that *n*-BFC exhibited the best DPPH radical scavenging activity (IC_{50} 93.3 µg/mL), whereas *n*-BFB and *n*-BFC showed a good hydroxyl radical scavenging activity (IC_{50} 113.2 µg/mL and 121.2 µg/mL, respectively) and a significant cupric ion reducing power (IC_{50} 2.7 µg/mL and 2.2 µg/mL, respectively) (Table 1). For the tyrosinase inhibitory activity, *n*-BFC exhibited the best result (IC_{50} 133.3 µg/mL). The elastase inhibition of *n*-BFA – *n*-BFE oscillates between 5 and 29% at the dose 10 µg/mL. Comparing the activity of the *n*-BF with that of fractions *n*-BFA – *n*-BFE, we observed for *n*-BFC a substantial increase of DPPH and hydroxyl radical scavenging activities, a higher cupric ion reducing power and a significant increase of tyrosinase inhibitory activity (Table 1).

Thus, *n*-BFC was further chemically profiled by dereplication with the same NMR-based strategy as described for the EAF. The CPC fractionation of *n*-BFC was performed with the biphasic solvent system EtOAc/CH_3CN/water (3/3/4, v/v/v), to afford seventeen CPC sub-fractions (*n*-BFC_{1-17}). The resulting HCA heat map containing correlated ^{13}C NMR signals is also drawn in Figure 1, and 11 major chemical shift clusters were revealed. The clusters 7 to 12 were identified as: a mixture of phenylethyl-β-D-glucopyranoside (compound **5**) [35], benzyl-β-D-glucopyranoside (compound **7**) [36] and (*Z*)-3-hexenyl-β-D-glucopyranoside [29]

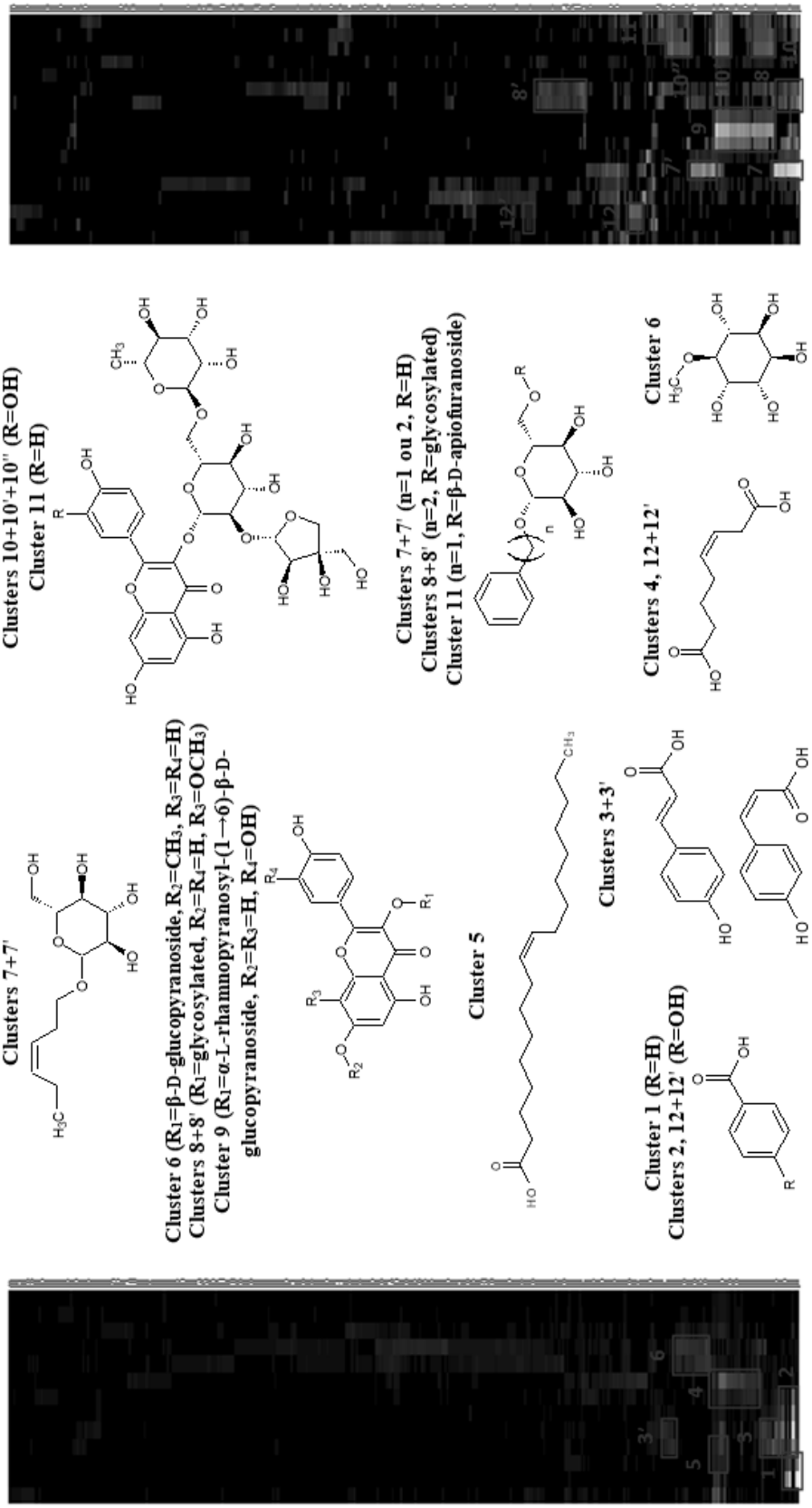

Figure 1. ^{13}C NMR chemical shift clusters obtained by applying HCA on EAF (left) and *n*-BFC (right) CPC fractions from *C. arborescens*.

(clusters 7 and 7'; fraction *n*-BFC$_6$), quercetin-3-*O*-α-L-rhamnopyranosyl- (1→6)-β-D-glucopyranoside (compound **10**) [37] (cluster 9; fractions *n*-BFC$_{8-10}$), quercetin-3-*O*-β-D-apiofuranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→6)]-β-D-glucopyranoside (compound **12**) [38] (Table SI) (clusters 10, 10' and 10"; fractions *n*-BFC$_{15-17}$), a mixture of benzyl-β-D-apiofuranosyl-(1→6)-β-D-glucopyranoside (compound **13**) [39] and kaempferol-3-*O*-β-D-apiofuranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→6)]-β-D-glucopyranoside (compound **14**) [38] (cluster 11; fractions *n*-BFC$_{16-17}$), and a mixture of *p*-hydroxybenzoic acid [29] and *Z*-3-octenedioic acid (clusters 12 and 12'; fractions *n*-BFC$_{2-3}$). For fractions *n*-BFC$_{11-12}$, the database proposed a mixture of phenylethyl-glycosylated and 8-methoxykaempferol-3-*O*-glycosylated [40], which could not be identified unambiguously (clusters 8 and 8').

Fractions *n*-BFC$_{2-17}$ were further screened for their biological activity. As shown in Table 1, fractions *n*-BFC$_5$ and *n*-BFC$_6$ best inhibited mushroom tyrosinase and *n*-BFC$_{15}$ had the best inhibition of human neutrophil elastase. In addition, these three fractions also had good antioxidant activities. The screening results indicate that the fractionation of *n*-BFC has increased the DPPH radicals scavenging potential and the elastase inhibitory activity.

Purification of the active fractions of *n*-BFC

Since active fractions *n*-BFC$_5$, *n*-BFC$_6$ and *n*-BFC$_{15}$ contained metabolites which were not unambiguously identified over the dereplication process, further purifications were performed using semi-preparative HPLC. Fraction *n*-BFC$_{17}$ was also purified by semi-preparative HPLC because it contains an original tri-glycosylated flavonoid. The chemical structures were assigned based on 2D-NMR and ESI-MS data to afford fourteen known compounds (**1-14**). Compounds **5**, **7**, **10**, **12**, **13** and **14** already identified during the dereplication process were also isolated in addition to β-carboline (compound **1**) [41], 1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (compound **2**) [42], *E*-(2,3-dihydroxy-2-methylbutanoic acid) caffeate (compound **3**) [43], phenylacetic acid (compound **4**) [44], quercetin-3-*O*-β-D-glucopyranoside (compound **6**) [45], 2-hydroxynaringin-5-*O*-β-D-glucopyranoside (compound **8**) [46]), isopentyl-β-D-glucopyranoside (compound **9**) [47], and kaempferol-3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (compound **11**) [48] (Figure 2).

Biological assays on compounds

Isolated compounds were evaluated for their antioxidant potential using the DPPH and hydroxyl radical scavenging assays, CUPRAC assay, and for their tyrosinase and elastase inhibitory properties. Some compounds have not been screened for these five assays due to their low available mass. For these compounds, their biological potential was discussed based on literature data when possible. As summarized in Table 1, compounds **6** [49], **10** [50] and **12** exhibited the highest antioxidant potential in DPPH (IC$_{50}$ 11.2, 28.8 and 45.9 µM, respectively) and CUPRAC tests (IC$_{50}$ 37.7, 13.9 and 38.8 µM, respectively). Compound **10** [50] showed also good activity in hydroxyl radicals scavenging (IC$_{50}$ 145.8 µM), and compound **1** [51] exhibited the highest capacity in hydroxyl scavenging (IC$_{50}$ 26.2 µM). Compounds **2**, **3** and **11** [51] showed also good antioxidant activities with hydroxyl radical assay (compound **2**: IC$_{50}$ 314.0 µM), DPPH test (compound **3**: IC$_{50}$ 103.5 µM), and CUPRAC test (compound **11**: IC$_{50}$ 54.7 µM).

The quercetin glycosides **10** and **12** had better antioxidant activities than the kaempferol glycosides **11** and **14**. In most cases, quercetin derivatives exhibited a higher capacity than kaempferol and isorhamnetin due to the presence of two free OH group at positions 3' and 4' of the B-ring [52]. Our results confirm this relationship since the most active compounds **6**, **10** and **12** are quercetin-type flavonoids. Moreover, the results indicated also that compounds **6** and **10** (possessing one or two sugars in position 3 of the aglycone) showed better activity in antioxidant assays when compared to compound **12** (with three sugars in position 3 of the aglycone), due to glycosylation at 2" and 6" positions of glucose. In addition, antioxidant capacity was very weak for glycosides **5** and **7** which have aromatic ring without free OH group necessary for the activity [53, 54].

Concerning the anti-tyrosinase tests, only compound **5** (phenylethyl-glucoside) [55] exhibited a

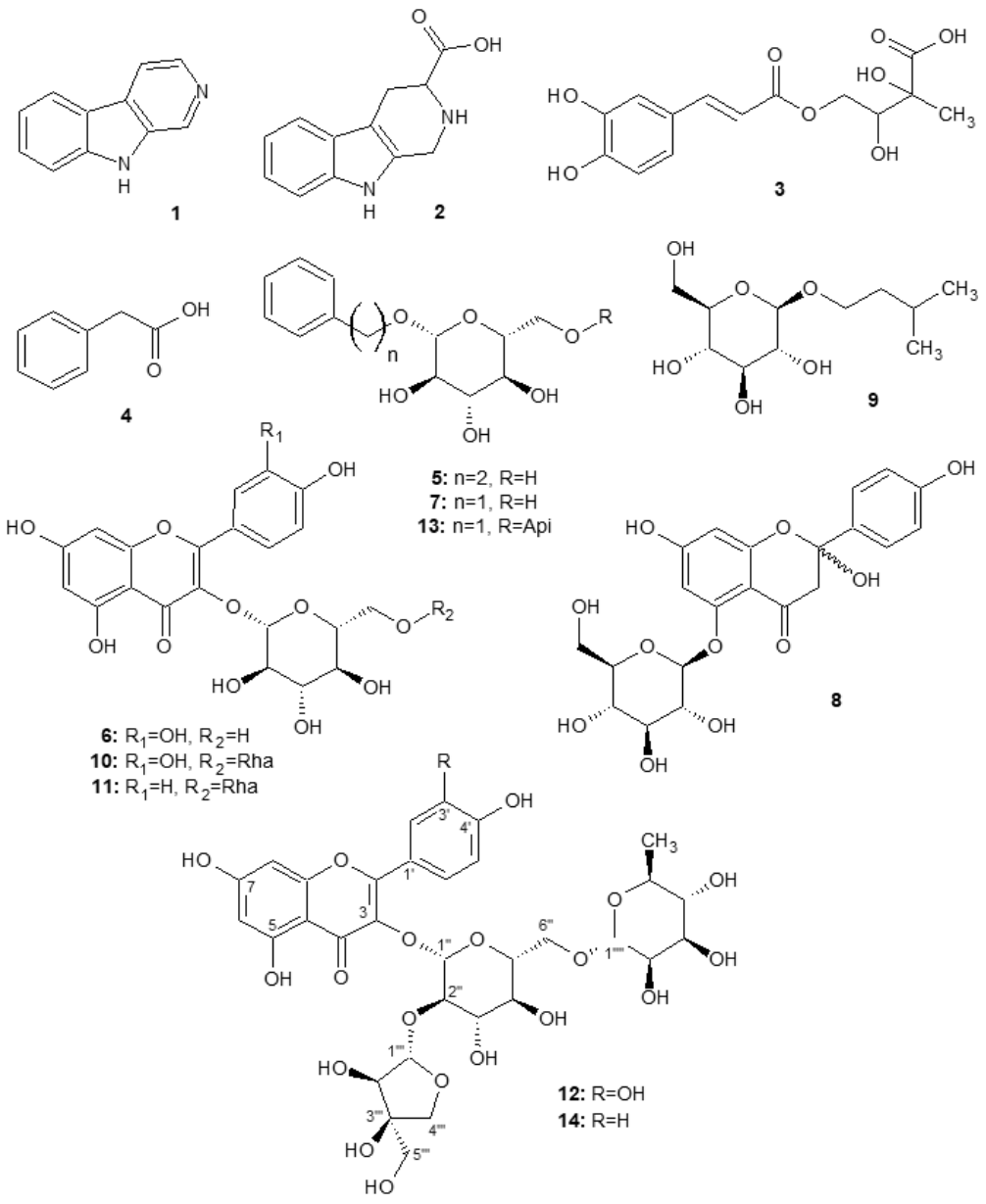

Figure 2. Chemical structures of compounds **1-14** isolated from *C. arborescens* aerial parts.

significant activity (IC_{50} 63.3 µM). It is more active than its analog compound **7** (benzyl-glucoside). This suggested another structure-activity relationship based on chain length that would increase anti-tyrosinase activity. Compounds **11** and **12** showed a moderate elastase inhibitory activity (IC_{50} 86.1 and 101.3 µM, respectively). However, the inhibitory capacity of compounds **6**, **10**, **11** and **12** is lower than that of quercetin found in the literature [56]. These results suggest that sugars attached to position 3 of the aglycone decrease anti-elastase activity.

CONCLUSIONS

Twenty-one secondary metabolites including flavonoids, phenolic acids, alkaloids and glycosides derivatives, were identified from the aerial parts of *Colutea arborescens* by ^{13}C NMR-based dereplication combined to bioactivity-guided fractionation. Our studies demonstrated that EtOAc

(EAF) and *n*-BFC fractions were characterized by the highest anti-tyrosinase and antioxidant activities among all prepared fractions.

From a chemotaxonomic viewpoint, among the compounds elucidated by dereplication, three were already known in the genus *Colutea*: pinitol [16], oleic acid [57] and rhamnocitrin-3-*O*-β-D-glucopyranoside [58]. The other compounds are reported here for the first time in the genus *Colutea*. In addition, *n*-BFC contains two tri-glycosylated flavonoids **12** (Table SI) and **14**, containing an apiose moiety.

The presence of *Z*/*E*-*p*-coumaric acid and *p*-hydroxybenzoic acid could explain in part the antioxidant activity [59] and the tyrosinase inhibitory activity, respectively observed for the EAF. In addition, quercetin-type flavonoids **6**, **10** and **12** showed a powerful antioxidant activity and phenylethyl-β-D-glucopyranoside (**5**) exhibited a mushroom tyrosinase inhibition.

Our investigation contributes to phytochemical database of *Colutea* species and highlighted the antioxidant activity of glycosylated flavonoids and more particularly quercetin-type flavonoids. The EAF, *n*-BF and the glycosylated flavonoids identified from *C. arborescens* could be valued in the dermo-cosmetic field for their interesting antioxidant activities.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

ABBREVIATIONS

HME : hydromethanolic extract
DCMF : dichloromethane fraction
EAF : ethyl acetate fraction
*n*BF : *n*-butanol fraction
*n*BFA : *n*-butanol fraction A
*n*BFB : *n*-butanol fraction B
*n*BFC : *n*-butanol fraction C
*n*BFD : *n*-butanol fraction D
*n*BFE : *n*-butanol fraction E

SUPPLEMENTARY MATERIAL

NMR spectroscopic data of the compound **12** in DMSO-d_6 is given in Table SI.

Table S1. NMR spectroscopic data of the compound **12** in DMSO-d_6.[a]

	12	
	δ_H m (*J* in Hz)	δ_C
2		156.6
3		133.3
4		177.6
5		161.4
6	6.15 d (1.6)	99.3
7		164.6
8	6.34 d (1.6)	94.1
9		156.8
10		104.1
1′		121.6
2′	7.49 d (2.1)	116.4
3′		145.3
4′		148.9
5′	6.81 d (8.4)	115.6
6′	7.59 dd (8.4, 2.1)	122.3
Glc		
1″	5.47 d (7.6)	99.2
2″	3.48	77.3
3″	3.38	77.4
4″	3.05	70.7
5″	3.22	76.1
6″	3.22	67.3
	3.67 d (9.7)	
Api		
1‴	5.33 d (1.1)	109.0
2‴	3.79 d (1.1)	76.6
3‴		79.7
4‴	3.48	74.4
	3.81 d (9.2)	
5‴	3.36	64.7
	3.44	
Rha		
1⁗	4.32 br s	101.1
2⁗	3.34	70.8
3⁗	3.25 dd (9.5, 3.1)	70.9
4⁗	3.05	72.2
5⁗	3.23	68.7
6⁗	0.96 d (6.2)	18.2

[a]Overlapping ^{1}H NMR signals are reported without designated multiplicity.

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