cosmetics

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Whitening Agents from *Reseda luteola* L. and Their Chemical Characterization Using Combination of CPC, UPLC-HRMS and NMR

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Received: 5 November 2017; Accepted: 23 November 2017; Published: 25 November 2017

Abstract: Skin whitening agents occupy an important part of the dermo-cosmetic market nowadays. They are used to treat various skin pigmentation disorders, or simply to obtain a lighter skin tone. The use of traditional skin bleachers (e.g., hydroquinone, corticoids) is now strictly regulated due to their side effects. When considering this and the growing consumers' interest for more natural ingredients, plant extracts can be seen as safe and natural alternatives. In this perspective, in vitro bioassays were undertaken to assess cosmetic potential of *Reseda luteola*, and particularly its promising whitening activities. A bioguided purification procedure employing centrifugal partition chromatography, Ultra Performance Liquid Chromatography-Mass Spectrometry (UPLC-HRMS) and NMR was developed to isolate and identify the whitening agents (i.e., luteolin and apigenin) from aerial parts of *R. luteola*. UPLC-HRMS also enabled the characterization of acetylated luteolin- and apigenin-O-glycosides, which occurrence is reported for the first time in *R. luteola*.

Keywords: *Reseda luteola*; UPLC-HRMS; biological activity; centrifugal partition chromatography; luteolin; apigenin

1. Introduction

Skin whitening agents occupy an important part of the cosmetic market nowadays, with Asia and Africa representing the largest shares of the sector. Skin bleachers are used to treat various skin pigmentation disorders or simply to obtain a lighter skin tone, as whiter skin may be synonymous of youth and/or beauty, so their usage is particularly widespread in countries where skin phototypes IV, V, and VI are predominant [1]. The active agents that lighten skin tone are either natural or synthetic substances, and may act at various levels of melanogenesis. Tyrosinase inhibitors are the most common approach to achieve skin hypo-pigmentation, as this enzyme catalyzes the rate-limiting step of pigmentation [1–3].

Exploring new sources of natural whitening products appears crucial nowadays, given the fact that the ancient practice of skin bleaching is more newsworthy than ever [1]. Despite their emblematic uses, whitening ingredients such as hydroquinone, retinoic acids, corticoids, or mercury salts are of safety concern as they display several severe side effects, resulting in their interdiction or restricted use under the European Cosmetic Regulation 1223/2009 [4] and several other international regulations.

One can observe a boom in the development of natural whitening agents over the last decade: the search for natural tyrosinase inhibitors in recent years has demonstrated that plant extracts could be potential sources of new whitening ingredients [1,5].

Reseda luteola L. (Resedaceae family) is a biennial species native from western Asia and is naturalized in the Mediterranean basin that grows up to 1.5 m by 0.5 m in sunny exposure [6]. Commonly named 'dyer's weld', the aerial parts of this herbaceous species were used to produce a mordant dye, especially for dyeing wool and silk during Medieval and early modern times: the word 'luteola' meaning 'yellowish' actually refers to the color of this dye [7,8]. The substances responsible for producing this color are two flavones: luteolin, principally concentrated in all of the upper green parts of the plant, e.g., the flowers and the seeds, on one hand, and apigenin on the other hand [7,8]. Furthermore, *O*-glycoside-forms of these two flavones are also inventoried in *R. luteola*: usually hydrolyzed to the parent aglycone flavone in the dyebath, they indirectly participate to the yellow color [8].

Displaying interesting agronomic characteristics, as well as good dyeing properties, *R. luteola*'s potential to be a promising new crop was investigated as part of the PrisCA project in Italy [9]. This little-demanding species is nowadays considered as a non-food crop, supporting the growing demand for natural colorants in the building (constituting an alternative to toxic paints) and DIY (Do It Yourself) sectors [10], as well as in the plastic industry [11], and is therefore cultivated in numerous small plots in the Mediterranean basin.

When considering this and the growing consumers' interest for more natural dermo-cosmetics, the anti-oxidant potential, as well as the whitening and anti-inflammatory activities of *R. luteola* extract were hence assessed using in vitro bioassays. From an extensive literature survey, it appeared that research on the chromatographic separation and quantification of flavonoids from *R. luteola* has already been undertaken [12–14]. However, no study on the preparative isolation of its bioactive agents by preparative chromatography technique has been reported to date, even given the widespread use over the last decade of countercurrent chromatography (CCC) to isolate natural bioactive, and often chemically sensitive, compounds [15,16]. These reasons prompted the use of CPC (Centrifugal Partition Chromatography), applied in elution-extrusion mode, and combined with UPLC-HRMS (Ultra Performance Liquid Chromatography-Mass Spectrometry) to separate, purify, and identify the major bioactive flavonoids from an ethyl acetate extract of *R. luteola* displaying promising whitening properties.

2. Material and Methods

2.1. Plant Material

Aerial parts of cultivated *R. luteola* were collected in September 2014 from the Conservatory Garden of Dye Plants, Lauris (Luberon Regional Nature Park, France). After a drying step of seven days in a ventilated greenhouse, *R. luteola* aerial parts were beaten to separate leaves and seeds from stems. The remaining leaves and seeds containing dying agents were then ground before extraction.

2.2. Materials

All the chemicals and analytical standards (apigenin and luteolin) were obtained from Sigma-Aldrich unless otherwise stated. DMSO (dimethyl sulfoxide) and acetone for NMR (nuclear magnetic resonance) experiments were purchased from Euriso-top.

Untreated 96-well plates were obtained from Thermo Nunc, whereas the UV-transparent ones were purchased from Costar. During incubation, the 96-well plates were sealed with adhesive polyester films (VWR). Samples for biological activity testing were prepared in 1.5 mL Eppendorf tubes, which were appropriate for the use of the automated pipetting system epMotion® 5075 (Eppendorf).

2.3. Plant Extraction

2.3.1. Soxhlet Extraction

A conventional soxhlet system was used to obtain large amounts of plant extracts to fractionate in order to isolate individual bioactive molecules: dried plant material (50 g) placed in a cellulose cartridge that was already impregnated with the solvent system used to perform the extraction, is installed in the soxhlet extractor, itself placed over a distillation flask filled with 1.5 L of the solvent system. Aerial parts of *R. luteola* were first soxhlet extracted for 6 h using a succession of five different solvent systems to cover the complete polarity range of its constituting secondary metabolites: cyclohexane, dichloromethane, ethyl acetate, ethanol/water (EtOH/H_2O) 90/10, and water (chromatography grade) were used to obtain, respectively, extracts RL1 to RL5, further assessed for bioactivity.

2.3.2. Liquid-Liquid Extraction (LLE)

To further characterize the bioactive RL3 extract that was obtained using ethyl acetate (AcOEt; chromatography grade), a liquid-liquid extraction was undertaken: 10 mL of a solution of methanol/hexane 1/1 (*v*/*v*; chromatography grade) were first added to 2 g of RL3 extract to solubilize the extract's components. The volume is then adjusted to 200 mL with the same solvent system and homogenized by means of repeated inversions of the container. The hexane phase containing apolar compounds was collected, and two further extractions of the MeOH fraction were performed, each with 100 mL hexane (chromatography grade). The three successive hexane phases were gathered together. Solvent traces in both methanol and hexane sub-extracts were eliminated under vacuum until getting residues of constant mass.

2.4. Biological Activity Assays

2.4.1. Instrumentation

An automated pipetting system Eppendorf epMotion® 5075 was used for the biological activity assays. Absorbance measurements were performed using a microplate reader (Spectramax Plus 384, Molecular Devices). Data were acquired with the SoftMaxPro software (Molecular Devices) and inhibition percentages were calculated with the Prism software (GraphPad Software). The results are presented as inhibition percentages (I%), calculated as follows:

$$I\% = [(OD_{control} - OD_{sample})/OD_{control}] \times 100 \text{ (with OD stating for optical density).}$$

Unless otherwise stated, all OD were corrected with the blank measurement corresponding to the absorbance of the sample before addition of the substrate (OD_{blank}).

2.4.2. DPPH Radical Scavenging Assay

The antioxidant activity of the extracts and resulting fractions, as well as of the analytical standards (altogether referred to as 'samples' in the following protocols), was measured on the basis of the scavenging activity of the stable 1,1-diphenyl-2-picrylhyorazyl (DPPH) free radical, according to one of the most widely used method to examine the anti-oxidant activity of plant extracts [17].

The assays were performed in 96-well plates as follows: 150 µL of a solution of EtOH/acetate buffer 0.1M pH = 5.4 (50/50) are distributed in each well, together with 7.5 µL of samples diluted at a concentration of 3.433 mg/mL in DMSO (chromatography grade; sample's final concentration per well: 100 µg/mL).

Vitamin C (3.433 mg/mL in dimethyl sulfoxide (DMSO)) was used as the positive control; DMSO alone was used as the negative control ($OD_{control}$).

After addition of 150 µL of buffer and 7.5 µL of sample, a first optical density reading was performed at 517 nm (OD_{blank}). Then, 100 µl of a DPPH solution (386.25 µM in EtOH, analytical grade)

were distributed in each well. The plate was sealed and incubated in the dark at room temperature (RT). After 30 min, the final OD reading was performed at 517 nm to assess the percentage of inhibition.

2.4.3. Tyrosinase Assay

Tyrosinase, an oxidase controlling the production of melanin, is mainly involved in the initial rate-limiting reactions in melanogenesis, e.g., the hydroxylation of L-tyrosine into L-DOPA (3,4-dihydroxyphenylalanine) and its subsequent oxidation to dopaquinone. Hence, the identification of tyrosinase inhibitors is of great concern in the development of skin whitening agents [1,18].

The assays were performed in 96-well plates as follows: 150 µL of a solution of mushroom tyrosinase prepared at a concentration of 171.66 U/mL in phosphate buffer (50 mM pH = 6.8) are distributed in each well (enzyme's final concentration per well: 100 U/mL), together with 7.5 µL of samples diluted at a concentration of 3.433 mg/mL in DMSO (chromatography grade; sample's final concentration per well: 100 µg/mL).

Kojic acid (3.433 mM in DMSO) was used as the positive control; DMSO alone was used as the negative control ($OD_{control}$).

The plate is filmed and incubated at RT for 20 min. Then, 100 µL of a solution of L-tyrosine 1 mM in phosphate buffer pH = 6.8 (L-tyrosine's final concentration per well: 0.388 mM) were distributed in each well. After 20 min of incubation, OD reading was performed at 480 nm to assess the percentage of inhibition.

2.4.4. Lipoxygenase Assay

Lipoxygenase is an iron-containing enzyme known to play a key role in inflammation [19].

The assays were performed in 96-well UV-transparent plates as follows: 150 µL of a solution of soybean lipoxygenase prepared at a concentration of 686.66 U/mL in phosphate buffer (50 mM pH = 8) are distributed in each well (enzyme's final concentration per well: 400 U/mL), together with 7.5 µL of samples diluted at a concentration of 3.433 mg/mL in DMSO (chromatography grade; sample's final concentration per well: 100 µg/mL).

Quercetin hydrate (343.33 µM in DMSO) was used as the positive control; DMSO alone was used as the negative control ($OD_{control}$).

The plate was sealed and after 10 min of incubation, a first OD reading was performed at 235 nm (OD_{blank}).

Then, 100 µL of a solution of linoleic acid that was prepared in phosphate buffer pH = 8 were distributed in each well. After 50 min of incubation, OD reading was performed at 235 nm to assess the percentage of inhibition

2.5. High-Performance-Liquid-Chromatography

Crude extracts and fractions were analyzed using an HPLC Agilent 1200 series system equipped with a diode array detector (DAD) and an evaporative light scattering detector (ELSD). Crude extracts and fractions were diluted at 10 mg/mL in MeOH (chromatography grade), and filtrated over 0.45 µm PTFE syringe filter before analysis on a Luna C18(2) 100 Å column (Phenomenex, 150mm × 4.6 mm; 5 µm). The injection volume was 20 µL and the flow rate was set at 1.0 mL/min. The mobile phase consisted in a gradient of chromatography grade water (A), acetonitrile (B) and 2-propanol (C), all acidified with 0.1% acid formic (analytical grade): 0–30 min, 10–70% B; 30–55 min, 0–100% C; 55–57 min, 100% C. The DAD was set at 254, 280 and 366 nm and ELSD conditions were set as follows: nebulizer gas pressure 3.5 bars, evaporative tube temperature 40 °C, and gain 3.

2.6. Centrifugal Partition Chromatography Purification

The Centrifugal Partition Chromatography (CPC) experiments were performed on an Armen Instrument SCPC-250-L apparatus, coupled with Spot Prep II system (Armen Instrument) equipped with an integrated quaternary pump, a UV/vis detector (scanning wavelengths from 200 to 600 nm)

and an LS-5600 fraction collector (Armen Instrument). The latter was operated thanks to the Armen Glider CPC software.

CPC separation was performed in descending mode using a biphasic solvent system consisting in chloroform, methanol, and water (4/3/2 $v/v/v$). The hydrophilic stationary phase is first loaded into the CPC column and kept stationary by the centrifugal force that is generated by the rotation of the system (2000 rpm, flow rate 3 mL/min). The organic mobile phase is then pumped through the stationary phase for 10 min until equilibrium is attained. To perform separation, 450 mg of RL3-MeOH dissolved in 8 mL of mobile phase/stationary phase mixture (50/50) were injected in the CPC system. The upper mobile phase was subsequently pumped into the CPC 50 mL-column. The fractions' elution was carried out for 40 min (2000 rpm, flow rate 3 mL/min), and was followed by an extrusion phase lasting 35 min and performed with fresh stationary phase in the same mode and experimental conditions (e.g., flow rate and rotation settings). CPC effluent was monitored at 254 and 280 nm, and 10 mL collection tubes were used. The composition of all the fractions was evaluated by HPLC-DAD-ELSD, and the fractions were pooled together according to their chemical profiles.

2.7. UPLC-ESI-HRMS Analyses of Fractions

The fraction fingerprints were obtained using an UPLC Acquity system (Waters). Separations were performed on an Acquity UPLC BEH C18 column (Waters, 100 mm × 2.1 mm I.D., 1.7 µm) at 25°C with a flow rate of 0.300 mL/min. An Acquity guard column (Waters, 5 mm × 2.1 mm, 1.7 µm) with the same stationary phase being placed before the column. The mobile phase consisted of water (solvent A) and ACN (solvent B) both acidified with 0.1% formic acid (all of chromatography grade), and was used in multistep gradient mode. The gradient was operated as follow: 0 min, 5% B; 8 min, 50% B; 10 min, 100% B; final isocratic step for 4 min at 100% B. The sample manager was thermostated at 6 °C, and the injection loop was set at 0.5 µL. The HRMS and HRMS/MS data were acquired using a XEVO-G2QTOF instrument (Waters) over a mass range of 100–1500 *m/z*. ESI conditions operated in negative and positive modes were set as follow: source temperature 150 °C, desolvation temperature 500 °C; capillary voltage ± 3 KV and cone voltage 10 V. Nitrogen was used as cone (10 L/Hr) and desolvatation gas (1000 L/Hr). Lockspray flow rate was set at 20 µL/min and lockspray capillary voltage at ± 2.5 KV. For the HRMS/MS acquisitions, a method including the detection in full scan and fragmentation of the most intense peaks per scan was used. Collision energy was varying from 10 to 35 V.

2.8. ^{1}H NMR Analysis of Isolated Compounds

^{1}H NMR spectra were recorded in d_6-acetone or d_6-DMSO at 25 °C on a 200 MHz Bruker® Avance NMR spectrometer (Bruker, Wissembourg, France). Chemical shifts were expressed in Hz relative to d_6-acetone or d_6-DMSO.

3. Results and Discussion

As already stated, *R. luteola* is species displaying interesting agronomic characteristics, as well as good dyeing properties, and is nowadays considered as a non-food crop that is supporting the growing demand for natural colorants in several industrial sectors [10,11]. Due to its growing economic interest, the anti-oxidant potential, as well as the whitening and anti-inflammatory activities of *R. luteola* extract, were hence assessed using in vitro bioassays. *R. luteola* extracted with ethyl acetate exhibiting interesting whitening (42%), anti-inflammatory (54%), and anti-oxidant (67%) activities appeared to be particularly compelling for further investigation. If the *R. luteola* anti-inflammatory and anti-oxidant activities, mainly credited to its luteolin content, have already been discussed [20], to our knowledge, the literature only alludes to its skin whitening briefly. In fact, *R. luteola*, like other species from the order Brassicales, contains glucosinolates (sulfur- and nitrogen-containing secondary metabolites) and notably glucobarbarin, the hydrolysis of which leads to barbarin (= (*R*)-5-phenyl-2-oxazolidinethione), which is identified as a potent inhibitor of mushroom

and murine tyrosinases [21,22]. The objective of this work was hence to assess whether barbarin or other compounds yet to identify, are responsible for the whitening activity of *R. luteola* extracts.

3.1. Fractionation Strategy

To identify the bioactive compounds, especially those that are liable with this whitening activity, a bio-guided fractionation was undertaken. Soxhlet extractions of *R. luteola* aerial parts were performed using a succession of solvents to cover the complete polarity range of its constituting secondary metabolites: RL1 (extraction yield: 5.4%), RL2 (2.2%), RL3 (1.0%), RL4 (11.1%), and RL5 (13.8%) extracts were, respectively, obtained by soxhlet extraction of 50 g of *R. luteola* using cyclohexane, dichloromethane, ethyl acetate, ethanol/water 90/10, and water.

The respective biological activities of these resulting extracts were assessed (Table 1): RL4 exhibits interesting anti-oxidant (53%) and anti-inflammatory activities (63%), whereas RL3 extract exhibits important anti-inflammatory activity (52%) and prominent whitening potential (45%).

Table 1. Mean inhibitions (±SEM, n = 3) of 1-diphenyl-2-picrylhyorazyl (DPPH), tyrosinase, and lipoxygenase by the *R. luteola* extracts RL1 to RL5.

Extract	Inhibition (%)		
	DPPH	Tyrosinase	Lipoxygenase
RL1	7.00 ± 0.05	12.00 ± 0.03	/
RL2	7.00 ± 0.05	38.00 ± 0.03	/
RL3	25.00 ± 0.05	45.00 ± 0.01	52.00 ± 0.05
RL4	53.00 ± 0.05	18.00 ± 0.01	63.00 ± 0.05
RL5	16.00 ± 0.05	/	/
Vitamin C	99.00 ± 0.05	-	-
Kojic acid	-	96.00 ± 0.00	-
Quercetin hydrate	-	-	92.00 ± 0.05

RL3 extract was then selected for further in-depth investigation. The HPLC fingerprinting of this extract revealed the presence of two groups of components: a series of flavonoid derivatives eluting between 6 and 19 min and a series of highly apolar compounds eluting between 42 and 58 min (Figure 1).

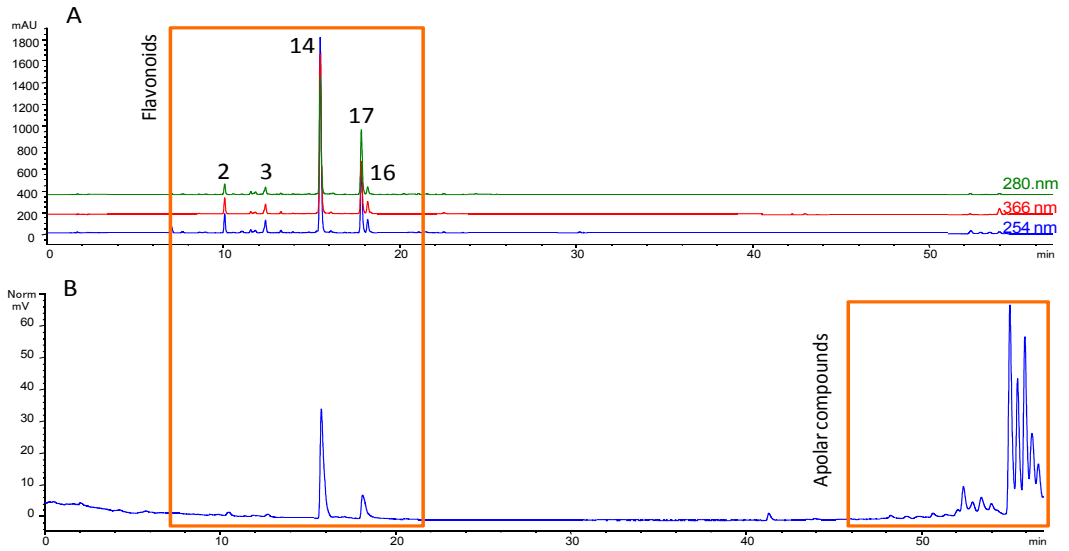

Figure 1. HPLC chromatograms obtained on a Luna C18(2) 100 Å column (150mm × 4.6 mm; 5 µm) at 254, 280 and 366 nm (**A**) and with ELSD (**B**), presenting the major families of compounds identified in RL-3 extract: flavonoids and hydrophobic compounds (peak identification as in Table 2).

Due to the important polarity difference between these two families of components, a liquid/liquid extraction was advocated to achieve their separation to determine which compound class was responsible for the whitening activity of the *R. luteola* extract. Hexane and methanol were selected to perform this liquid/liquid extraction, yielding, respectively, the fractions RL3-Hexane (extraction yield: 53%) and RL3-MeOH (extraction yield: 42%), the whitening and anti-inflammatory activities of which were tested (Figure 2). The sub-extract RL3-Hexane consisting in apolar compounds only displays very low anti-inflammatory activity, and no whitening potential. On the contrary, the sub-extract RL3-MeOH constituted of phenolic components displays increased whitening and anti-inflammatory activities compared to the initial RL3 extract.

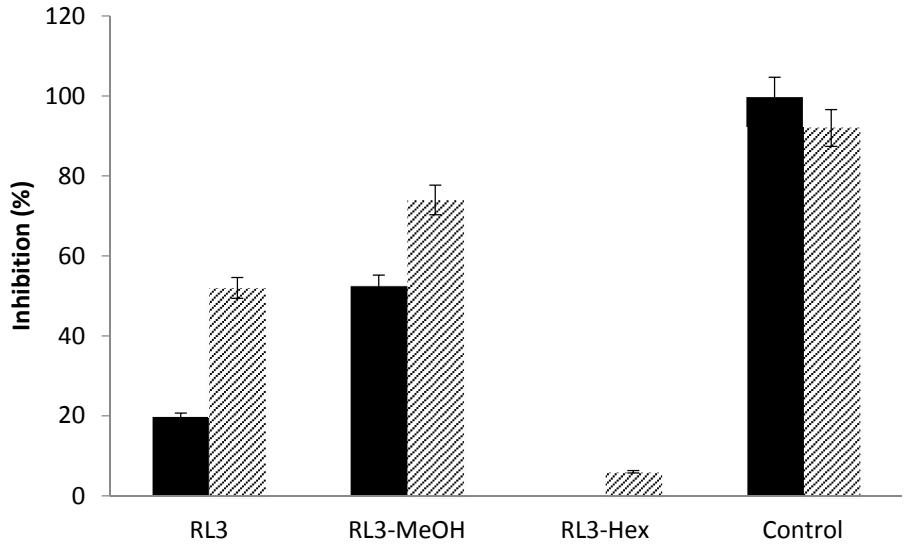

Figure 2. Tyrosinase (plain black) and lipoxygenase (hatched) inhibition of *R. luteola* sub-extracts RL3-Methanol and RL3-Hexane in comparison with those of the RL3 crude extract and control (kojic acid and quercetin were used as control for whitening and anti-inflammatory assays, respectively).

3.2. Putative Identification of Compounds

To characterize the metabolites composing the RL3-MeOH sub-extract, UPLC-HRMS analysis were undertaken. Briefly, all of the peaks well resolved in base peak ion chromatograms (BPI) that were ionized both in negative and positive modes were selected. To reduce the possible elemental compositions (EC) candidates, mass tolerance was set below 3 ppm, elemental formulas consisting solely of C, H, O, N, and S atoms were selected for calculations and only consistent ring double bond equivalent (RDBeq) values were considered. Additionally, HRMS/MS data, as well as bibliographic information, were employed to identify the compounds detected in sub-extract RL3-MeOH. Those components including their retention time (rt), their EC, their monoisotopic mass (*m/z*), the RDBeq and their major HRMS/MS fragments are listed in Table 2. Crossing those MS data (notably the parent ion and fragmentation patterns) with the literature [23,24], a number of flavones and flavonoid glycosides have tentatively been identified. However, the exact nature of some isomers could not be solved. For convenience, only negative mode will be described hereafter.

Table 2. Compounds identified in RL3-MeOH extract by Ultra Performance Liquid Chromatography-Mass Spectrometry (UPLC–HRMS).

Compound	rt (min)	Positive Mode			Negative Mode			RDBeq	Putative Identification
		$[M + H]^+$ *m/z*	EC	Fragment ions (*m/z*)	$[M - H]^-$ *m/z*	EC	Fragment Ions (*m/z*)		
1	6.53	527.0884	$C_{18}H_{23}O_{18}$	365.0335 153.0188	525.0695	$C_{18}H_{21}O_{18}$	363.0218 299.0564 150.9901 91.0100	8	Unknown
2	6.62	611.1619	$C_{27}H_{31}O_{16}$	449.1084 287.0568 365.0343	609.1461	$C_{27}H_{29}O_{16}$	447.0848 285.0420	14	Luteolin-3',7-diglucoside
3	7.67	449.1079	$C_{21}H_{21}O_{11}$	287.0555	447.0927	$C_{21}H_{19}O_{11}$	285.0382	12	Luteolin glycoside
4	8.67	433.1132	$C_{21}H_{21}O_{10}$	301.0723 271.0615 153.0190 119.0504	431.0975	$C_{21}H_{19}O_{10}$	268.0375	12	Apigenin-7-*O*-glucoside
5	9.12	491.119	$C_{23}H_{23}O_{12}$	287.0566 153.0566	489.1016	$C_{23}H_{21}O_{12}$	285.0397 429.0814	13	Acetylated luteolin-*O*-glycoside
6	9.30	449.1084	$C_{21}H_{21}O_{11}$	287.0558 153.0184	447.0927	$C_{21}H_{19}O_{11}$	285.0390	12	Luteolin glycoside
7	9.57	365.0328	$C_{12}H_{13}O_{13}$	286.0471 258.0539 153.0193	363.0173	$C_{12}H_{11}O_{13}$	299.0561 211.005 173.0255 151.0023 125.0230 109.0312	7	Unknown
8	9.91	491.119	$C_{23}H_{23}O_{12}$	287.0558 153.0184	489.1033	$C_{23}H_{21}O_{12}$	285.0413	13	Acetylated luteolin-*O*-glycoside
9	10.04	491.119	$C_{23}H_{23}O_{12}$	287.0558 153.0184	489.1033	$C_{23}H_{21}O_{12}$	285.0468	13	Acetylated luteolin-*O*-glycoside
10	10.21	475.124	$C_{23}H_{23}O_{11}$	271.0617 153.0184	473.1084	$C_{23}H_{21}O_{11}$	268.0369 269.0456 199.1416	13	Acetylated apigenin-*O*-glycoside
11	11.00	475.1247	$C_{23}H_{23}O_{11}$	271.0605 153.0184	473.107	$C_{23}H_{21}O_{11}$	285.0338 268.0369 269.0456	13	Acetylated apigenin-*O*-glycoside

Table 2. *Cont.*

Compound	rt (min)	Positive Mode			Negative Mode			RDBeq	Putative Identification
		$[M + H]^+$ *m/z*	EC	Fragment ions (*m/z*)	$[M - H]^-$ *m/z*	EC	Fragment Ions (*m/z*)		
12	11.40	533.1298	$C_{25}H_{25}O_{13}$	287.0558 153.0184	531.1096	$C_{25}H_{23}O_{13}$	327.2147 285.0399	14	Unknown
13	11.60	287.0561	$C_{15}H_{11}O_6$	269.0455 241.0492 213.0549 179.0345 153.0190 135.0447 117.0338	285.0401	$C_{15}H_9O_6$	241.0473 217.0513 199.0341 175.0378 151.0019 133.0285 107.0126	11	Luteolin
14	12.17	555.0931	$C_{30}H_{19}O_{11}$	403.0839 287.0559 153.0180	553.0783	$C_{30}H_{17}O_{11}$	459.0345 433.0530 391.0460 285.0389	22	Unknown
15	12.43	301.0718	$C_{16}H_{13}O_6$	286.0484 258.0535 229.0504 153.0199 105.0707	299.0553	$C_{16}H_{11}O_6$	284.0312 256.0360 227.0360 199.0379 151.0028	11	*O*-Methylluteolin
16	12.56	271.0609	$C_{15}H_{11}O_5$	229.0520 187.0404 153.0187 119.0499	269.0451	$C_{15}H_9O_5$	225.0554 201.0554 183.0455 151.0032 149.0236 117.0343 107.0128 83.0142	11	Apigenin
17	13.02	495.1291	$C_{26}H_{23}O_{10}$	409.0918 287.0552 257.0459	493.1097	$C_{26}H_{21}O_{10}$	403.3051 285.0408	16	Unknown
18	13.18	465.1179	$C_{25}H_{21}O_9$	447.1079 287.0549 286.0464 257.0454	463.102	$C_{25}H_{19}O_9$	283.0240 255.0293	16	Unknown

Compounds 13 (*m/z* 285.0401, rt = 11.6 min) and 16 (*m/z* 269.0451, rt = 12.56 min) were identified as luteolin and apigenin, respectively. Their identification was further confirmed by their typical HRMS fragmentation in negative mode [25]. Compounds 3 (*m/z* 447.0927, rt = 7.67 min) and 6 (*m/z* 447.0927, rt = 9.30 min) were identified as luteolin glycosides. In fact, they both exhibit a fragment ion at *m/z* 285.03, indicating the presence of a luteolin aglycone part together with the typical loss of a 162-unit corresponding to a hexose moiety (either glucose or galactose). Mass fragmentation patterns actually do not enable the resolution of the "isomers issue": indeed, one cannot determine the exact nature of sugar substituents (e.g., glucose vs. galactose, or whether the sugar is the α- or β-anomer) or it position on the aglycone part. Compounds 3 and 6 might hence correspond to luteolin-7-glucoside and luteolin-3′-glucoside, both already identified in *R. luteola* [12,13,26,27]. Luteolin-4′-glucoside, also identified in *R. luteola* [13], might be another possibility. Finally, the occurrence of galactosides, and notably luteolin-7-galactoside, identified in *R. phyteuma,* cannot be ruled out [28].

The fragmentation pathway of compound 15 (*m/z* 299.0553, rt = 12.43 min) is characterized by the loss of a methyl group and displays shared fragment ions with luteolin (*m/z* 199.0379 and *m/z* 151.0028) [25]. This compound could hence correspond to 7-*O*-methylluteolin or to 3′-*O*-methylluteolin (also known as chrysoeriol), both have already been identified in *R. luteola* [24,29].

Compound 2′s fragmentation pattern (*m/z* 609.1461, rt = 6.62 min) exhibiting the fragment ion at *m/z* 285.03 and the loss of two 162-units, this compound corresponds to a luteolin diglycoside. It was tentatively identified as luteolin-3′,7-diglucoside, already identified in *R. luteola* [12,13,27].

Compounds 5 (*m/z* 489.1016, rt = 9.12 min), **8** (*m/z* 489.1033, rt = 9.91 min) and 9 (*m/z* 489.1033, rt = 10.04 min) were identified as further luteolin derivatives, i.e., acetylated luteolin-*O*-glycosides. In fact, they exhibit a fragment ion at *m/z* 285.03 indicating the presence of luteolin as the aglycone part together with the loss of a 204-unit corresponding to a hexose moiety plus an acetyl group (neutral loss of 42 Da). To our knowledge, such derivatives were not previously identified in *R. luteola;* the occurrence of such a derivative, i.e., luteolin-7-*O*-(6″-*O*-acetyl)-glucoside, was reported in the leaves of another tinctorial plant, i.e., *Carthamus tinctorius* [30].

Similarly, the mass spectrum of compound 4 (*m/z* 431.0975, rt = 8.67 min) encloses a main fragment ion at *m/z* 268.0375, indicating the presence of apigenin as the aglycone part together with the loss of a 162-unit corresponding to a hexose moiety. Even if the exact position and nature of the sugar moiety could not be determined, further comparison with the literature enabled its tentative identification as apigenin-7-*O*-glucoside [13,23].

Compounds 10 (*m/z* 473.1084, rt = 10.21 min) and 11 (*m/z* 473.1070, rt = 11.00 min) exhibit a fragment ion at *m/z* 268.0369 indicating the presence of apigenin as the aglycone part, together with the loss of a 204-unit corresponding to a hexose moiety plus an acetyl group. To our knowledge, such derivatives were not previously identified in *R. luteola;* the occurrence of apigenin-7-(4″-*O*-acetyl)-glucoside and apigenin-7-(6″-*O*-acetyl)-glucoside was reported in *Chamomilla recutita* florets [31].

Finally, the remaining compounds, i.e., compounds 1, 7, 12, 14, 17, and 18 could not be identified based on their fragmentation patterns and the literature survey.

3.3. Final Purification and Identification of Active Compounds

To characterize the molecules that were responsible for the prominent whitening activity observed in RL3-MeOH sub-extract, purification of active compounds was undertaken by CPC. This versatile technique offer several advantages, the major ones being the almost complete sample recovery (permanent chemisorption is avoided by the elimination of the solid support matrix) and the decrease of the potentiality of individual compounds' decomposition [16]. First, the appropriate biphasic solvent system was determined experimentally: seven solvent systems that were previously used for flavonoids separation were selected from a literature survey [32–35], and their extraction effectiveness were evaluated by the determination of the distribution coefficient (K_d) and separation factor (α), calculated for four target analytes (compounds 2, 3, 14 and 17, Figure 1) in RL3-MeOH sub-extract

(Supplementary data 1). Theoretically, compounds from a mixture are eluted through the column according to their respective K_d, which is defined as the ratio of the molecule's concentrations in the upper and the lower phases, respectively ($C_{upper\ phase}/C_{lower\ phase}$). The nearest from 1 the K_d is, the more equally partitioned between both phases the molecules are and the most accurate the separation is. If the K_d is too small, compounds will elute very quickly and coelutions might happen. On the contrary, a K_d too large is consistent with an increased elution time. In addition, the separation factor α must be superior to 1.5 to ensure a suitable compounds separation.

The solvent system, consisting in chromatography grade chloroform, methanol, and water (4/3/2 *v*/*v*/*v*), already used to separate *Ginkgo biloba* and *Hippophae rhamnoides* flavonoids using high-speed counter-current chromatography [36] was hence selected as the one that gave suitable K_d and α values for an optimized RL3-MeOH sub-extract's fractionation. A total of 90 tubes were collected and gathered together following the CPC chromatogram (Supplementary data 2) to obtain 18 fractions (named RL3-F1 to RL3-F18 respectively), the whitening activities of which were again tested (RL3-F1 actually corresponding to the CPC solvent dead space was not tested for bioactivity; Figure 3).

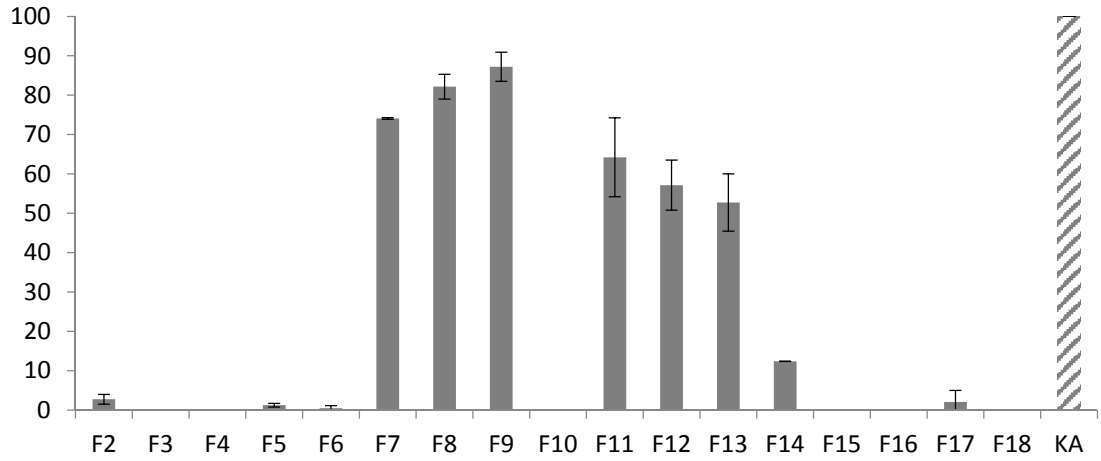

Figure 3. Whitening activity of *R. luteola* fractions F2 to F18 obtained by Centrifugal Partition Chromatography (CPC) fractionation of the sub-sample RL3-Methanol (mean whitening activity ± SD on 2 independent tyrosinase assays; KA: kojic acid, inhibition control).

The whitening activity from the RL3-MeOH extract appears to be concentrate into fractions F7 to F9 and F11 to F13 (Figure 3). Subsequent HPLC-DAD analysis of these fractions reveals that F7 to F9 actually correspond to compound 13 (Figure 4A), and that F12 and F13 correspond to compound 16 (Figure 4B). On the contrary, F11 consists in a mixture of molecules.

Consecutive HRESIMS and ^{1}H-NMR experiments (Supplementary data 3) confirmed that compounds 13 and 16, respectively, correspond to luteolin and apigenin.

Both of the molecules have been widely studied; their bioactivities have extensively been discussed. Luteolin's anti-allergic, anti-oxidant, anti-carcinogenic, anti-inflammatory, and notably whitening properties have already been largely documented [20,37–40]. Apigenin, particularly promising for cancer prevention [41], also displays a variety of engaging activities and notably anti-inflammatory and anti-oxidant properties [39,42]. Only weak whitening activity was reported for apigenin so far, but its bad solubilization in the water-based assay medium might distort the tests' results [43,44]. To confirm our results, the whitening activities of apigenin and luteolin were assessed in vitro following the same protocol as the one used previously to evaluate the activity of the *R. luteola* extracts and resulting fractions. Both of the analytical standards dissolved well in DMSO and present convincing tyrosinase inhibitory activities (luteolin: 76.28 ± 0.04% and apigenin: 60.60 ± 0.07%).

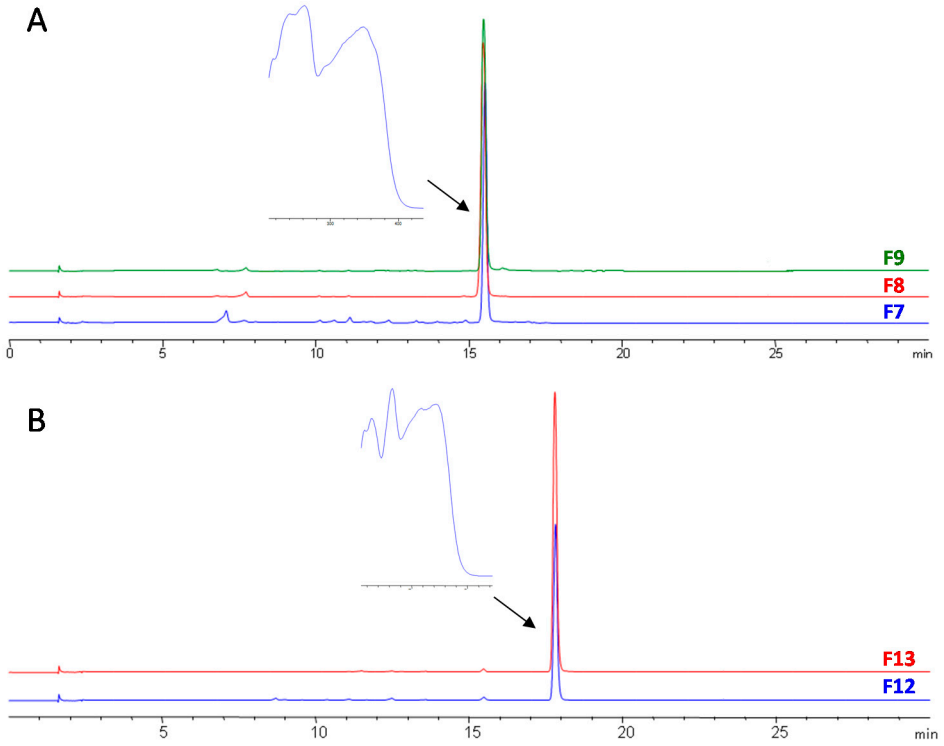

Figure 4. HPLC chromatograms obtained on a Luna C18(2) 100 Å column (150mm × 4.6 mm; 5 µm) at 254 nm, presenting the compositions of fractions F7 to F9 (**A**) and those of F12 and F13 (**B**).

4. Conclusions

This article reports for the first time the promising whitening activity of *R. luteola* extract, and the optimization of the extraction and CPC separation conditions that are suitable for the recovery of the flavones that are responsible for this activity. Such a purification procedure is highly engaging as no adsorption of components is likely to occur in such a liquid/liquid environment regarding to fractionation methods requiring solid matrices. Analysis being performed at room temperature also assured higher recovery rates as the probability of compounds' decomposition decreases [16]. Support-free partitioning techniques, such as CPC, are furthermore considered as time- and solvent-saving procedures. Finally, the method scale-up for industrial purposes appears to be possible with great accuracy [45].

Following this bioguided fractionation, the structural and molecular elucidation using UPLC-HRMS enabled, among others, the characterization of acetylated luteolin- and apigenin-*O*-glycosides, which occurrence is reported for the first time in *R. luteola*. UPLC-HRMS also facilitated the identification of the two bioactive principles as luteolin and apigenin. If luteolin was already known as a skin-lightening agent [37], apigenin was only reported to be a weak tyrosinase inhibitor, an observation that might be biased by its low solubility in water-based medium [43,44]. Their whitening potencies were hence further confirmed by tyrosinase inhibitory assays that were performed on analytical standards in the same experimental conditions as the ones that were used to assess the activities of the *R. luteola* extracts and fractions: both luteolin and apigenin appear to be potent tyrosinase inhibitors. Flavonoids have previously been identified as competitive tyrosinase inhibitors thanks to their ability to chelate the copper from the enzyme's active site, leading to the enzyme's irreversible inactivation [1,44].

Skin bleachers occupy an important part of the cosmetic market nowadays, and their usage is particularly widespread in countries with skin phototypes IV, V, and VI. The use of chemical substances (hydroquinone, corticoids, mercury salts, etc.) to lighten skin tone is authorized for hyperpigmented lesions' treatment under careful dermatological supervision. However, causing

severe side effects, the use of such substances as cosmetic ingredients has been banned by the EU Cosmetic Regulation 1223/2009 [46]. Nevertheless, facing the constant consumers' demand for bleaching agent providing an even skin complexion, the cosmetic industry is eagerly seeking natural alternatives [1]. The data presented here suggest that beyond its use as a dyestuff, *R. luteola* is potentially of great concern in the development of a natural whitening ingredient that is dedicated to cosmetics, cosmeceuticals, and eventually pharmaceutics, once its safety and tolerability are assessed. Furthermore, besides its lightening skin tone action, *R. luteola* extract also displays other interesting properties, e.g., anti-oxidizing anti-inflammatory effects, thus enlarging even more the marketing opportunities for a cosmetic formulation integrating it.

Supplementary Materials: The following are available online at www.mdpi.com/2079-9284/4/4/51/s1, Supplementary data 1; Supplementary data 2 and Supplementary data 3.

Acknowledgments: This work was made possible thank to the financial support provided by the 'Université Européenne des Senteurs et des Saveurs' (UESS). The authors would like to thank the ERINI institute (European Research Institute on Natural Ingredients) where UPLC-HRMS analysis was undertaken.

Author Contributions: O.B., P.C., S.A., X.F. and T.M. conceived and designed the experiments; O.B. and P.C. sourced the plant material; P.B., A.M. and T.M. performed the experiments and analyzed the data; P.B. and T.M. wrote the article; and all authors have equally contributed to its revision.

Conflicts of Interest: The authors declare no conflict of interest.

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