SUPPLEMENTARY MATERIAL

Chemical profile, antibacterial and antioxidant properties of Rubia tinctorum L. essential oils

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Abstract

Plants have gained great importance in the drug discovery and development process thanks to their bioactive secondary metabolites. *Rubia tinctorum* L. essential oil was obtained and analysed. Antioxidant and antibacterial activities were evaluated. Essential oils were extracted from the plant by steam distillation and the compounds were identified by GC-MS analysis. DPPH free radical scavenging and ferric reducing antioxidant power (FRAP) were used as a measure of antioxidant activity. Total antioxidant capacity (TAC) was also presented. The disc diffusion method was employed for testing antibacterial activity. Cyclohexanone (88.74 %) was found as a major product in essential oil. The essential oil did not show significant antioxidant capacity, while it demonstrated antimicrobial effect against Bacillus cereus ATCC 6633 (>13 mm of inhibition; 500 mg/ml) and Shigella ATCC 12022 (≥ 12 mm of inhibition; 500 mg/ml). Rubia tinctorum L. is new source of cyclohexanone.

Keywords: Natural products; bioactivity; GC-MS; *Rubia tinctorum*

Experimental

Experimental Chemical

The solvents, DPPH (1,1-diphenyl-2-picryl-hydrazyl), potassium ferricyanide [K3Fe (CN)6], trichloroacetic acid, ferric chloride (FeCl3), sodium phosphate (Na3PO4), ammoniummolybdate, sulfuric acid, gallic acid, sodium carbonate, potassium acetateand quercetin were obtained from Sigma–Aldrich GmbH (Sternheim, Germany).

Plant materials

Rubia tinctorum L. was harvested in June 2018 in Sidi Bel Abbes (Algeria). Botanical identification was executed by Dr. Righi K. at the Faculty of Natural and Life Sciences, Mustapha Stambouli University, Mascara (Algeria). The collected plant was dried at room temperature for 15 days and was finely powdered. A voucher specimen of the plant has been placed in the herbarium of the Faculty of Sciences at Mustapha Stambouli University (HAM 02 74).

Essential oil generation

The essential oil was isolated by hydrodistillation using a Clevenger type apparatus. The dry leaves of *Rubia tinctorum* (300 g) were mixed with distilled water (600 ml) and steam distillation was performed for 3 hours. The distillate was treated with diethyl ether and the organic phase was separated by decantation to obtain the essential oil that will be stored in a refrigerator at 4°C until its use (*Karan, Yildiz, et al., 2018*).

GC-MS analysis

The analysis of essential oils was performed on a Trace 1310 gas chromatograph equipped with an ISQ single quadrupole mass spectrometer (Thermo Fisher Scientific, Austin, TX). The process was initially set at 60°C for 6 min, then set at 1°C/min to 145°C, maintained at 145°C for 0.50 min, then held at 20°C/min to 230°C and finally for 20 min at 230°C.The mass detector was configured with an electron impact (EI) mode at 70 eV (Türkmen et al.,2014). The ion source and detector temperatures were 250°C.1 mL of sample was dissolved in 5 mL of 100% CHCl3 and then filtered through a 0.22 µm disposable syringe filter. A diluted sample of 1.0 µL was injected in the split mode (20:1). Separation of the sample was performed on a Thermo TG- WAXMS GC column (60 m × 0.25 mm ID × 0.25 µm) using helium as carrier gas at 1.2ml/min. Mass spectral scan range was set at the rate of 55–550 m/z (Adams, 2007). Retention indices determined regarding homolog series of normal alkanes. The relative amounts of the individual components of the total essential oils were reported as a percentage of the peak area relative to the total peak area. The quantification was performed by the external standard methodusing calibration curves generated by GC analysis of representative compounds (Sara

et al., 2021). Peak identification was carried out by comparing known components stored in the NIST Demo, Wiley7, Wiley9, redlip, mainlip, WinRI.

Antioxidant activity

DPPH free radical scavenging assay

The antioxidant activity of essentials oils was measured by the method DPPH (1, 1- diphenyl-2-picrylhydrazyl) according to the protocol described in the literature (*Elmastas et al., 2004*) with some modifications. 200 µL of essentials oils diluted in methanol at varying concentrations (0.031-1mg/mL-1) were added to 2 mL of 0.004 % DPPH solution. Ascorbic acid was used as a positive control. The negative control was prepared by the same method by mixing 200 µL of methanol with 2 mL of a methanolic solution of DPPH. After stirring with a vortex, all the mixtures were left in the dark for 30 minutes and the optical density was measured at 517 nm using a UV-visible spectrophotometer (Shimadzu) against a blank which contains 200 µL of each concentration of the extract or standard and 2 mL of methanol. The scavenging ability of the plant on DPPH was calculated using the following equation (1)

Scavenging ability (%) = (A0 -A1) / A0) × 100 (1) Where A0 is the control and A1 is the sample absorbance.

Ferric reducing antioxidant power (FRAP)

The reducing power of *Rubia tinctorum* was determined according to the method given (*Erenler et al., 2015*). 0.5 mL of different concentrations of oils (0.093-4 mg/mL) was mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL (1 %) of potassium ferricyanide [K3Fe (CN)6]. Afterwards, the mixture was heated at 50 °C in a water bath for 20 minutes. After the incubation, 2.5 mL of trichloroacetic acid (10 %) was added and themixture was centrifuged for10 min at 3000 rpm. 2.5 mL of supernatant was removed and mixed with 2.5 mL of distilled water and 1.0 mL of ferric chloride (1 %). The absorbance was measured at 700 nm against a blank without a sample prepared under the same conditions. Ascorbic acid was used as a positive control. The higher absorbance represents the higher power of reduction.

Total antioxidant capacity (TAC)

Preparation of Molybdate Reagent Solution: 1 mL of 0.6 M sulfuric acid, 28 mM of sodium phosphate and 4.0 mM of ammonium molybdate were added to 20 mL of distilled water and the volume was raised to 50 mL by addition of distilled water (*Phatak & Hendre, 2014*). The total antioxidant capacity (TAC) of *Rubia tinctorum* essentials oils was spectrophotometrically evaluated by the phosphomolybdenum assay using the technique as described in (*Idris et al., 2017*). A volume of 3 mL of reagent (28 mM sodium phosphate, 0.6

M sulfuric acid and 4 mM ammonium molybdate) was added to 0.3 mL of plant sample extract and standard ascorbic acid at different range of concentrations (0.031-1 mg/mL). The mixture was heated in a water bath for 90 min at 95 °C. The absorbance was measured against the blank (solvent) at 695 nm. Ascorbic acid was used as the positive control. The percentage of inhibition of different extract was calculated using the following equation (2):

$$TAC = ((A0 - A1) / A0)\ 100 \quad (2)$$

Where A0denotesabsorbance of the control reaction and A1 represents Absorbance in the presence of sample.

Antibacterial activity

The antibacterial activity of the essential oil was determined by the disk diffusion method according to the National Committee for Clinically Laboratory standard (NCCLS, National Committee for Clinical Laboratory Standards, 1997) which is based on the spread of antimicrobial compound in solid medium.The microorganism strains including *Staphylococcus aureus* ATCC 25923, *Bacillus cereus* ATCC 6633, *Salmonella* enteritidis ATCC 13076, *Shigella* ATCC 12022 and *Pseudomonas aeruginosa* ATCC 27853 microorganisms were obtained from Pasteur Institute of Algeria. Bacterial suspensions were adjusted by comparing against 5.0 McFarland and spread in Mueller Hinton agar using sterile swabs. Subsequently, filter paper discs (6 mm) were placed on solid media plates and impregnated with 20 µL of the essential oil (500 mg /mL,250 mg/ml, 125 mg/mL, 62.5 mg/mL). Negative controls were prepared with DMSO (20 µL), while positive controls were prepared with nalidixic acid and vancomycin. All Petri dishes were kept at 4 °C for 2 h and then incubated at 37 °C for 24 h. Thediameters (mm) of the inhibition zones were measured.

Statistical analysis

The experiments were repeated three times. The relations between the experimental parameters were investigated using one-way ANOVA. Differences were considered statistically significant when $P < 0.05$.

Legends of tables and figures

Table -S1: Chemical composition of *Rubia tinctorum* essential oil.

Table -S2; Antimicrobial activity of *Rubia tinctorum* essential oil using agar diffusion method.

Figure S1. Chemical constituents of *Rubia tinctorum* essential oil.

Figure S2. Antioxidant activity of *Rubia tinctorum* essential oil using the DPPH assay.

Figure S3. Antioxidant activity of *Rubia tinctorum* essential oil using the FRAP assay.

Figure S4. Antioxidant activity of *Rubia tinctorum* essential oil using Phosphomolybdete assay

Table -S1. Chemical composition of *Rubia tinctorum* essential oil.

No	Compounds	[a]RT	[b]RI	[c]RI	[d]%
1	Cyclohexanone	23.85	1285	1295	88.74
2	Bicyclohexyliden-2-one	95.23	1551	1564	1.22
3	Diplodialide B	96.45	1656	1660	3.49
4	Hexahydrofarnesyl acetone	97.25	1838	1845	1.19
5	Methyl-10-octadecenoate	97.75	2085	2092	1.97
6	Phytol	105.67	2593	2601	2.40

[a]RT: Retention time, [b]Retention index of references, [c]Retention index calculated from retention times relatives to that of the n-alkane series, [d]% Compound percentages.

Table -S2. Antimicrobial activity of *Rubia tinctorum* essential oil using agar diffusion method.

Microbial strains	**Inhibition zones (mm)**					
Concentration in mg. mL^{-1}	**500**	**250**	**125**	**62.5**	**NA**	**VA**
Bacillus cereus ATCC 6633	13.5±0.9^{a}	11.0±0.2^{a}	9.0±0.2^{a}	ND	29±1.2^{d}	21±0.9^{b}
Staphylococcus aureus ATCC 25923	12.0±0.7^{b}	9.0±0.4^{d}	8.0±0.6^{b}	ND	30±0.9^{c}	25±0.4^{a}
Pseudomonas aerugenosa ATCC 27853	11.0±0.4^{c}	9.5±0.7^{c}	9.0±0.1^{a}	ND	35±1.4^{a}	12±0.2^{e}
Salmonella entreritidis ATCC 13076	11.0±0.3^{c}	9.0±0.9^{d}	ND	ND	32±1.6^{b}	15±0.6^{d}
Shigella ATCC 12022	12.5±0.5^{b}	10.5±0.3^{b}	9.0±0.2^{a}	ND	30±1.3^{c}	19±0.8^{c}

*ND: no detected, NA: nalidixic acid, VA: voncomicyn acid. The values were analysed by the unpaired Tukey test; each value is expressed as the mean ± SD (n = 3). Values in the same column with different superscripts are highly significant ($P < 0.05$) different.

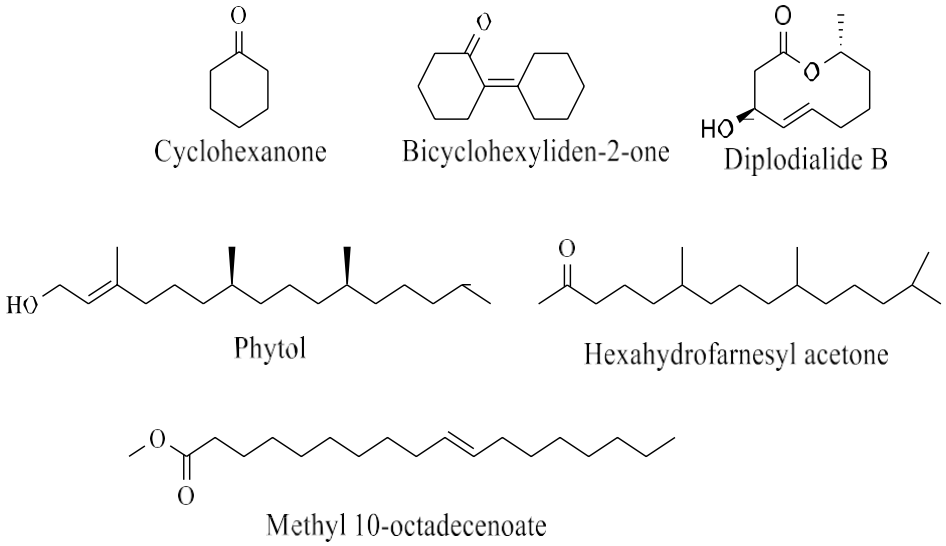
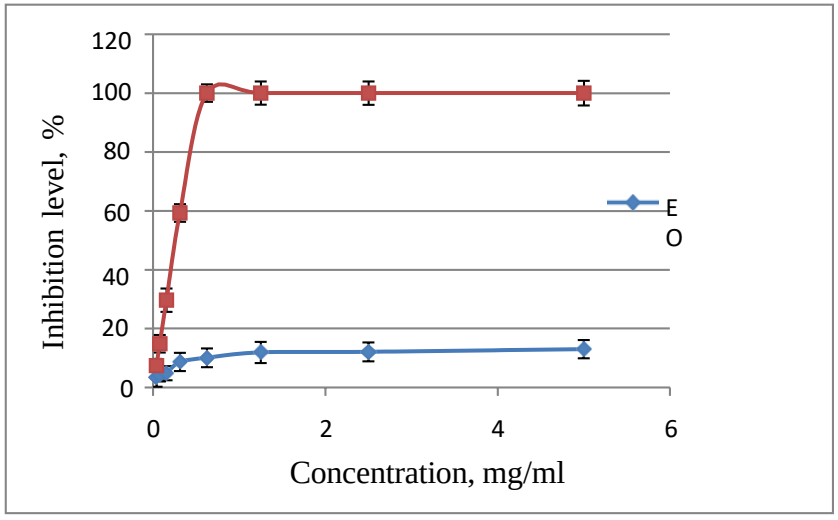
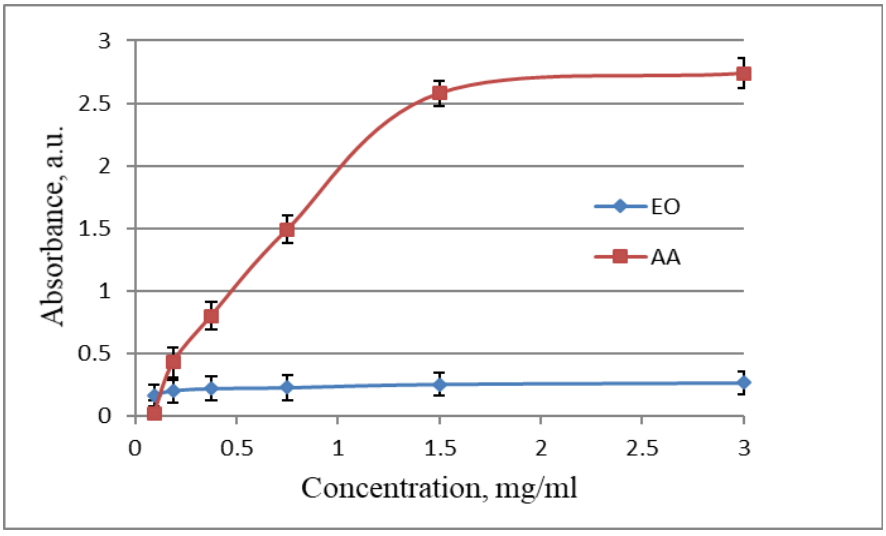


Figure S1. Chemical constituents of *Rubia tinctorum* essential oil.



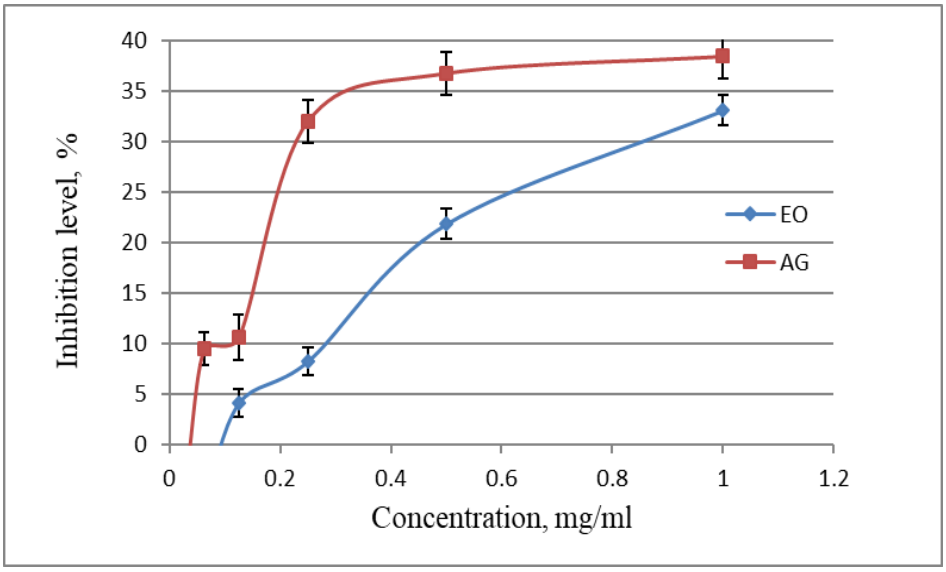
All value is expressed as the mean ± SD (n = 3).

Figure S2. Antioxidant activity of *Rubia tinctorum* essential oil using the DPPH assay.



All value is expressed as the mean ± SD (n = 3).

Figure S3. Antioxidant activity of *Rubia tinctorum* essential oil using the FRAP assay.



All value is expressed as the mean ± SD (n = 3).

Figure S4. Antioxidant activity of *Rubia tinctorum* essential oil using Phosphomolybdete assay.

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