SUPPLEMENTARY MATERIAL

Phytochemical Constituents and Biological Activities of *Cleome iberica* DC.

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Abstract: Phytochemical investigation of *Cleome iberica* DC. aerial parts led to the isolation and characterization of six triterpenoid including glutinol (**1**), lupeol (**2**), β -sitosterol (**3**), cabraleadiol monoacetate (**4**), cabraleadiol (**6**), and daucosterol (**8**), one flavonoid, calycopterin (**5**) along with a fatty acid, tricosanoic acid (**7**). While studied samples showed weak potency in antimicrobial assessment, the acetone extract and some isolated compounds exhibited considerable cytotoxicity against cervical cancer cell line (HeLa) in MTT assay, among them calycopterin with IC_{50} equals to 5.1 μ M was the strongest. The extract showed moderate antioxidant capacity in DPPH assay method (IC_{50} = 100.2 μ g/ml) and total phenolics and total flavonoids contents of the dried extract were also determined as 39.6 mg/g and 90.7 mg/g, respectively.

Keywords: *Cleome iberica* DC., Cleomaceae, triterpenoid, flavonoid, MTT assay, antioxidant

1. Experimental

1.1. Plant material and extraction

Aerial parts of *Cleome iberica* DC. were collected in August 2011 from Kashan, Isfahan province and a voucher specimen was deposited under the number 1352 at the herbarium of Kashan Botanical Garden (KBGH). The air-dried and powdered aerial parts (3kg) were macerated with acetone (5×20L) at room temperature. Evaporation of the solvent under reduced pressure at 45°C provided acetone extract.

1.2. Isolation and purification of compounds

The obtained acetone extract (85g) was chromatographed on a 5.5×110 cm silica gel column (70-230 mesh, Merck, Germany), eluted with hexane-ethylacetate (100:0 to 0:100) and then ethylacetate-methanol (95:5 – 90:10) to give thirty seven fractions. Fractions A8 and A9 were separately precipitated with hexane to give compounds 1 (30mg) and 2 (40mg) as white powders. Compound 3 (35mg) was isolated by recrystallization of fraction A12 from hexane. Isolation of compound 4 (8mg) was achieved via chromatography of fraction A13 (1g) over a 2×30 cm silica gel column (70-230 mesh), eluted by chloroform-methanol (95:5). Fraction A24 was washed and recrystallized with acetone to afford compound 5 as white precipitate (21mg). Fraction A18 was separated by a 2×35 cm silica gel column (70-230 mesh), eluting with chloroform-methanol (85:15) to obtain compounds 6 (7mg) and 7 (5mg). Recrystallization of fraction A37 by methanol resulted in purification of compound 8 (42mg).

1.3. Total phenolics content estimation

Total phenolics content of the extract was calculated by a colorimetric method using Folin-Ciocalteu reagent. An aliquot of 1.5 ml from freshly prepared aqueous Folin-Ciocalteu reagent solution (10% v/v) was added to 200 µl of the extract solution (500 µg/ml in methanol) and kept at room temperature. After 5 min, 1.5 ml of sodium bicarbonate solution (60 mg/ml) was added to the mixture and stored for another 90 min at room temperature. Absorbance of the prepared solution was measured by spectrophotometer (Cintra 6, GBC, Australia), at 725 nm.

A calibration curve was obtained at the detection wavelength of 725 nm for gallic acid using a series of standard solutions over the concentration range from 10 to 200 µg/ml. The line equation was used to calculate total phenolic content.

1.4. Total flavonoids content estimation

Total flavonoids content was measured by a spectrophotometric assay based on Dowd method. In brief, equal volumes of the extract solution (500 µg/ml in methanol) and AlCl₃ 2% in methanol was mixed together and absorption was recorded at 415 nm after 10 min against a blank consisting of same volumes of extract solution and methanol without AlCl₃. The total flavonoid content was calculated using a standard curve with rutin (5 – 100 µg/ml) as standard.

2. Biological evaluations

2.1. MTT assay

Cytotoxicity potentials of acetone extract from aerial parts of *C. iberica*, and compounds 1, 2, 5 and 7 were evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. HeLa cells (5000 cells/well) were plated in a 96 well microplate at 37°C. After attachment, the cells were treated by the mentioned test samples and Paclitaxel as positive control for 24 hours. 200µL fresh medium containing 20µL MTT was added to each well, then incubated at 37°C for an additional 4 hours. The resultant formazan crystals were dissolved in 200µL DMSO and the absorbance intensity was measured at 545nm with a reference wavelength of 630nm, by a microplate reader spectrophotometer.

2.2. Disk Diffusion assay

Antimicrobial evaluation of extract and some of the isolated compounds including 1, 2, 5 and 8 against selected microorganisms (11 strains) was performed via disk diffusion method. The microbial strains which were obtained from Iranian Research Organization for Science and Technology (IROST) were as follow: *Escherichia coli*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Salmonella enterica subsp. enterica serovar Paratyphi*, *Shigella dysenteriae*, *Klebsiella pneumonia*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Candidia albicans*, *Aspergillus niger* and *Aspergillus brasiliensis*.

The solutions of extract and the compounds were prepared in DMSO (30 mg/ml) and filtered through 0.45 µm Millipore filters to sterilize. 100 µl of microbial suspensions containing 10⁸ CFU/ml of bacteria, 10⁶ CFU/ml of yeasts and 10⁴ spore/ml of molds were spread on to the nutrient agar (NA), sabouraud dextrose agar (SDA) media and potato dextrose agar (PDA) media respectively. The discs (6 mm in diameter) impregnated with 10 µl of the test solutions (300 µg/disc) and DMSO (as negative control) were placed on the inoculated agar surfaces. The plates were then incubated at 37°C for 24 hours for bacterial strains and 48 and 72 hours at 30°C for yeast and mold isolates respectively. Gentamicin (10 µg/disc) and rifampicin (5 µg/disc) for bacteria and nystatin (100 I.U.) for fungi were used as positive controls. The antimicrobial activity was evaluated by measuring the diameter of inhibition zone.

2.3. DPPH free radical-scavenging assay

The extract stock solution (1.0 mg/ml) was diluted to give final concentrations of 500, 250, 100, 50, 20 and 10 µg/ml, in methanol. A total of 1 ml of a freshly prepared 80 µg/ml DPPH in methanol solution was added to 1 ml of each diluted sample solution and were kept in dark place at room temperature and allowed to reaction for 30 min. Butylated hydroxytoluene (BHT), a commercial well-known antioxidant, was used as positive control. Absorbance of solutions were measured by spectrophotometer (Cintra 6, GBC, Australia), at 517 nm. Inhibition percent of free radical DPPH was calculated as follow:

$$I\% = [(A_{b_a} - A_{b_b}) / A_{b_a}] \times 100 \quad (A_{b_a}: \text{absorbance of DPPH solution (40 } \mu\text{g/ml)}, A_{b_b}: \text{absorbance of sample solution})$$

IC₅₀ values were calculated from the equation of lines obtained by plotting a graph of concentration (µg/ml) versus inhibition percent.

Figure S1 (H NMR spectrum of Glutinol)

Figure S2 (HMQC-DEPT spectrum of glutinol)

Figure S3 (Detail of the COSY spectrum of glutinol)

Figure S4 (Detail of the HMBC spectrum of glutinol)

Figure S5 (H NMR spectrum of lupeol)

Figure S6 (COSY spectrum of lupeol)

Figure S7 (HMQC-DEPT spectrum of lupeol)

Figure S8 (Detail of HMBC spectrum of lupeol)

Figure S9 (H NMR spectrum of cabraleadiol monoacetate)

Figure S10 (Detail of COSY spectrum of cabraleadiol monoacetate)

Figure S11 (Detail of HMBC spectrum of cabraleadiol monoacetate)

Figure S12 (¹H NMR spectrum of cabraleadiol)

Figure S13 (HMQC-DEPT spectrum of cabraleadiol)

Figure S14 (HMQC-DEPT spectrum of calycopterin)

Figure S15 (Detail of HMBC spectrum of calycopterin)

Figure S16 (H NMR spectrum of tricosanoic acid)

Figure S17 (COSY spectrum of tricosanoic acid)