PHYTOCHEMICAL SCREENING AND BIOLOGICAL EVALUATION OF DYPSIS LEPTOCHEILOS LEAVES EXTRACT AND MOLECULAR DOCKING STUDY OF THE ISOLATED COMPOUNDS

HAITHAM ALI IBRAHIM1*, FATEHIA SAYED ELSHARAWY2, MAHMMOUD ELHASSAB2, SAMAH SHABANA3, EMAN GABER HAGGAG1

1Department of Pharmacognosy, Faculty of Pharmacy, Helwan University, Ein Helwan, 11795, Cairo, Egypt, 2Department of Pharmaceutical chemistry, School of Pharmacy, Badr University, Badr City, 31669, Cairo, Egypt, 3Department of Pharmacognosy, Faculty of Pharmaceutical Sciences and Drug Manufacturing, Misr University for Science and Technology, 6th of October, 12556, Cairo, Egypt

Email: haithamali081@gmail.com

Received: 20 Aug 2020, Revised and Accepted: 28 Sep 2020

ABSTRACT

Objective: phytochemical investigation of the ethyl acetate fraction (EAF) of 80% aqueous methanol extract (AME) of Dyopsis leptocheilos leaves, in addition to evaluation of the antioxidant, cytotoxic and antimicrobial activities of the AME and EAF. Docking was used to predict and understand cytotoxicity of the isolated compounds.

Methods: The ethyl acetate fraction (EAF) of Dyopsis leptocheilos leaves was subjected to different chromatographic separation techniques. Structures of the isolated compounds were established by different spectroscopic techniques (1H/13C NMR). Antioxidant activity was evaluated by DPPH assay, while cytotoxicity was evaluated by MTT cell viability assay. Antimicrobial activity was evaluated by agar diffusion method. The docking study was conducted using Auto Dock Vina, the estrogen receptor (PDB 5r92) was used as a receptor for the docking.

Results: Chromatographic separation techniques were led to the isolation of five phenolic compounds; these compounds were identified to be apigenin 8-C-β-D-glucopyranoside (Vitexin) (1), apigenin 6-C-β-D-glucopyranoside (Isovitexin) (2), luteolin 7-O-β-D-glucopyranoside (3), luteolin 8-C-β-D-glucopyranoside (Orientin) (4), luteolin 6-C-β-D-glucopyranoside (Isoorientin) (5). They were isolated and identified for the first time from this plant species. The AME and EAF showed moderate activity against Gram positive and Gram negative bacteria, while both of them showed similar and powerful antioxidant activity with SC50 = 12.8±0.56 µg/ml and SC50 = 17±0.77 µg/ml respectively, compared to ascorbic (reference drug) SC50 = 1.42±0.35 µg/ml. The EAF showed higher cytotoxic activity on the MCF-7 cells (human breast cancer cell line), with IC50 = 12.3 ± 1.82 µg/ml, compared to Vinblastine Sulfate (reference drug). All isolated compounds showed good binding affinity to the estrogen receptors existed in the MCF-7 cell.

Conclusion: Five phenolic compounds were isolated for the first time from the EAF of Dyopsis leptocheilos leaves. The AME and EAF extracts showed variable antioxidant, antimicrobial and cytotoxic activities.

Keywords: Antimicrobial, Antioxidant, Cytotoxicity, Docking, Dyopsis leptocheilos, Polyphenolic

INTRODUCTION

Medicinal plants are the richest bio-resource of drugs related to the traditional medical system, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates, and chemical entities for synthetic drugs [1]. The medicinal plants are beneficial for curing human diseases because of the presence of phytochemical constituents. Several important medicinal components are derived from plants like alkaloids, flavonoids tannins, terpenoids, steroids etc [2].

Dyopsis is one of the largest single genera in the palm family, with over 170 species ranging from tiny understory plants to massive emergent canopy palms and just about every size in between. Dyopsis are an extraordinary phenomenon of evolutionary diversity; this genus is composed of numerous former separate palm genera, including Vonitra, Chrysalidocarpus, Phloga, Neophloga, Phugella, Trichophyta, Naphodysia, Adelodypsia, Antotiglia, and Neodypsia. Dyopsis were catalogued and described in great detail in “The Palms of Madagascar” a seminal work by Dr. John Dransfield and Henri Beentje, first published in 1995 by Kew Botanical Gardens [3]. By reviewing the current literature, it was found that no reports dealt with the phytochemical study of D. leptocheilos and this encouraged our team to work for isolation and identification of its Phyto-constituents in addition to evaluation of its therapeutic activities.

On the basis of the fact, that said the development of antibiotic resistance in microorganisms is a global challenge for the clinicians, pharmacist, and research scientists leading to the development of new medicinal formulations that are effective and easily consumable [1] we decided to study antimicrobial activities of D. leptocheilos. As Anti-oxidants play an important role in protecting and safeguarding health problem especially in the disease such as cancer [4] that encouraged our team to measure antioxidant activity of D. leptocheilos. Molecular docking of isolated compounds was carried out as it is a method which predicts the preferred orientation of one molecule to a second when bound to each other to form a stable complex [5]. Molecular docking research focuses on computationally simulating the molecular recognition process. It aims to achieve an optimized confirmation for both the protein and ligand and relative orientation between protein and ligand such that the free energy of the overall system is minimized [6].

In this study, the isolation of five phenolic compounds from D. leptocheilos leaves extract was reported and their molecular docking study was performed, in addition to the evaluation of the antioxidant, cytotoxic and antimicrobial activities of the AME and EAF extracts.

MATERIALS AND METHODS

Plant material

The leaves of Dyopsis leptocheilos (Arecaaceae) were collected from Al-Abed garden, Alexandria-Egypt, desert road in August 2017. The plant material was identified by Dr. Teras Labib, Department of Flora and Taxonomy, Al-Orman Botanical Garden, Giza, Egypt. A
voucer specimen No.01/De/2017 was kept in the herbarium in the Department of Pharmacognosy, Faculty of Pharmacy, Helwan University, Egypt.

Materials for biological studies

Mammalian cell lines: MCF-7 cells (human breast cancer cell line), HepG2 (human hepatocellular carcinoma) and HeLa cells (human cervical cancer cell line), were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Fungi: Aspergillus fumigatus (RCMB 002008) and Candida albicans (RCMB 005003) (1 ATCC 10231), Gram-positive bacteria: Staphylococcus aureus (RCMB010010), Bacillus subtilis (RCMB 015) (1 NRRL B-543), Gram-negative bacteria: Salmonella typhiurium (RCMB 006 (1 ATCC 14028), Escherichia coli (RCBM 010052) were supplied from the Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University, Cairo, Egypt.

Instruments and chemicals for biological studies

All cell lines were cultured in DMEM media supplemented with 10% (v/v) foetal bovine serum, 2 mmol glutamine and 50 μg/ml penicillin/streptomycin solution (all from Invitrogen, Paisley, UK). Dimethyl sulfoxide (DMSO), MTT and trypan blue dyes were purchased from Sigma-Aldrich Co., UK. Authentic reference flavonoid compounds were supplied by Pharmacognosy department, Faculty of Pharmacy, Helwan University. Authentic sugars were purchased from Sigma-Aldrich Co., UK. A microplate reader (Sunrise, TEGAN, Inc, USA), the 96-well plate used for cytotoxicity evaluation using cell viability assay. UV-visible spectrophotometer (Milton Roy, Spectronic 1201), used for measuring the absorbance in the antioxidant assay.

Instruments and materials for chromatographic techniques

The NMR spectra were recorded using Bruker a 400 MHz for 1H NMR and 100.40 MHz for 13C NMR. The spectra were run in DMSO, and chemical shifts were given as δ ppm relative to tetramethylsilane (TMS) as an internal standard. Negative ES-MS were run on LQc deca MS and LTQ-FT-MS spectrometers for MS analysis (Thermo Electron, Finnigan, Germany). For column chromatography, silica gel G60 for column chromatography (70-230 mesh, Merk), silica gel G60 for thin layer chromatography (E. Merk, Germany), sheets of Whatman filter paper (1 mm) for paper Chromatography (Whatman11, Maid stone, Kent, England), microcrystalline cellulose (E. Merk-Darmstadt, Germany) and polyamide 65 (Riedel-De-Haen AG, SeelzeHanver, Germany). Solvent systems for paper chromatography: Si (n-BuOH:H2O:Ac-H2O 4: 1: 5, top layer), Si5: (15% aqueous H2O) were used [7].

Extraction and isolation

Air-dried leaves of D. leptochellos (1 kg) were coarsely ground and extracted exhaustively with 80% methanol/H2O (5 L x 4, 60 °C, 4 h). Then, the total extract was evaporated to dryness under reduced pressure to yield 115 g of the dark brown residue. This residue was reconstituted with 300 ml H2O then fractionated with 3 x 300 ml of petroleum ether and ethyl acetate by liquid-liquid phase separation yielding four fractions weighing (15 g petroleum ether fraction, 17 g ethyl acetate fraction and 65 g aqueous fraction). 2D-PC of the fractions revealed the presence of a pronounced number of flavonoid spots in the ethyl acetate fraction, which were detected under UV-light and spray reagents. The EAF (10 g) was fractionated on silica gel column (5 x 90 cm), the column was eluted using 25:75 petroleum ether/CH2Cl2 mixtures with increasing polarity till 100% CH2Cl2 then increasing polarity using methanol up to 25:75 (MeOH/CH2Cl2), yielding 46 individual fractions, collected into 6 fractions (I-VI). Fraction I was found to be polyphenolic free. Fraction II (450 mg) was rechromatographed on polyamide column (2.5 x 50 cm), the column was eluted using water then H2O/MeOH mixtures (0-40% MeOH) to yield 6 subfractions (i-vi), the promising fraction was subfraction iii, (89 mg), showed one dark purple spot which was further purified on cellulose column using H2O/MeOH, to afford pure compound 1 (14 mg). Fraction III (650 mg) was fractionated on polyamide column (3 x 65 cm), the column was eluted using water then H2O/MeOH mixtures with decreasing polarity to yield 5 subfractions (i-v), the promising fractions were subfractions iii and v, which were further individually purified on cellulose column using H2O/MeOH, to afford pure compound 2 (15 mg) compound 3 (20 mg). Fraction IV (320 mg) was fractionated on a cellulose column using H2O-MeOH (1:5-90%) mixtures, giving two subfractions, i and ii, which were further individually purified on sephadex LH-20 columns, using BrijOH, to afford pure samples of 4 (12 mg) and 5 (10 mg), respectively.

Antioxidant activity (DPPH radical scavenging activity)

The antioxidant activity of the AME and EAF was determined at the Regional Center for Mycology and Biotechnology (RCMB) at Al-Azhar University by the DPPH free radical scavenging assay in triplicate and mean values were considered [8]. Freshly prepared (0.004% w/v) methanol solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was prepared and stored at 10 °C in dark. A methanol solution of the tested extracts were prepared with sample concentrations (0, 10, 20, 40, 80, 160, 320, 640, 1280 μg/ml). A 40 μl aliquot of the methanol solution was added to 3 ml of DPPH solution. Absorbance measurements were recorded immediately with a UV-visible spectrophotometer. The decrease in absorbance at 515 nm was determined continuously, with data being recorded at 1 min intervals until the absorbance stabilized (16 min). The absorbance of the DPPH radical without antioxidant (control) and the reference compound ascorbic acid were also measured. All the determinations were performed in three replicates and averaged. The percentage of DPPH radical scavenging was calculated according to the formula: 

\[
\text{scavenging activity} = \left( \frac{\text{AC} - \text{AT}}{\text{AC}} \right) \times 100 \%
\]

Where AC: Absorbance of the control at t = 0 min and AT: absorbance of the sample+DPPH at t = 16 min.

Antimicrobial activity

The AME and EAF of D. leptochellos leaves were assayed for antimicrobial activity using the susceptibility tests. Screening tests regarding the inhibition zone were carried out by the well diffusion method [9]. The inoculum suspension was prepared by painting colonies grown overnight on an agar plate and inoculated into Mueller-Hinton broth (fungi) using malt broth. A sterile swab was immersed in the suspension and used to inoculate Mueller-Hinton agar plates (fungi using malt agar plates). The extracts were dissolved in dimethyl sulfoxide (DMSO) with different concentrations (10, 5, 2.5 mg/ml). The inhibition zone was measured around each well after 24h at 37 °C. Controls using DMSO were adequately done.

Evaluation of cytotoxicity

The cytotoxic activity of the AME and EAF of D. leptochellos leaves were evaluated against (HepG2), (MCF-7) and HeLa cell lines using the MTT cell viability assay.

Cell line propagation

The cells were grown on RPMI-1640 medium supplemented with 10% inactivated fetal calf serum and 50 μg/ml gentamicin +ampicillin. The cells were maintained at 37 °C in a humidified atmosphere with 5% CO2 and were subcultured two to three times a week.

Cytotoxicity evaluation using viability assay

For cytotoxicity assay, the cancer cell lines were suspended in media at concentration 5x104/cell/well in Coating® 96 well tissue culture plates, and the inoculation suspension was prepared by painting colonies grown for 24 h. The total AME and EAF were then added into 96-well plates (three replicates) to achieve twelve concentrations for each extract. Six vehicle controls with media or 0.5 % DMSO were run for each 96-well plate as a control. After incubating for 24 h, the numbers of viable cells were determined by the MTT test. Briefly, the media was removed from the 96-well plates and replaced with 100 μl of fresh culture RPMI 1640 medium without phenol red then 10 μl of the 12 mmol MTT stock solution (5 mg of MTT in 1 ml of PBS) to each well, including the untreated controls. The 96-well plates
were then incubated at 37 °C and 5% CO₂ for 4 h. An 85 µl aliquot of the media was removed from the wells, and 50 µl of DMSO was added to each well and mixed thoroughly with the pipette and incubated at 37 °C for 10 min. Then, the optical density was measured at 590 nm with the microplate reader (SunRise, TECAN, Inc, USA) to determine the number of viable cells and the percentage of viability was calculated as [(ODt/ODc)×100%] where ODt is the mean optical density of wells treated with the tested sample and ODc is the mean optical density of untreated cells. The relation between surviving cells and drug plotted to get the survival curve of each cell line. The 50% inhibitory concentration (IC50) required to cause toxic effects in 50% of intact cells, was estimated from graphic plots of the dose-response curve for each concentration. Using Graph pad Prism software (San Diego, CA, USA) [10,11].

Statistical analysis of data

All the experimental results were expressed as mean±SD. Analysis of variance was performed by ANOVA procedures. Correlation coefficient (R²) was used to determine two variables. SPSS software was used for statistical calculations. The results with p<0.05 were regarded to be statistically significant.

Docking studies

The docking study was conducted using Auto Dock Vina, M. G. L tools 1.5.7 and Discovery Studio 4.5 as a visualizer. The estrogen receptor (PDB 5t92) was used as a receptor for the docking of the co-crystallized ligand and the docked pose were calculated. The active site of the estrogen receptor has been determined from the binding of co-crystallized ligand. The energy minimized estrogen receptor, the co-crystallized ligand and the three isolated compounds were finally prepared in the right format using MGL tools 1.5.7 for conducting docking study by Auto Dock Vina that requires both the receptor and the ligands in pdbqt format [12]. Grid was generated for the receptor using MGL tools 1.5.7. Auto Dock Vina achieves approximately two orders of magnitude speed-up compared to the molecular docking software Auto Dock 4, while also significantly improving the accuracy of the binding mode predictions. Further speed-up is achieved from parallelism, using multithreading on multi-core machines. Auto Dock Vina uses Auto Dock score that calculates free binding energies and iterated local search global optimization algorithm [13-15]. The result of docking was visually inspected by discovery studio 4.5 visualizer. The evaluation of candidates was based on binding affinity and interaction with the receptor.

RESULTS AND DISCUSSION

Characterization and identification of isolated compounds

Chromatographic separation of EAF of D. leptochelis leaves resulted in five compounds. Structures of the isolated compounds (Fig. 1) were identified by different spectral techniques including 1H NMR, 13C NMR, ESI-MS and CoPC against standard authentic after complete acid hydrolysis.

![Fig. 1: Chemical structures of the isolated compounds from D. leptochelis leaves](image)

Table 1: 1H NMR (400 MHz, DMSO-d6), 13C NMR (100.40 MHz, DMSO-d6) of compound 1, 2, 4 and 5

| C-Number | Compound 1 | Compound 2 | Compound 4 | Compound 5 |
|----------|------------|------------|------------|------------|
| a C      | a C        | a C        | a C        | a C        |
| Hs       | Hs         | Hs         | Hs         | Hs         |
| 2        | 164.31     | 163.23     | 2          | 164.17     | 164.57     |
| 3        | 102.85     | 102.96     | 3          | 103.11     | 6.68 s      | 102.75     | 6.65 s     |
| 4        | 182.48     | 181.81     | 4          | 182.40     | 182.54     |
| 5        | 161.72     | 161.76     | 5          | 163.73     | 160.99     |
| 6        | 98.75      | 6.19 s     | 98.48      | 6.28 s     |
| 7        | 161.68     | 161.13     | 7          | 160.97     | 162.98     |
| 8        | 106.08     | 94.64      | 8          | 93.91      | 6.49 s      | 104.91     |
| 9        | 156.46     | 156.97     | 9          | 156.68     | 156.33     |
| 10       | 104.32     | 102.96     | 10         | 104.09     | 104.31     |
| 1'       | 122.02     | 121.57     | 1'         | 121.75     | 122.33     |
| 2'/5'    | 128.89     | 128.73     | 2'         | 113.91     | 7.41 d (2.4) | 114.48     | 7.49 d (2.4) |
| 3'/5'    | 116.29     | 116.49     | 3'         | 146.14     | 146.51     |
| 4'       | 150.07     |              | 4'         | 150.07     | 150.04     |
| 5'       | 116.54     | 116.06     | 5'         | 116.06     | 6.86 d (8.97) |
| 6'       | 73.81      | 73.50      | 6'         | 119.23     | 7.44 d (2.4 and 2.85) | 119.97 | 7.54 d (2.4 and 8.97) |
| 7'       | 71.01      | 71.14      | 7'         | 71.14      | 4.05 t-like | 71.17      | 4.99 s     |
| 8'       | 182.75     | 79.18      | 8'         | 79.18      | 3.15-3.89 m | 79.03      | 3.15-3.89 m |
| 9'       | 61.75      | 61.82      | 9'         | 70.41      | 62.10      | remaining of sugar |

Table 1: 1H NMR (400 MHz, DMSO-d6), 13C NMR (100.40 MHz, DMSO-d6) of compound 1, 2, 4 and 5
Compound 1 and 2 were isolated as yellow amorphous powder, chromatographic properties, R-values (0.43 S, 0.49 S) and (0.57 S, 0.55 S) respectively, they gave dark purple spot under UV-light turned to yellowish-green on exposure to NH3 vapors, grayish-yellow fluorescence on exposure to Natursstoff and green color with FeCl3 spray reagents. Based on chromatographic properties, they were expected to be an apigenin structure [7]. This expectation was supported by H NMR spectrum of 1 and 2 (table 1) which showed an AX2 spin coupling system of two ortho-doublets each integrated for two aryl protons (J=8.22 Hz, H-2’/6’c) and 6.90 (2H, d, J= = 8.61 Hz, H-3’/5’) for 1, 7.90 (2H, d, J= = 8.51 Hz, H-2’/6’) and 6.92 (2H, d, J = 8.58 Hz, H-3’/5’) for 2 indicated 4’-hydroxy B-ring. The glycoside moiety in both compounds was identified as β-D-glucoside from doublets at δ 4.72 and 4.59 with large J values (~9 Hz) for compound 1 and 2 respectively. Absence of H-8 and H-6 signals from H NMR spectrum of 1 and 2 led us to conclude that the C-glycosylation must be on C-8 in case of 1 and C-6 in case of 2. This evidence was confirmed from the downfield shift of C-8 to 106.08 and C-6 to 109.59 (Δ+10 ppm) in 13C NMR spectra (table 1) of compound 1 and 2 respectively; moreover, the C-glycoside moiety in the structures were confirmed as β-D-glucoside depending on the characteristic up field location of C-1’ at 73.50 and 73.48 ppm for compound 3 and compound 4 respectively, and downfield location of both C-5’” and C-3” to 81.92 and 79.18 ppm for compound 3 and δ 82.37 and 79.03 ppm for compound 4, with respect to those of Cglycosides [18]. The C-glycosidation at C-6 in 4 and at C-8 in 5 was concluded from the downfield shift of δC-signals of C-6 to 109.19 and of C-8 to 104.91 (Δ+10 ppm) for 4 and 5 respectively. The assignment of all other 13C NMR resonances was achieved by comparison with the corresponding data of structural related compounds [22-24]. Negative ESI-MS spectra of 4 and 5 exhibited the molecular ion peak at m/z 447 (M+H) corresponding to the Mwt of 448 and molecular formula of C19H24O3. Thus according to the above-discussed data, compound 4 was confirmed as luteolin-6,7-dihydroxy-8-glucoside (Isoorientin), while compound 5 was confirmed as luteolin-8-C-β-D-glucopyranoside (Orientin). However, both compounds were isolated for the first time from D. leptocheilos.

Compound 3, isolated as yellow amorphous powder, δH NMR: δ ppm 7.46 (1H, dd, J = 7.94, 1.8 Hz, H-6), 7.14 (1Hd, J = 1.8 Hz, H-2’), 6.92 (1Hd, J = 7.94 Hz, H-5’), 6.80 (1H, d, J = 1.8 Hz, H-8), 6.75 (1H, H-3’), 6.45 (1H, d, J = 1.8 Hz, H-6), 5.09 (1H, d, J = 6.83 Hz, H-1’), 3.73-3.17 (5 H, m, H-2’, 3’, 4’, 5’ and 6’). 13C NMR (100.40 MHz, DMSO-d6): δ 182.36 (C-6), 164.95 (C-2), 163.40 (C-7), 161.59 (C-5), 157.41 (C-9), 150.46 (C-4’), 146.27 (C-7’), 121.77 (C-1’), 119.64 (C-6’), 116.47 (C-5’), 113.28 (C-2’), 105.80 (C-10), 103.58 (C-3’), 100.36 (C-1’), 100.52 (C-6’), 95.91 (C-8), 77.61 (C-5’), 76.88 (C-3’), 73.75 (C-7’), 70.02 (C-4’), 61.08 (C-6’). Negative ESI-MS spectra of compound 3 exhibited the molecular ion peak at m/z 447 (M+H) corresponding to the Mwt of 448 and a molecular formula of C19H24O3. The H NMR spectrum showed two meta-coupled doublets (J = 1.9 Hz) at δ 6.80 and 6.45, each integrating for one proton, and were assigned to H-6 and H-8, respectively of ring A of 5,7-dihydroxyflavones. The presence of ABR system at δ 7.46 (dd, J = 7.94, 1.8 Hz), 7.43 (d, J = 1.8 Hz) and 6.92 (d, J = 7.94 Hz) characteristic of 2, 4-trisubstituted phenyl unit. The only singlet at δ 6.75, integrating for one proton, was attributed C-3 to proton of flavonoids. These spectral data revealed the presence of luteolin skeleton. In addition, the H NMR spectrum showed a series of signals between δ 3.73-3.15, attributable to the sugar moiety. The coupling constant (J = 6.83 Hz) of the anomeric proton located at δ 5.09 and the 13C NMR chemical shifts of the sugar carbons (δ 100.36, 77.61, 76.83, 73.75, 70.02 and 61.08) revealed the presence of β-D-glucoside unit in luteolin-7-Oglucoside. The 13C NMR data showed the presence of a ketone carbonyl (δ 182.36), two définic carbons (δ 164.95 and 103.58 C), and four hydroxyl carbons (δ 163.40 C-7, 161.59 C-5, 150.46 C-4’ and 146.27 C-7’). The assignment of all other 13C resonances of 3 was achieved by comparison with the corresponding data of structurally related compounds [25]. According to the above-discussed data as well as comparison with authentic samples, compound 3 was confirmed as luteolin-7-O-glucoside, which was isolated for the first time from D. leptocheilos.

Table 2: Antioxidant activity (scavenging activity) of the AME and EAF of D. leptocheilos leaves

| Sample conc. (μg/ml) | Mean of DPPH scavenging % | Mean ± SD | Mean of EAE % | Mean ± SD | Mean of Ascorbic % | Mean ± SD |
|----------------------|---------------------------|-----------|----------------|-----------|-------------------|-----------|
| 1.28                 | 98.11                     | 1.33      | 85.04          | 2.76      | 89.35             | 1.04      |
| 640                  | 86.64                     | 0.68      | 87.03          | 0.25      | 88.64             | 0.99      |
| 320                  | 84.28                     | 0.46      | 85.48          | 0.51      | 80.92             | 1.64      |
| 160                  | 81.86                     | 0.59      | 83.28          | 1.39      | 73.68             | 1.50      |
| 80                   | 80.46                     | 0.74      | 81.66          | 1.04      | 66.13             | 4.45      |
| 40                   | 76.40                     | 1.02      | 78.67          | 3.03      | 53.21             | 1.81      |
| 20                   | 74.38                     | 0.70      | 75.87          | 1.54      | 50.71             | 3.11      |
| 10                   | 72.67                     | 0.57      | 72.97          | 1.42      | 47.09             | 2.05      |

Results are means ± SD (n=3; P<0.05)
Antioxidant activity

Free radicals are known as Reactive Oxygen Species (ROS) are produced by the human body. Plants are potential sources of natural antioxidants that protects the cells against the damaging effects of reactive oxygen species (ROS) [26]. The scavenging ability of DPPH radical is widely used for antioxidant evaluation of natural products besides other several in vitro complementary assays based on inactivation of O2 and NO radicals [27]. DPPH assay was selected to evaluate the antioxidant power of the extracts. The AME and EAF of D. leptochellos leaves showed similar and powerful antioxidant activity with $SC_{50} = 128 \pm 0.56 \, \mu g/ml$ and $SC_{50} = 17 \pm 0.77 \, \mu g/ml$ respectively when compared to ascorbic acid ($SC_{50} = 14.2 \pm 0.35 \, \mu g/ml$ and (table 2).

Antimicrobial activity

Results which compiled in table 3, showed that The AME and EAF of D. leptochellos leaves displayed moderate activity against Gram-positive and Gram-negative bacteria; however, both of them possess no anti-fungal activity.

Evaluation of cytotoxicity

Results of the cytotoxic activity of the AME and EAF of D. leptochellos leaves against MCF-7, HepG2 and HeLa cell lines are represented in tables (4, 5, 6 and 7).

### Table 3: Antimicrobial activity of the AME and EAF of D. leptochellos leaves

| Sample tested microorganisms | AME Mean (µg/ml) | EAF Mean (µg/ml) | Control Mean (µg/ml) |
|------------------------------|------------------|------------------|---------------------|
| **AME**                      | **EAF**          | **Control**      |
| Fungi                        |                  |                  |                     |
| Aspergillus fumigatus        | NA               | NA               | 21.01               |
| Candida albicans             | NA               | NA               | 23.03               |
| Staphylococcus aureus        | 10.00            | 0.90             | 30.01               |
| Bacillus subtilis            | 8.50             | 0.50             | 26.02               |
| Gram Positive Bacteria       | 10.50            | 11.50            | 33.02               |
| Salmonella typhimurium       | NA               | NA               | 27.09               |
| Escherichia coli             | 9.50             | 0.50             | 0.91                |

*NA: No activity. The sample was tested at concentration 10 mg/ml. (n=3)*

### Table 4: Cytotoxic activity (viability %) of the AME and EAF of D. leptochellos leaves against MCF-7 cell line compared to vinblastine sulfate

| Sample conc. (µg/ml) | Mean of viability % MCF-7 cell line | Mean of viability % MCF-7 cell line |
|----------------------|----------------------------------|----------------------------------|
| AME                  | EAF                              | Vinblastine                      |
| Mean (µg/ml)         | Mean (µg/ml)                     | Mean (µg/ml)                     |
| 500                  | 4.57                             | 2.78                             |
| 250                  | 6.36                             | 3.63                             |
| 12.5                 | 12.54                            | 8.24                             |
| 62.5                 | 23.44                            | 12.39                            |
| 31.25                | 33.15                            | 26.40                            |
| 15.6                 | 44.79                            | 37.05                            |
| 7.8                  | 59.50                            | 43.96                            |
| 3.9                  | 74.41                            | 53.96                            |
| 2.00                 | 100                              | 61.38                            |
| 1.00                 | 69.21                            | 2.52                            |

Results are means±SD (n=3) P<0.05

### Table 5: Cytotoxic activity (viability %) of the AME and EAF of D. leptochellos leaves against HepG-2 cell line compared to vinblastine sulfate

| Sample conc. (µg/ml) | Mean of viability % HepG-2 cell line | Mean of viability % HepG-2 cell line |
|----------------------|-----------------------------------|-----------------------------------|
| AME                  | EAF                              | Vinblastine                      |
| Mean (µg/ml)         | Mean (µg/ml)                     | Mean (µg/ml)                     |
| 500                  | 4.44                             | 3.89                             |
| 250                  | 7.67                             | 7.15                             |
| 12.5                 | 11.82                            | 13.64                            |
| 62.5                 | 22.88                            | 24.52                            |
| 31.25                | 33.22                            | 38.20                            |
| 15.6                 | 41.87                            | 47.09                            |
| 7.8                  | 54.86                            | 63.97                            |
| 3.9                  | 69.29                            | 79.62                            |
| 2.00                 | 100                              | 56.84                            |
| 1.00                 | 65.76                            | 2.48                            |

Results are means±SD (n=3) P<0.05

### Table 6: Cytotoxic activity (viability %) of the AME and EAF of D. leptochellos leaves against hela cell line compared to vinblastine sulfate

| Sample conc. (µg/ml) | Mean of viability % HeLa cell line | Mean of viability % HeLa cell line |
|----------------------|----------------------------------|----------------------------------|
| AME                  | EAF                              | Vinblastine                      |
| Mean (µg/ml)         | Mean (µg/ml)                     | Mean (µg/ml)                     |
| 500                  | 6.35                             | 5.48                             |
| 250                  | 11.62                            | 10.62                            |
| 12.5                 | 23.11                            | 19.26                            |
| 62.5                 | 34.29                            | 31.53                            |
| 31.25                | 45.13                            | 39.24                            |

Results are means±SD (n=3) P<0.05
Docking is the most widely used drug design tool in which the exact binding mode between the receptor and the ligand could be predicted. The docking result was analyzed using Discovery Studio 4.5. Because of the different results of the NW-docking and the W-docking, the water molecules were kept in the docking procedure [28]. Finally, the pure isolated compounds were docked in the estrogen receptor. The docking result was analyzed using Discovery Studio 4.5.

The binding of the five compounds with the receptor was strong and involved many types of interactions. Table 8 summarizes the binding affinity scores of the three compounds and their most significant interactions with the receptor. The good binding mode of the three compounds is shown in (Fig. 2).

![Docking diagram](image-url)

**Docking study**

Docking is the most widely used drug design tool in which the exact binding mode between the receptor and the ligand could be predicted. But docking procedure should always be validated and compared to a reference crystal structure. Thus the co-crystalized ligand in Estrogen receptor (PDB ID: 5t92) was docked to its corresponding active site. The calculated RMSD between the co-crystalized ligand and the docked pose was 1.55 Å in the NW-docking (docking without water) and less than 0.2 Å in the W-docking (docking with water), indicating a valid docking protocol. Because of the different results of the NW-docking and the W-docking, the water molecules were kept in the docking procedure [28]. Finally, the pure isolated compounds were docked in the estrogen receptor. The docking result was analyzed using Discovery Studio 4.5.

The binding of the five compounds with the receptor was strong and involved many types of interactions. Table 8 summarizes the binding affinity scores of the three compounds and their most significant interactions with the receptor. The good binding mode of the three compounds is shown in (Fig. 2).

| Sample     | MCF-7        | HeLa         | HepG2      |
|------------|--------------|--------------|------------|
| AME        | IC₅₀ = 12.8 ± 1.04 µg/ml | IC₅₀ = 26.3 ± 2.83 µg/ml | IC₅₀ = 10.7 ± 0.37 µg/ml |
| EAF        | IC₅₀ = 12.3 ± 1.82 µg/ml | IC₅₀ = 18.5 ± 3.29 µg/ml | IC₅₀ = 14.2 ± 0.60 µg/ml |
| Vinblastine Sulfate | IC₅₀ = 5.44±0.57 µg/ml | IC₅₀ = 6.54±0.39 µg/ml | IC₅₀ = 3.48±0.22 µg/ml |

**Results are mean±SD (n=3) P<0.05**

**Table 7: IC₅₀ values of the standard drug, the AME and EAF of D. leptocheilos leaves**
Fig. 2: The 2D interaction diagram of the three compounds with the estrogen receptor. (A) Vitexin, (B) Isovitexin, (C) Luteolin-7-O-glucoside (D) Orientin and (E) Isoorientin

Table 8: The binding affinity scores of isolated compounds and their most significant interactions with the receptor

| Compound name       | Vina score (Kcal/Mol) | Residues involved in hydrogen bonds interactions                  | Residues involved in hydrophobic interactions |
|---------------------|-----------------------|---------------------------------------------------------------|---------------------------------------------|
| Vitexin             | -6.8                  | GLU353, LEU346 and ASP351                                      | MET421, ALA350 and LEU525                  |
| Isovitexin          | -5.8                  | GLU353                                                         | MET343, LEU346, THR347, ALA350 and LEU525  |
| Luteolin-7-O-glucoside | -7.5 | GLU353, LEU346, THR347 and H30705                             | MET343, LEU346, ALA350, ASP351, LEU354 and LEU525 |
| Orientin            | -4.6                  | ASP351 and THR347                                             | MET343, TRP383 and LEU525                  |
| Isoorientin         | -4.6                  | THR347, TRP383, HIS524 and GLU339                              | MET343, TRP383 and LEU525                  |

Results of the molecular docking of vitexin, isovitexin, luteolin-7-O-glucoside, orientin and isoorientin in estrogen receptor revealed a favorable bind mode with high docking score, which may confirm the higher cytotoxic activity of the EAF.

CONCLUSION

Chromatographic separation of the EAF of D. leptochelis leaves led to isolation and identification of five phenolic compounds for the first time from this plant species, namely apigenin 8-C-β-D-glucopyranoside (Vitexin) (1), apigenin 6-C-β-D-glucopyranoside (Isovitexin) (2), luteolin 7-O-β-D-glucopyranoside (3), luteolin 8-C-β-D-glucopyranoside (Orientin) (4), luteolin 6-C-β-D-glucopyranoside (Isoorientin) (5). The AME and EAF were evaluated for their antioxidant activity using DPPH assay; both showed similar and powerful antioxidant activity which attributed to the polyphenolic content. The antimicrobial activity of two extracts was found to be moderate against bacteria and very weak against fungi. Cytotoxic activity also has been evaluated for both extracts.

ACKNOWLEDGMENT

The authors are thankful to Ass. Prof. Dr. Radwan Elhaggar, Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Helwan University, Cairo, Egypt for his valuable effort during the molecular docking study.

FUNDING

Nil

AUTHORS CONTRIBUTIONS

Eman G. Haggag revised the manuscript; Haitham A. Ibrahim, Fateha S. Elsharawy and Samah S. Shabana conducted the chromatographic separation, performed the structure elucidation of the pure isolated compounds and were responsible for drafting and writing the final version of the manuscript. Mahmoud A. Elhassab performed the molecular docking study. All authors performed the antimicrobial, antioxidant and cytotoxicity assays and their data analysis in addition, they read and approved the final manuscript.

CONFLICTS OF INTERESTS

Authors declared no conflict of interest.

REFERENCES

1. Villamil PA, Burbano AC, Osippa LP, Palacios JA, Aguirre OE. Determination of antimicrobial activity in leaves and flowers of Chromolaena odorata (L. F.) R. M. king and H. Rob. Asian J Pharm Clin Res 2020;13:53-6.
2. Pavithra S, Sekar T. Phytochemical profiling, free-radical scavenging and anti-inflammatory activities of melissosia simplicifolia (L.). Int J Pharm Pharm Sci 2020;12:62-9.
3. Dransfield J, Beentje H. The palms of Madagascar, Royal Botanic gardens, Kew and the International palm society; 1995.
4. Imtillena A, Barhe V, Barbhuiya SB, Saio L. Preliminary phytochemical screening and in vitro antioxidant activity of the methanolic extract of Lindernia ruellioides (Colom.) pennisl. Asian J Pharm Clin Res 2020;13:141-6.
5. Lengauer T, Carey M. Computational methods for biomolecular docking. Curr Opin Struct Biol 1996;6:402-6.
6. Meng EC, Shoichet BK, Kuntz ID. Automated docking with grid-based energy evaluation. J Comput Chem 1992;13:505-24.
7. Mahry TJ, Markham KR, Thomas MB. Ultraviolet spectra of isoflavones, flavonones and dihydroflavonols in the systematic identification of flavonoids. Springer Berlin Heidelberg; 1979. p. 165-226.
8. Yen GC, Duh PD. Scavenging effect of methanol extracts of peanut hulls on free radical and active oxygen species. J Agric Food Chem 1994;42:629-32.
9. Hindler JA, Howard BJ, Keiser JF. Antimicrobial agents and susceptibility testing. In: Howard BJ (Editor). Clinical and Pathogenic Microbiology. Mosby-Year Book Inc. St. Louis, MO, USA; 1994.
10. Mansfield T. Rapid colorimetric assay for cellular growth and survival measurement to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55-63.
11. Gomha SM, Riyad SM, Mahmoud EA, Elssasser MM. Synthesis and anticancer activities of thiazoles, 1, 3-thiazines, and thiazolidine using chitosan-grafted-poly (vinyl pyridine) as basic catalyst. Heterocycles 2015;91:1227-43.
12. Trotz O, Olson AJ. Auto dock vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization and multi thread. J Comput Chem 2010;31:455-61.
13. Abagyan R, Totrov M, Kuznetsov D. ICM-a new method for protein modeling and design: Applications to docking and structure prediction from the distorted native conformation. J Comput Chem 1994;15:488-506.
14. Baxter J. Local optimisation in deposit location. J Oper Res Soc 1981;32:815-9.
15. Blum A, Blesa M, Sampels M. Hybrid metaheuristics: an emerging approach to optimization (Eds.). Berlin, Heidelberg: Springer-Verlag; 2008. p. 26-1-99.
16. Nassar MI, Gaara AH, Marzouk MS, El-Din E, El-Khrisy AM. A new gentisic acid glycoside and C-glycosyl flavones from Erythrina indica with the antioxidant activity evaluation. Bull Fac Pharm Cairo Univ 2003;41:207-9.

17. El-Toumy SA, Omara EA, Nada SA, Bermejo J. Flavone C-glycosides from Montana bipinnatifida stems and evaluation of hepatoprotective activity of extract. JMPR 2011;5:1291-6.

18. Kubacek TM, Haggag EG, El-Toumy SA, Ahmed AA, El-Ashmawy BM, Youns MM. Biological activity and flavonoids from Centaurea alexanderina leaf extract. J Pharm Res 2012;5:3352-61.

19. Li H, Zhou P, Yang Q, Shen Y, Deng J, Li L, et al. Comparative studies on anxiolytic activities and flavonoid compositions of Passiflora edulis 'edulis' and Passiflora edulis 'flavicarpa'. J Ethnopharmacol 2011;133:1085-90.

20. Choo CY, Sulong NY, Man F, Wong TW. Vitexin and isovitexin from the leaves of Ficus deltoidea with in vivo a-glucosidase inhibition. J Ethnopharmacol 2012;142:776-81.

21. Mohamed TK, Kamal AM, Nassar MI, Ahmed MA, Haggag EG, Ezzat HA. Phenolic contents of Gleditsia triacanthos leaves and evaluation of its analgesic anti-inflammatory, hepatoprotective and antimicrobial activities. Life Sci 2013;10:3445-66.

22. Agrawal PK, Thakur RS, Bansal MC. In 13C NMR of flavonoids agraval (ed) Elsevier; New York; 1989;6:287-93.

23. Zhang Y, Jiao J, Liu C, Wu X, Zhang Y. Isolation and purification of four flavon C-glycosides from antioxidant of bamboo leaves by macroporous resin column chromatography and preparative high-performance liquid chromatography. Food Chem 2009;1:07:1326-36.

24. Sezik E, Aslan M, Yesilada E, Ito S. Hypoglycaemic activity of Gentiana olivieri and isolation of the active constituent through bioassay-directed fractionation techniques. Life Sci 2005;76:1223-38.

25. Chiruvella KK, Mohammed A, Dampuri G, Ghanta RG, Raghavan SC. Phytochemical and antimicrobial studies of methyl angolensate and luteolin-7-O-glucoside isolated from callus cultures of Strychnos nuxvomica. Int J Biomed Sci 2007;3:269.

26. Chandrika M, Chellaram M. Efficacy of antioxidation and anti-inflammation of the leaf extracts of Borresmania hispida. Int J Pharm Pharm Sci 2016;8:369-72.

27. Ibrahim RR, Ibrahim HA, Shabana SS, El-Hosari DG, Ali SA, Mahgoub S, et al. New phenolic compounds from calothamnus quadrijudus R.Br. aerial parts and their antioxidant activity. Nat Prod Res 2020;8:1-9.

28. Wei Y, Li J, Qing J, Huang M, Wu M, Gao F, et al. Discovery of novel hepatitis c virus NS5B polymerase inhibitors by combining random forest, multiple e-pharmacophore modeling and docking. PLoS ONE 2016;11(Suppl 2):e0148181.