**Heliotropium ramosissimum** metabolic profiling, in silico and in vitro evaluation with potent selective cytotoxicity against colorectal carcinoma

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*Heliotropium* is a genus of the Boraginaceae family. Its members are used in many traditional and folklore medicines to treat several ailments. Despite this widespread usage, only a few evidence-based scientific studies investigated and identified its phytoconstituents. Herein, we documented the chemical profile of the *Heliotropium ramosissimum* methanolic extract using gas chromatography-mass spectrometry (GC–MS) and liquid chromatography-tandem mass spectrometry (LC–ESI–MS/MS) and assessed its antioxidant and cytotoxic effects. The methanolic extract exhibited high phenolic content (179.74 ± 0.58 µg/mL) and high flavonoid content (53.18 ± 0.60 µg/mL). The GC–MS analysis of the lipoidal matter allowed us to identify 41 compounds with high percentages of 1,2-benzenedicarboxylic acid, bis(2-methoxyethyl) ester (23.91%), and 6,10,14-trimethylpentadecan-2-one (18.74%). Thirty-two phytomolecules were tentatively identified from the methanolic extract of *H. ramosissimum* using LC–MS/MS. These compounds belonged to several phytochemical classes such as phenolic acids, alkaloids, coumarins, and flavonoids. Furthermore, we assessed the antioxidant activity of the methanolic extract by DPPH assay and oxygen radical absorbance capacity assay, which yielded IC50 values of 414.30 µg/mL and 170.03 ± 44.40 µM TE/equivalent, respectively. We also assessed the cytotoxicity of the methanolic extract on seven different cell lines; Colo-205, A-375, HeLa, HepG-2, H-460, and OEC showed that it selectively killed cancer cells with particularly potent cytotoxicity against Colo-205 without affecting normal cells. Further studies revealed that the extract induced apoptosis and/or necrosis on Colo-205 cell line at an IC50 of 18.60 µg/mL. Finally, we conducted molecular docking on the LC–ESI–MS/MS-identified compounds against colon cancer antigen 10 to find potentially cytotoxic compounds. Binding score energy analysis showed that isochlorogenic acid and orientin had the highest affinity for the colon cancer antigen 10 protein, with binding scores of (−13.2001) and (−13.5655) kcal/mol, respectively. These findings suggest that *Heliotropium ramosissimum* contains potent therapeutic candidates for colorectal cancer treatment.

**Abbreviations**

A-375       Human melanoma cell line
AlCl3       Aluminum chloride
Colo-205    Colorectal cancer cell line
DPPH       2,2-Diphenyl-1-picrylhydrazyl
GAE         Gallic acid equivalents
GC–MS       Gas chromatography-mass spectrometry
H-460       Large cell lung cancer cell line
HeLa        Cervical cancer cell line
HepG-2      Hepatocellular carcinoma cell line
LC–ESI–MS/MS Liquid chromatography–electrospray ionization–mass spectrometry

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was determined using a Trace GC-TSQ mass spectrometer (Thermo Fisher Scientific, Austin, TX, USA) with a direct capillary column TG–5MS (Thermo Fisher Scientific, Austin, TX, USA) (30 m × 0.25 mm × 0.25 µm film thickness). The initial column oven temperature was 50 °C. It was then increased to 250 °C at 5 °C/min, held for 2 min, increased to the final temperature of 300 °C at 30 °C/min, and held for 2 min. The injector and MS transfer line were kept at 270 °C and 260 °C, respectively. The carrier gas (helium) had a constant flow rate of 1 mL/min. The solvent delay was 4 min, and diluted 1 µL samples were injected automatically using Autosampler AS1300 coupled with GC in split mode. We collected electron ionization mass spectra at an ionization voltage of 70 eV over the m/z range 50–650 in full scan mode. We set the

Materials and methods

Statement. All experiments and methods including collection of the plant were performed following relevant national, and international guidelines and legislation of the Faculty of Pharmacy, University of Sadat City, Sadat City, Egypt.

Plant material. We collected Heliotropium ramosissimum (Lehm.) DC. flowering aerial parts (Fig. 1) in March 2020 from the El-Sadat City desert region, Sadat City, Egypt with license approval from the Faculty of Pharmacy, University of Sadat City, Sadat City, Egypt according to relevant guidelines and regulations. The plant material was kindly identified by Prof. Dr. A. A. Fayed, Professor of Plant Taxonomy, Faculty of Science, Assiut University, Assiut, Egypt. We deposited a voucher sample (alphabetically ordered under the letter “H”) in the Herbarium of the Faculty of Science, Assiut University, Assiut, Egypt.

Extraction and fractionation. The air-dried powdered aerial parts of H. ramosissimum (Lehm.) DC. (1 kg) were defatted with n-hexane (3 × 1.5 L) to prepare the lipoidal matter and then extracted with methanol (3 × 1.5 L) to prepare the total methanolic extract used in this study. We performed the extractions in each solvent until exhaustion. After completing the process, we removed each solvent under reduced pressure using a rotary evaporator (Acculab, USA) at 50 °C. The n-hexane yielded 5 g of residue, and the total methanolic extracts weighed 10 g. We stored the extracts in a vacuum desiccator until further use.

Phytochemical studies. Phytochemical screening. We identified the presence of phytochemical classes in freshly prepared crude extracts of the flowering aerial parts of H. ramosissimum using standard colorimetric procedures.

Estimation of the total phenolic and flavonoid contents. The total phenolic content was calculated as gallic acid equivalents (GAE) per g of the sample using the Folin–Ciocalteu reagent and a calibration curve prepared with gallic acid. Moreover, the total flavonoid content was determined as rutin equivalents (RE) per g of the sample using aluminum chloride (AlCl₃) colorimetric assay.

Metabolomic analysis. GC–MS analysis of the lipoidal matter. The chemical composition of the lipoidal matter of the aerial parts of H. ramosissimum was determined using a Trace GC-TSQ mass spectrometer (Thermo Fisher Scientific, Austin, TX, USA) with a direct capillary column TG–5MS (Thermo Fisher Scientific, Austin, TX, USA) (30 m × 0.25 mm × 0.25 µm film thickness). The initial column oven temperature was 50 °C. It was then increased to 250 °C at 5 °C/min, held for 2 min, increased to the final temperature of 300 °C at 30 °C/min, and held for 2 min. The injector and MS transfer line were kept at 270 °C and 260 °C, respectively. The carrier gas (helium) had a constant flow rate of 1 mL/min. The solvent delay was 4 min, and diluted 1 µL samples were injected automatically using Autosampler AS1300 coupled with GC in split mode. We collected electron ionization mass spectra at an ionization voltage of 70 eV over the m/z range 50–650 in full scan mode. We set the
To separate the ions, we used a temperature of 200 °C. We identified the components by comparing their mass spectra with those of the WILEY 09 and NIST 14 mass spectral libraries.

**LC–ESI–MS/MS profiling.** We performed the LC–ESI–MS/MS analysis of the methanolic extract on an ExionLC AC system coupled with a SCIEX Triple Quad 5500+ MS/MS system equipped with an electrospray ionization (ESI) system. The samples were eluted on an Ascentis C18 Column (4.6×150 mm, 3 µm). Mobile phases consisted of eluent A (0.1% formic acid) and eluent B (acetonitrile, LC grade). The mobile phase gradient was programmed as follows: 10% B at 0–1 min, 10%–90% B at 1–21 min, 90% B at 21–25 min, and 10% at 25.01–28 min. The flow rate was 0.5 mL/min, and the injection volume was 10 µL. MS/MS analysis used positive and negative ionization modes with a scan (EMS-IDA-EPI) from 100 to 1000 Da for MS1 with the following parameters: curtain gas, 25 psi; Ion Spray voltage, 5500 and −4500 V for positive and negative modes, respectively; source temperature, 500 °C; ion source gas 1 & 2, 45 psi and from 50 to 800 Da for MS2; declustering potential, 80; collision energy, 35 and −35 for positive and negative modes, respectively; and collision energy spread, 2021. We identified the compounds using MS-DIAL software version 4.70.

**Free radical scavenging activity assessment by DPPH assay.** We prepared 1000 and 100 µg/mL solutions from the methanolic extract to identify a range within which the inhibitory concentration 50 (IC50) lay. We serially diluted the solutions exceeding 50% five times. We prepared a 100 µM Trolox stock solution in methanol. From this stock solution, we prepared 50, 40, 30, 20, 15, 10, and 5 µM solutions. We performed the 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) free radical assay as described by Boly et al., 201617,20. Briefly, we added 100 µL of freshly prepared DPPH reagent (0.1% in methanol) to 100 µL of the sample in a 96-well plate (n = 6) that we incubated at room temperature for 30 min in the dark. Next, we measured the reduction in DPPH color intensity at 540 nm using the microplate reader FluoStar Omega. We presented the data as mean ± standard deviation according to the following equation:

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\text{Percentage inhibition} = \frac{\text{Average absorbance of blank} - \text{Average absorbance of the test}}{\text{Average absorbance of blank}} \times 100.
\]

**Assessment of the oxygen radical absorbance capacity (ORAC assay).** We prepared a 1 mM Trolox stock solution and performed nine serial dilutions to obtain 400, 300, 200, 150, 100, 75, 50, 25, and 12.5 µM solutions. We prepared a 400 µg/mL sample solution in MeOH. We performed the assay as described by Liang et al., 201422, with minor modifications. Briefly, we incubated 12.5 µL of the prepared sample(s) with 75 µL of fluorescein (10 nM) for 30 min at 37 °C. Next, we measured background fluorescence (485 nm excitation and 520 nm emission) for three cycles (cycle time: 90 s). Then, we immediately added 12.5 µL of freshly prepared 2,2′-azobis(2-aminodopropane) (240 mM) to each well and continuously measured fluorescence (485 nm excitation and 520 nm emission) for 2.5 h (100 cycles of 90 s each). We presented the data as mean (n = 3) ± standard deviation and calculated the antioxidant effect of the extract as µM Trolox equivalents by substitution in the linear regression equation: Y = 32,356.3X + 989,769.9 (R2 = 0.9957).

**Assessment of cytotoxic activity.** We obtained colorectal cancer (Colo-205), human melanoma (A-375), cervical cancer (HeLa), hepatocellular carcinoma (Hep G-2), large cell lung cancer (H-460), and oral epithelial (OEC) cells from Nawah Scientific Inc. (Mokatam, Cairo, Egypt). We maintained them in RPMI medium supplemented with 100 mg/mL of streptomycin, 100 units/mL of penicillin, and 10% of heat-inactivated fetal bovine serum and incubated them in humidified 5% (v/v) CO2 atmosphere at 37 °C.

**Figure 1.** A Photo of the *H. ramosissimum* (Lehm.) DC. flowering aerial from the place of collection (taken by the corresponding author in March 2020 from El-Sadat City desert region, Sadat City, Egypt).
Cytotoxicity assay. We assessed cell viability through a sulforhodamine B (SRB) assay. We added 100 µL of cell suspension (5 × 10^6 cells) to 96-well plates and incubated them in a complete medium for 24 h. We then treated the cells with 100 µL of medium containing samples at different concentrations (10 and 100 µg/mL). After 72 h of exposure, we fixed the cells by replacing the medium with 150 µL of 10% trichloroacetic acid and incubated them at 4 °C for 1 h. Next, we removed the trichloroacetic acid solution and washed the cells five times with distilled water. We then added 70 µL of SRB solution (0.4% w/v) and incubated the mixture in a dark place at room temperature for 10 min. We washed the plates three times with 1% acetic acid and allowed them to air-dry overnight. Then, we added 150 µL of TRIS (10 mM) to dissolve the protein-bound SRB stain and measured the absorbance at 540 nm using a BMG LABTECH-FLUOstar Omega microplate reader (Ortenberg, Germany)\(^{23}\).

Annexin-based apoptosis assay. Colo-205 cells were treated with either doxorubicin (10 µM) as a positive control or the total methanolic extract of H. ramosissimum for 48 h. Next, we collected the cells (10^6 cells) by trypsination and washed them twice with ice-cold phosphate-buffered saline (PBS, pH 7.4). We then resuspended the cells in the dark with 0.5 mL of Annexin V-FITC/propidium iodide (PI) solution for 30 min at room temperature according to the manufacturer's protocol (Annexin V-PI staining apoptosis detection kit from Abcam Inc., Cambridge Science Park, Cambridge, UK). After staining, we injected the cells into an ACEA Novocyte flow cytometer (ACEA Biosciences Inc., San Diego, CA, USA) and detected the FITC and PI fluorescent signals using FL1 and FL2 signal detectors, respectively (\(\lambda_{\text{ex/em}} = 488/530\) nm for FITC and \(\lambda_{\text{ex/em}} = 535/617\) nm for PI). For each sample, we acquired 12,000 events and quantified the FITC- and PI-positive cells by quadrant analysis using ACEA NovoExpress software (ACEA Biosciences Inc., San Diego, CA, USA)\(^{23}\).

Cell cycle distribution analysis. After treating the cells (10^6 cells) with the total methanolic extract of H. ramosissimum for 48 h or paclitaxel (1 µM) for 24 h, as a positive control, we collected them by trypsination and washed them twice with ice-cold PBS (pH 7.4). We then resuspended the cells in 2 mL of 60% ice-cold ethanol and incubated them at 4 °C for 1 h for fixation. Next, we washed the fixed cells twice with PBS (pH 7.4) and resuspended them in 1 mL of PBS containing 50 µg/mL RNAase A and 10 µg/mL PI. After 20 min of incubation in the dark at 37 °C, we analyzed the cells' DNA contents by flow cytometry using an FL2 (\(\lambda_{\text{ex/em}} = 535/617\) nm) signal detector (ACEA Novocyte flow cytometer, ACEA Biosciences Inc., San Diego, CA, USA). For each sample, 12,000 events were acquired. We determined cell cycle distribution using ACEA NovoExpress software (ACEA Biosciences Inc., San Diego, CA, USA)\(^{23}\).

Statistical analysis. We carried out triplicate experiments and analyzed data using Microsoft Excel. We determined the IC\(_{50}\) using GraphPad Prism 5 by converting the concentrations to their logarithmic value and using the “non-linear inhibitor regression equation (log (inhibitor) vs. normalized response–variable slope equation)” function\(^{26}\).

Molecular docking. We performed docking studies using Molecular Operating Environment (MOE, 2014.0901)\(^{27}\). We retrieved the three-dimensional (3D) structures of serologically defined colon cancer antigen 10 (PDB ID: 2HQ6) determined by X-ray diffraction (resolution: 1.75 Å) from the RCSB Protein Data Bank (https://www.rcsb.org/) and used it as a target in our molecular docking experiments\(^{28}\). We prepared and optimized the structure of the receptor protein using MOE's ligx function with default settings. We drew the structures of the identified compounds in Chemdawr 17.0.0.206 in MOL format and performed 3D protonation, partial energy correction, and energy minimization using Merck molecular force field (MMFF94x). We determined the receptor active sites using MOE's site finder tool. We carried out the molecular docking analysis to predict the receptor–ligand interaction using flexible ligand-fixed receptor docking parameters with Triangle Matcher placement (scoring: London dG; retain: 30) and force field refinement (rescoring: London dG; retain: 10). We selected the most stable protein–ligand interactions based on their S-score minimum energy and root mean square deviation (RMSD). We recorded the docking score, RMSD, and 2D and 3D interactions\(^{29,30}\).

Results and discussion

Phytochemical screening. We carried out phytochemical screening on H. ramosissimum to identify the different chemical classes of the total methanolic extract active constituents using different reagents. The preliminary screening revealed the presence of alkaloids, terpenoids, saponins, tannins, flavonoids, coumarins, polyphenolics, and reducing sugars.

Total phenolic and flavonoid contents. We estimated the total phenolic content in the methanolic extract of H. ramosissimum to be 179.74 ± 0.58 µg/mL, in gallic acid equivalents. The equation for the standard curve was \(Y = 0.0031x + 0.0564\) (Fig. S1), with \(R^2 = 0.99961\). Moreover, we measured a total flavonoid content of 53.18 ± 0.60 µg/mL, in rutin equivalents. The equation for the standard curve was \(Y = 0.0032x + 0.0398\) (Fig. S2), with \(R^2 = 0.9981\).

Metabolomic analysis. GC–MS analysis of the lipoidal matter. We performed a GC–MS analysis of the plant's lipoidal matter to address the lack of information regarding its phytoconstituents. The GC–MS chromatogram (Fig. S3) of the lipoidal matter of H. ramosissimum revealed the presence of 41 compounds, and we identified 30 (91.73%) of them by comparing their mass spectra data (retention time and peak area) with the Wiley spectral library. We left 11 (8.25%) compounds unidentified. The most abundant compound was 1, 2-benzenedi-
carboxylic acid, bis (2-methoxyethyl) ester (23.91%), followed by 6,10,14-trimethylpentadecan-2-one (18.74%), ethyl hexadecanoate (13.68%), and hexadecanoic acid methyl ester (11.83%) (Table 1 and Fig. S4).

LC–ESI–MS/MS profiling. We identified the compounds of the *H. ramosissimum* methanolic extract by LC–MS/MS. We compared their retention time (R<sub>t</sub>), mass, and MS2 with standards-reported literature, and data-bases (TMIC and MassBank). In total, we tentatively identified 32 compounds, including alkaloids, flavonoids, coumarins, phenolic acids, and their derivatives. We identified 17 compounds in negative mode and 15 in posi-
tive mode. Tables 2 and 3 list all identified compounds ordered by relative retention times and their structures were supplied (Figs. S5, S6, S74, S8).

Free radical scavenging activity. The DPPH spectrophotometric assay is one of the most reliable and widely used methods to estimate the free radical scavenging effect of different plant extracts. The percent of inhibition values in the initial screening step of the two concentrations, namely, 1000 and 100 µg/mL, were 80.26 ± 1.50 (>50) and 6.50 ± 0.73 (<50), respectively. We serially diluted the extract that exceeded 50% inhibition (1000 µg/mL) to provide five concentrations that we tested to determine that the IC50 was 414.30 µg/mL (Fig. S9). For reference, Trolox has an IC50 of 24.42 ± 0.87 µM. The *H. ramosissimum* methanolic extract showed a potent radical-scavenging effect, which may be attributed to its total phenolic and flavonoid contents.

Assessment of ORAC assay. We evaluated the antioxidant activity of the *H. ramosissimum* methanolic extract using the ORAC assay (Fig. S10). The antioxidant activity of the extract (Fig. 2) was 170.03 ± 44.45 µM TE/equivalent, which is higher than that of Trolox.

### Table 2. Metabolites identified in the methanolic extract of *H. ramosissimum* (Lehm.) DC. using positive mode LC–ESI–MS/MS.

| No | Rt (min) | [M+H]+ | Fragments | Identified Compound | Chemical Class | Ref |
|----|----------|---------|-----------|--------------------|---------------|-----|
| 1  | 0.42     | 149     | 79, 105, 131 | Cinnamic acid       | Phenolic acids | 60  |
| 2  | 9.28     | 191     | 115, 119, 147, 148 | 7-Methoxy-4-methylcoumarin | Coumarins | 60  |
| 3  | 10.36    | 291     | 273, 139, 123 | (epi)-Catechin | Flavonoids | 60  |
| 4  | 10.94    | 300     | 138, 139, 157 | Lycopsamine | Alkaloids | 61-63 |
| 5  | 11.22    | 314     | 138, 156, 269 | Heliospathine | Alkaloids | 61-62 |
| 6  | 13.20    | 286     | 153, 127, 109 | Trachelanthamine | Alkaloids | 64  |
| 7  | 15.70    | 185     | 126, 80 | Methyl gallate | Phenolics | 65  |
| 8  | 15.95    | 277     | 134, 175, 217, 241 | Stearidonic acid | Fatty acids | 66  |
| 9  | 18.09    | 289     | 153, 127, 109 | Eriodictyol | Flavonoids | 67  |
| 10 | 19.57    | 287     | 241, 261, 213, 153 | Kaempferol | Flavonoids | 68  |
| 11 | 19.85    | 305     | 287, 259 | Taxifolin | Flavonoids | 68  |
| 12 | 19.89    | 330     | 121, 139, 241 | Europine | Alkaloids | 69,70 |
| 13 | 22.72    | 398     | 107, 121, 139 | Heliosupine | Alkaloids | 61,71,72 |
| 14 | 25.80    | 303     | 285, 275, 257, 229 | Quercetin | Flavonoids | 70  |
| 15 | 27.15    | 156     | 81, 112, 139 | Retronecine | Alkaloids | 61,63,73,74 |

### Table 3. Metabolites identified in the methanolic extract of *H. ramosissimum* (Lehm.) DC. using negative mode LC–ESI–MS/MS.

| No | Rt (min) | [M-H]- | Fragments | Identified Compound | Chemical Class | Ref |
|----|----------|---------|-----------|--------------------|---------------|-----|
| 1  | 2.21     | 115     | 97, 71 | Maleic acid | Dicarboxylic acids | 59,75 |
| 2  | 2.59     | 117     | 73, 98, 116 | Succinic acid | Dicarboxylic acids | 76  |
| 3  | 4.14     | 181     | 125, 137, 153 | 2-Ethoxy-4,5-dihydroxybenzaldehyde | Phenolics | 77  |
| 4  | 4.18     | 241     | 105, 195, 225 | 6-Deoxycochinoïde | Benzofuran | 78  |
| 5  | 4.48     | 175     | 113, 115, 131, 157 | 2-Isopropylmalic acid | Organic acids | 79,74 |
| 6  | 4.61     | 138     | 79, 107, 110 | 7-Hydroxy-1-methylenepyrrrolizidine | Alkaloids | 80  |
| 7  | 4.70     | 153     | 153, 135, 123, 109 | Protocatechusic acid | Phenolic acids | 80,81 |
| 8  | 5.55     | 137     | 93, 109, 119 | 4-Hydroxybenzoic acid | Phenolic acids | 80,81,82 |
| 9  | 5.6      | 179     | 135, 143, 161, 107 | Caffeic acid | Phenolic acids | 83  |
| 10 | 9.39     | 177     | 105, 133, 146, 176 | 4-Methoxycinnamic acid | Phenolic acids | 79  |
| 11 | 10.01    | 289     | 243, 245 | Filifolinic acid | Phenolic acids | 84,85 |
| 12 | 10.77    | 161     | 105, 117, 133 | Umbelliferone | Coumarins | 80,84 |
| 13 | 16.92    | 515     | 515, 353, 179, 173 | 4,5-di-O-caffeoylquinic acid (isochlorogenic acid) | Phenolic acids | 87  |
| 14 | 20.05    | 337     | 337, 191, 163 | 3-O-p-coumaryl quinic acid | Phenolic acids | 87,88 |
| 15 | 24.9     | 447     | 313, 319, 327, 357 | Luteolin 8-C-Glucoside (Orientin) | Flavonoids | 89  |
| 16 | 26.73    | 367     | 367, 191 | 3-O-feruloyl quinic acid | Phenolic acids | 80  |
| 17 | 27.89    | 233     | 109, 121, 145, 175 | Tournefolin C | Flavonoids | 77  |
Cytotoxicity assay. We screened the cytotoxicity of the *H. ramosissimum* methanolic extract at 10 and 100 µg/mL on various cell lines (Table 4 and Fig. 3). We performed an SRB quick screening assay on six cancer cell lines, namely, colorectal cancer (Colo-205), human melanoma (A-375), cervical cancer (HeLa), hepatocellular carcinoma (HepG-2), large cell lung cancer (H-460) cells, and a normal cell line, that is, oral epithelial cells (OEC). The *H. ramosissimum* methanolic extract exhibited potent activity on all tested cell lines. The most sensitive cell line was Colo-205, followed by A-375, HeLa, and H-460, and the least sensitive was the MCF-7 cell line. Because the *H. ramosissimum* methanolic extract showed the highest activity against colorectal carcinoma (Colo-205) cells—in excellent agreement with the in silico study—we further investigated its IC$_{50}$ and mechanism of action through cell cycle and apoptosis flow cytometry assays on this cell line.
We assessed cell viability at five different concentrations (0.01, 0.1, 1, 10, and 100 µg/mL) and used doxorubicin as a standard cytotoxic drug. The SRB assay (Figs. 4, 5) revealed that the *H. ramosissimum* methanolic extract possesses a dose-dependent cytotoxic effect with an IC$_{50}$ of 18.60 µg/mL (Fig. 4A) and that of doxorubicin is 0.08 µg/mL (Fig. 4B). The *H. ramosissimum* extract decreased cell viability from 75.95% ± 0.81% at 10 µg/mL to 2.24% ± 0.37% at 100 µg/mL (Table 4).

The optical microscope staining images (Fig. 5) show the results of the SRB cytotoxicity assay against Colo-205 cell line of both total *H. ramosissimum* DC. aerial parts for two methanolic extract concentrations (0.01 and 100 µg/mL), doxorubicin at the same concentrations, and negative control. The figure clearly shows that at 0.01 µg/mL, neither the extract nor doxorubicin caused significant morphological changes. Meanwhile, significant changes occurred at 100 µg/mL, confirming the dose-dependent character of the cytotoxicity of the extract.

To investigate the safety of the *H. ramosissimum* methanolic extract on normal cells and the selectivity of its cytotoxicity on cancer cells, we performed the cytotoxic activity assay on OECs. The viability of OECs treated with 10 µg/mL methanolic extract was 98.94% ± 0.77%. Meanwhile, this concentration showed a potent cytotoxic effect on Colo-205 cells by reducing cell viability to 75.95% ± 0.81%.

**Figure 4.** *In-vitro* SRB cytotoxicity assay of (A) the total methanolic extract of *H. ramosissimum* (Lehm.) DC. aerial parts; (B) Doxorubicin; in increasing concentrations (0.01–100 µg/mL) against colorectal carcinoma (Colo-205) cell lines. Data points are expressed as mean ± SD (n = 3).

**Figure 5.** Optical microscope stained images of SRB cytotoxicity assay against Colo-205 cell line (A) Negative control, (B) Doxorubicin (0.01 µg/mL), (C) Doxorubicin (100 µg/mL), (D) the total methanolic extract of *H. ramosissimum* (Lehm.) DC. aerial parts (0.01 µg/mL), (E) the total methanolic extract of *H. ramosissimum* (Lehm.) DC. aerial parts (100 µg/mL), magnification power: X 100.
To further investigate the mechanism responsible for the cytotoxic activity of the *H. ramosissimum* methanolic extract, we carried out an apoptosis assay on Colo-205 cells. We used Annexin V-FITC/PI double staining and evaluated the apoptotic rates using flow cytometry. As illustrated in Fig. 6, the apoptotic rate was 56.14% in the positive control group using doxorubicin, whereas treating the cells with 18 µg/mL of methanolic extract (which is equal to its IC₅₀) significantly increased the apoptotic rate to 71.76%. These results suggest that the extract reduced Colo205 cell viability by inducing apoptosis.

To examine whether the cytotoxicity of the *H. ramosissimum* methanolic extract on Colo-205 was associated with cell cycle arrest, we conducted a PI-metric cell cycle analysis using flow cytometry (Fig. 7). The cell cycle histograms revealed that cells treated with the extract (18 µg/mL) had a markedly higher sub-G₁ population (63.01%) than those treated with the positive control, paclitaxel (37.33%). This suggests that the extract induced apoptosis and/or necrosis in Colo-205 cells.

We reviewed the literature on the cytotoxic activity of different *Heliotropium* species and found that the *n*-hexane fraction of the ethanolic extract of *H. subulatum* aerial parts displayed cytotoxic activity at 3 mg/mL. An in vitro assay against MRC5 human cells revealed that the methanolic extract of *H. zeylanicum* aerial parts exerted significant cytotoxicity with an IC₅₀ of 13 µg/mL. Furthermore, the methanolic extract of the dried roots of *H. indicum* showed different mortality rates at different concentrations, with a lethal concentration (LC₅₀) of 47.86 µg/mL and LC₉₀ of 75.85 µg/mL in a brine shrimp lethality bioassay. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay revealed that the whole plant ethanolic extract of *H. indicum* had significant antiproliferative activity against SKBR-3 human breast adenocarcinoma cells. The IC₅₀ of the extract was 34 ± 9.09 µg/mL, and that of the standard drug paclitaxel was 22.20 ± 2.30 µg/mL. These findings suggest that the *H. ramosissimum* methanolic extract exerts a potent cytotoxic effect, especially against colorectal carcinoma, by inducing apoptosis. These results suggest that this extract is a promising candidate for the treatment of this type of cancer.

**Molecular docking.** In silico molecular docking, techniques have enhanced drug discovery and development by allowing the structure-based exploration of ligand-receptor interactions. The cytotoxicity assays
revealed that the cytotoxicity of the methanolic extract was more potent against colon cancer than against the other cell lines. We investigated the possible interactions between the 32 phytochemical compounds identified by LC–ESI–MS/MS and the colon cancer antigen 10 binding sites and compared them to the standard anticancer drug doxorubicin. Table 5 shows the binding energies of the compounds. The computed binding score energy values range from −6.4244 kcal/mol (hydroxybenzoic acid) to −14.0088 kcal/mol (doxorubicin).

Isochlorogenic acid, orientin, kaempferol, and taxifolin are the top-scoring compounds with pose scoring values of −13.200103, −13.5655, −11.836662, and −11.228658 kcal/mol, respectively. Isochlorogenic acid and orientin (flavonol glycoside) showed the best interactions with the receptor, with the lowest binding energy values of −13.2001 kcal/mol (RMSD = 1.46 Å) and −13.5655 kcal/mol (RMSD = 1.16 Å), respectively. These values are close to that of doxorubicin (−14.0088 kcal/mol, RMSD = 1.71 Å), indicating that they could inhibit the receptor. Isochlorogenic acid interaction with the receptor involved hydrogen bonds with Thr71 (H-donor), Gln64 (H-acceptor), and Arg56 (H-acceptor) in addition to ionic interactions with Arg 56AA (Fig. 8). Orientin formed interactions with Ser73AA (H-donor) and π-H interaction with Glu 76 (Fig. 9).

Kaempferol (Fig. 10) formed hydrogen bonds with Gly75AA (H-donor), and taxifolin (Fig. 11) interacted with Gly75AA and Ser73AA (H-donor). The cytotoxicity of the tested extract may be attributed to the phenolic compounds and flavonoids with the best binding affinity in the molecular docking study.

The previous experimental studies are matched with our molecular docking results. Chlorogenic acid was reported to exhibit potential effects on cytotoxicity and inhibited human colon cancer cell proliferation through cell-cycle arrest and apoptosis95. In addition, orientin exhibited remarkable cytotoxicity and antiproliferative activity against HT-29 colon cancer cells, induced G0/G1 cell cycle arrest, regulated cyclin and cyclin-dependent protein kinases, and mediated apoptosis in human colorectal cancer HT-29 cells96. Further, taxifolin showed human colorectal cancer cell growth arrest in the G2 phase of the cell cycle and apoptosis in a concentration-dependent approach97. Besides, kaempferol's cytotoxic effects and induction of apoptosis in different human colorectal cancer cell lines have been reported98.

Figure 7. Schematic representation of cell cycle arrest at Sub G1 and G2 phase (A) Control, (B) the total methanolic extract of *H. ramosissimum* (Lehm.) DC. aerial parts.
| No | Compound                                    | Binding score (kcal/mol) | RSMD_refine (Å) | E_conf (kcal/mol) |
|----|--------------------------------------------|--------------------------|-----------------|------------------|
| 1  | Cinnamic acid                              | −6.7353                  | 1.07            | −75.77           |
| 2  | Methyl gallate                             | −8.4939                  | 1.36            | −99.08           |
| 3  | Taxifolin                                  | −11.2287                 | 1.32            | −17.49           |
| 4  | 7-Methoxy-4-methylcoumarin                 | −6.5481                  | 1.12            | 34.50            |
| 5  | Quercetin                                  | −10.6524                 | 0.92            | 35.93            |
| 6  | epicatechin                                | −10.6177                 | 1.26            | 23.60            |
| 7  | Catechin                                   | −10.3107                 | 1.28            | 23.49            |
| 8  | Lycopsamine                                | −8.8984                  | 1.89            | 9.61             |
| 9  | Heliospathine                              | −7.9719                  | 0.88            | −7.98            |
| 10 | Trachelanthamine                           | −8.5378                  | 1.44            | 18.25            |
| 11 | Protocatechuic acid                        | −8.0570                  | 0.98            | −71.46           |
| 12 | Stearidonic acid                           | −7.8434                  | 1.81            | 53.25            |
| 13 | Eriodictyol                                | −10.5128                 | 1.31            | 9.4              |
| 14 | Kaempferol                                 | −11.8367                 | 0.66            | −52.05           |
| 15 | Europine                                   | −9.2788                  | 1.29            | 39.07            |
| 16 | Heliosupine                                | −8.5358                  | 1.06            | 117.43           |
| 17 | Retronecine                                | −7.7943                  | 1.82            | −60.09           |
| 18 | Maleic acid                                | −7.1007                  | 0.66            | −177.99          |
| 19 | Caffeic acid                               | −9.5269                  | 1.34            | 96.22            |
| 20 | 2-Ethoxy-4,5-di-hydroxybenzaldehyde        | −9.6644                  | 0.65            | 16.05            |
| 21 | 6-Deoxycochinsolide                        | −8.2384                  | 0.89            | 1.1              |
| 22 | 2-Isopropylmalic acid                      | −9.5676                  | 0.84            | 36.78            |
| 23 | 7-Hydroxy-1-methylenepyrrulizidine         | −7.5278                  | 0.90            | 4.06             |
| 24 | 4-Hydroxybenzoic acid                      | −6.4245                  | 1.48            | −68.26           |
| 25 | Succinic acid                              | −6.8769                  | 0.83            | −176.13          |
| 26 | 4-Methoxyxycinnamic acid                   | −7.3200                  | 1.52            | −72.06           |
| 27 | Filifolinoic acid                          | −8.7365                  | 0.84            | 3.57             |
| 28 | Umbelliferone                              | −9.2712                  | 1.42            | 12.29            |
| 29 | 4,5-di-O-cafeoylquinic acid (Isochlorogenic Acid) | −13.2001        | 1.46            | −4.03            |
| 30 | 3-O-p-coumarylquinic acid                  | −9.5033                  | 1.09            | 10.87            |
| 31 | 3-O-Feruloylquinic acid                    | −10.1228                 | 0.85            | 23.34            |
| 32 | Orientin                                   | −13.5655                 | 1.16            | 53.38            |
| 33 | Tournefolin C                              | −9.0987                  | 0.97            | 9.53             |
| 34 | Donorubicin                                | −14.0088                 | 1.71            | −79.03           |

Table 5. Docking scores of identified compounds against colon cancer antigen 10 (PDB ID: 2HQ6).

Figure 8. 2D and 3D interactions complex of Isochlorogenic acid against colon cancer antigen 10 (generated by using Molecular Operating Environment, MOE, 2014.090199).
Conclusion

In conclusion, the phytochemical screening of the total methanolic extract of the aerial parts of *H. ramosissimum* (Lehm.) DC. revealed the presence of alkaloids, terpenoids, saponins, tannins, flavonoids, coumarins, polyphenolics, and reducing sugars. It is worthy to mention that all the identified compounds in the *n*-hexane fraction are the first to be reported in the genus *Heliotropium*, in addition, thirty-two compounds of different chemical classes were tentatively identified by LC–ESI–MS/MS analysis. Besides, our findings revealed that the total methanolic extract of *H. ramosissimum* aerial parts exhibits a potent in vitro antitumor effect against several cell lines especially colorectal carcinoma (Colo-205) in a dose-dependent manner and with selectivity in comparison to normal cell lines (OEC). The methanolic extract reduces the viability of invasive Colo-205 cell lines by inducing apoptosis and/or necrotic cell death. Furthermore, a docking study for the identified compounds from the LC–ESI–MS/MS analysis to find out the candidates responsible for the cytotoxic activity where isochlorogenic acid and orientin showed the highest binding scores with colon cancer antigen 10. Our findings can aid in the creation of a new alternative candidate for the selective treatment of early stages of colorectal cancer with safety on normal cells. Future studies are needed to isolate the active constituent/constituents responsible for this potent cytotoxic activity.
Data availability
All data generated or analyzed during this study are included in this published article [and its supplementary information files].

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All authors shared Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Software; Supervision; Validation; Visualization; Roles/Writing – original draft; Writing – review & editing.

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