SUPPLEMENTARY MATERIAL

Metabolomic profiling and anti-infective potential of Zinnia elegans and Gazania rigens (family Asteraceae).

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Abstract

The present study evaluates the chemical composition of Zinnia elegans and Gazania rigens based on their metabolomic profiles using liquid chromatography coupled with high-resolution mass spectrometry (LC-HR-MS), along with the anti-infective activities of their ethanol extracts, as well as, different fractions. A significant difference was observed between the LC-MS profiles of the two plants such as, coumarins, sesquiterpene lactones and phenylethanoids which were characteristic for Z. elegans, while amides and phenolic acid derivatives were characteristic for G. rigens. These results highlight the chemical potential of Z. elegans and G. rigens. Furthermore, the ethyl acetate fraction of Z. elegans showed a significant antimalarial activity with IC\textsubscript{50} values of 21.03 and 13.72 \textmu g/mL against Plasmodium falciparum D\textsubscript{6} and P. falciparum W\textsubscript{2}, respectively.

Keywords: Zinnia elegans, Gazania rigens, metabolomics, LC-MS, anti-infective.
Experimental

Plant material

*Zinnia elegans* (leaves, stems and roots) and *Gazania rigens* (whole plant) were collected in November and December 2015, respectively, from the Nursery of the Faculty of Agriculture, Minia University, Egypt and identified by Prof. Dr. Mahmoud Abdelhady Hassan, Professor of Horticulture, Faculty of Agriculture, Minia University. The voucher samples (Mn-ph-Cog-026) and (Mn-ph-Cog-025) for *Zinnia elegans* and *Gazania rigens* were kept in the Herbarium of the Pharmacognosy Department, Faculty of Pharmacy, Minia University, Minia, Egypt.

Sample preparation

The air-dried powdered leaves and stems (5 kg), roots (600 g) of *Z. elegans* and the whole plant material (4.5 Kg) of *G. rigens* were extracted with 95% ethanol and concentrated under reduced pressure to yield 400, 20 and 500 g dry extract, respectively. The concentrated ethanol extracts of *Z. elegans* (leaves and stems) and *G. rigens* were suspended in the least amount of distilled water, transferred to a separating funnel and partitioned successively with petroleum ether (3 L) and ethyl acetate (3L). The fractions were concentrated under reduced pressure to afford petroleum ether (80 and 153 g) and ethyl acetate fractions (57 and 19 g). The remaining mother liquor was concentrated to give the aqueous fraction (225 and 260 g), respectively.

**LC-HR-MS analysis**

The crude extracts (1 mg/mL) in MeOH were analyzed on an Accela HPLC (Thermo Scientific, Germany) coupled to a UV detector at 280 and 360 nm and an Exactive-Orbitrap high-resolution mass spectrometer (Thermo Fisher Scientific, Germany). The HPLC column was an ACE (Berkshire, UK) C18, 75 mm × 3.0 mm, 5 μm column. The mobile phase was composed of purified water (A) and acetonitrile (B) with 0.1 % formic acid in each solvent. The gradient method started with 10 % B linearly increased to 100 % B at a flow rate of 300 μL/min for 30 min and remained isocratic for 5 min before linearly decreasing back to 10 % B in 1 min. The column
was then re-equilibrated with 10% B for 9 min before the next injection. The total analysis time for each sample was 45 min., where 10 µL were injected and the tray temperature was maintained at 12 °C. High-resolution mass spectrometry was carried out in both positive and negative ESI ionization modes with a spray voltage at 4.5 kV and capillary temperature at 320 °C. The mass range was set from m/z 150-1500.

**Metabonomic analysis**

In MZmine, the raw data was imported by selecting the ProteoWizard-converted positive or negative files in mzML format. The peaks in the samples and blanks were detected using the chromatogram builder. Mass ion peaks were isolated with a centroid detector threshold that was greater than the noise level set to $1.0 \times 10^4$ and an MS level of one. Following this, the chromatogram builder was used with a minimum time span set to 0.2 min, and the minimum height and m/z tolerance to $1.0 \times 10^4$ and 0.001 m/z or 5.0 ppm, respectively. Chromatogram deconvolution was then performed to detect the individual peaks. The local minimum search algorithm (chromatographic threshold: 95%, search minimum in RT range: 0.4 min, minimum relative height: 5%, minimum absolute height: 3.0 × 104, the minimum ratio of peak top/edge: 3, and peak duration range: 0.2–5 min) was applied. Isotopes were also identified using the isotopic peaks grouper (m/z tolerance: 0.001 m/z or 5.0 ppm, retention time tolerance: 0.1 absolute (min), maximum charge: 2, and representative isotope: most intense). The chromatographic alignment and gap-filling were then used to reduce inter-batch variation. The peak lists were all aligned using the join aligner parameters set to m/z tolerance: 0.001 m/z or 5.0 ppm, weight for m/z: 20, retention time tolerance: 5.0 relative (%), weight for retention time: 20. The values for the weight of m/z and retention time should be kept the same; this means that both retention time and m/z are given equal importance. Missing peaks were reported using the gap filling peak finder with an intensity tolerance of 25%, m/z tolerance of 0.001 m/z or 5.0 ppm, and retention time tolerance of 0.5 absolute (min). An adduct search was performed for Na-H, K-H, NH₄, formate, and ACN + H (RT tolerance: 0.2 absolute (min), m/z tolerance: 0.001 m/z or 5.0 ppm, max relative adduct peak height: 30%). Additionally, a complex search was performed (ionization method: [M + H]$^+$ for ESI positive mode and [M − H]$^-$ for ESI negative mode, retention time tolerance: 0.2 absolute (min), m/z tolerance: 0.001 m/z or 5.0 ppm, and with maximum complex peak height of 50%). Adjust parameters with heuristics element count with all three
sub-options to get the isotope pattern filter working with all features with isotope peaks.

Excel macros were written to enable the subtraction of background peaks and to combine positive and negative ionization mode data files generated by MZmine. Peaks originating from the culture medium were extracted. By applying an algorithm to calculate the intensity of each m/z in both plant extracts and different fractions. The positive and negative ionization mode data sets from each of the respective extracts or fractions were combined with the macro enabling ion peaks that were observed in either or both positive and negative modes to be overlaid for further statistical analysis. The Excel macro was used to dereplicate each m/z ion peak with compounds in the customized database (using RT and m/z threshold of ±3 ppm) which provided details on the putative identities of all metabolites in plant fraction and sequentially sorted the number of the remaining unknowns for each fraction. (El-Sayed et al., 2018).

**Evaluation of antimalarial activity**

All fractions were investigated for their *in vitro* antimalarial activity and were evaluated for their ability to inhibit the chloroquine – sensitive (D6, Sierra Leone) *Plasmodium falciparum* protozoan and the chloroquine– resistant (W2) strains. The samples were tested against a suspension of red blood cells infected with *P. falciparum*. A 200 μL, with 2% parasitemia and 2% hematocrit in RPMI-1640 medium supplemented with 10% human serum and 60 μg/mL amikacin was added to the wells of a 96-well plate containing 10 μL of test samples at 15.87 μg/mL in duplicates and the percentage of inhibition was calculated relative to the negative and positive controls. The samples that showed % inhibition ≥ 50% were further proceeded to the second phase assay. In the second phase assay, the tested samples examined at 47.6, 15.87 and 5.29 μg/mL and the tested concentrations that afforded 50% inhibition of the protozoan relative to positive and negative controls (IC<sub>50</sub>) against the chloroquine – sensitive (D6) and the chloroquine– resistant (W2) strains were reported.

Concomitantly, all samples were tested against the VERO mammalian cell lines as an indicator of general cytotoxicity. The selectivity indices (SI) and the ratio of VERO
IC$_{50}$ to D$_6$ or W$_2$ IC$_{50}$ were calculated. The standard antimalarial drug chloroquine (0.079 μg/mL) was used as the positive control and DMSO (0.25%) was used as a vehicle. All IC$_{50}$ were calculated using the XLfit curve (Makler and Hinrichs, 1993).

**Evaluation of antileishmanial activity**

The anti-leishmanial activity of the samples was screened against *Leishmania donovani*, a fly-borne protozoan that causes visceral leishmaniasis. The promastigotes were grown in RPMI 1640 medium supplemented with 10 % fetal calf serum (GibcoChem. Co.) at 26 °C. A three-day-old culture was diluted to 5 x 10$^5$ promastigotes/mL. Drug dilutions were prepared directly in cell suspension in 96-well plates. Plates were incubated at 26 °C for 48 h, and growth of *Leishmania* promastigotes was determined by the Alamar Blue$^{TM}$ assay. Standard fluorescence was measured on a Fluostar Galaxy plate reader (BMG Lab Technologies) at an excitation wavelength of 544 nm and an emission wavelength of 590 nm. Amphotericin B and Alpha-difluoromethylornithine were used as standard antileishmanial agents. IC$_{50}$ values were computed from the dose-response curve (Ma et al., 2004).

**Evaluation of antitrypanosomal activity**

Blood stage forms of *Trypanosoma brucei* were grown in IMDM medium supplemented with 10 % fetal bovine serum. The assays were set up in a clear 96 well microplates. A two days old culture of *T. brucei* in the exponential phase was diluted with IMDM to 5000 parasites/mL. For primary screening (Single concentration of 20 μg/mL in duplicates) samples dilutions (1 mg/mL) were prepared from the stock samples (20 mg/mL) in IMDM medium. Each well received 4 μl of the diluted sample and 196 μl of the culture volume (total culture volume 200 μl). The plates were incubated at 37 °C in 5 % CO$_2$ for 48 h. Alamar blue (10 μl) (ABD Serotec, catalog number BUF012B) was added to each well and the plates were further incubated overnight. Standard fluorescence was measured on a Fluostar Galaxy fluorometer (BMG LabTechnologies) at 544 nm excitation, 590 nm emission. α-difluoromethyl ornithine (DFMO) was tested as standard. The samples that have shown more than 90
% inhibition of T. brucei growth in primary screening were subjected to secondary screening for dose-response analysis (Jain et al., 2016).

**Evaluation of antimicrobial activity**

All organisms used for the biological evaluation were obtained from the American Type Culture Collection (Manassas, VA) and include the following fungi, *Candida albicans* ATCC 90028, *Candida glabrata* ATCC 90030, *Candida krusei* ATCC 6258, *Cryptococcus neoformans* ATCC 90113, and *Aspergillus fumigatus* ATCC 90906 and the following bacteria, methicillin-resistant *Staphylococcus aureus* ATCC 43300 (MRS), *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, and *Mycobacterium intracellulare* ATCC 23068. Susceptibility testing was performed using a modified version of the CLSI methods. Samples were serially diluted in 20 % DMSO/saline and transferred in duplicates to 96-well flat-bottom microplates. Microbial inocula were prepared by correcting the OD630 of microbe suspensions in incubation broth to afford final target inocula. Amphotericin B (ICN Biomedicals, Ohio) for fungi and ciprofloxacin (ICN Biomedicals, Ohio) for bacteria is included as positive controls in each assay. All organisms were read at either 630 nm using the EL-340 Biokinetics Reader (Bio-Tek Instruments, Vermont) or 544ex/590em (*M. intracellulare, A. fumigatus*) using the Polarstar Galaxy plate reader (BMG Lab Technologies, Germany) prior to and after incubation. Percentage growth was plotted versus test concentration to afford the IC\textsubscript{50} (Samoylenko et al., 2009).

**Results and discussion**

The metabolomic analysis was performed according to Elsayed et al. 2018 Metabolomic profiling of *Z. elegans* and *G. rigens* revealed that the presence of various classes of metabolites in extracts and different fractions of both plants. Coumarins, sesquiterpene lactones and phenylethanoids were detected in *Z. elegans*, whereas amides and phenolic acid derivatives were characteristic for *G. rigens* (Figure 1). Identification of compounds was established using METLIN database (Table S1 and S2).

The LC-MS profiling of *Z. elegans* showed the mass ion peak at m/z 163.039 [M+H]\textsuperscript{+} in agreement with the molecular formula C\textsubscript{9}H\textsubscript{6}O\textsubscript{3} which was dereplicated as umbelliferone, which was isolated formerly from *Diplostephium foliosissimum*
Moreover, the LC-MS profiling of *G. rigens* revealed mass ion peak at \(m/z\) 242.151 [M+H]^+, corresponding to the predicted molecular formula C_{16}H_{19}NO which was characterized as undeca-2E,4E-diene-8,10-diynoic acid piperidide that was previously detected in *Achillea ptarmica* (Veryser et al. 2017). Another mass ion peak at \(m/z\) 313.070 [M-H]^- for the predicted molecular formula C_{17}H_{14}O_{6} was dereplicated as cirsimaritin. This flavone is a potential anti-cancer drug candidate against breast and prostate cancer formerly reported from *Centaurea kilaea* (Sen et al. 2017).

Additionally, two phenolic acid derivatives were detected in *G. rigens*, of which one mass ion peak at \(m/z\) 515.119 [M-H]^- in agreement with the molecular formula C_{25}H_{24}O_{12} was characterized as 3,5-di-O-cafeoylquinic acid that was formerly isolated from the same plant and *G. longiscapa* (Desoukey et al. 2016), the other at \(m/z\) 529.134 [M-H]^- corresponding to the molecular formula C_{26}H_{26}O_{12} was dereplicated as 3,5-di-O-cafeoyl quinic acid methyl ester previously obtained from *G. nivea* (El-Alfy et al. 2008).

Besides, both plants showed mass ion peak at \(m/z\) 338.342 [M+H]^+ for the suggested molecular formula C_{22}H_{24}NO which was identified as 13Z-docosenamide that was previously detected in *Vernonia amygdalina* (Oyugi et al. 2011).

Moreover, the results of the antimalarial activity of the examined samples revealed that the ethyl acetate fraction of *Z. elegans* exhibited a significant antimalarial activity of a 63% inhibition, followed by the petroleum ether fraction of the
same plant, ethyl acetate fraction of G. rigens and the total ethanol extract of roots of Z. elegans (39, 28 and 25%, respectively). All the other tested extracts and fractions showed weaker activity (Table S3). In the secondary phase assay, the ethyl acetate fraction of Z. elegans showed an IC₅₀ value of 21.03 and 13.72 µg/mL against P. falciparum D₆ and P. falciparum W₂, respectively. Furthermore, no cytotoxic activity against the VERO mammalian cell lines was observed up to the maximum dose tested 47.6 µg/mL, indicating the safety of the above mentioned plants (Table S4). Additionally, the antileishmanial activity of the tested samples was determined against Leishmania donovani promastigotes, where only the petroleum ether and the aqueous fractions of Z. elegans exhibited the highest percentage of inhibition (20%), while the other extracts and fractions showed weaker activities. Also, all the examined extracts and fractions showed weak antitrypansomal activity and none of the investigated extracts or fractions showed any antimicrobial activity (Table S5).

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Figure S1: Total ion chromatogram of the total ethanol extract of *Z. elegans* (leaves& stems).

Figure S2: Total ion chromatogram of the petroleum ether fraction of *Z. elegans* (leaves& stems).
Figure S3: Total ion chromatogram of the ethyl acetate fraction of *Z. elegans* (leaves & stems).

Figure S4: Total ion chromatogram of the aqueous fraction of *Z. elegans* (leaves & stems).
Figure S5: Total ion chromatogram of the total ethanol extract of *Z. elegans* (roots).

Figure S6: Total ion chromatogram of the total ethanol extract of *G. rigens.*
Figure S7: Total ion chromatogram of the petroleum ether fraction of *G. rigens*.

Figure S8: Total ion chromatogram of the ethyl acetate fraction of *G. rigens*.
Figure S9: Total ion chromatogram of the aqueous fraction of *G. rigens*.
Table S1. Identification of chemical composition of *Zinnia elegans* by LC/MS.

| Detected Compounds | Molecular Formula | m/z        | R_t (min) | Peak intensity | Extract / fraction                  |
|--------------------|-------------------|------------|-----------|----------------|-------------------------------------|
| Umbelliferone      | C₉H₆O₃            | 163.03988  | 2.3036    | 9.5 x10⁵       | Petroleum ether fraction (leaves & stems) |
| Unidentified       | —                 | 163.03999  | 3.5816    | 11.8 x10⁵      | Petroleum ether fraction (leaves & stems) |
|                    |                   |            |           |                | Total extract (roots)               |
| Esculetin          | C₉H₆O₄            | 177.0546   | 8.1619    | 9.5 x10⁵       | Total extract (leaves & stems)       |
|                    |                   |            |           |                | Petroleum ether fraction (leaves & stems) |
| Unidentified       | —                 | 222.09232  | 8.9535    | 23.0 x10⁵      | Total extract (leaves & stems)       |
|                    |                   |            |           |                | Petroleum ether fraction (leaves & stems) |
|                    |                   |            |           |                | Ethyl acetate fraction (leaves & stems) |
|                    |                   |            |           |                | Aqueous fraction (leaves & stems)    |
|                    |                   |            |           |                | Total extract (roots)               |
| Zaluzanin C        | C₁₅H₁₈O₃          | 245.11732  | 8.1638    | 1.0 x10⁶       | Total extract (leaves & stems)       |
|                    |                   |            |           |                | Petroleum ether fraction (leaves & stems) |
| 13Z-docosenamide   | C₂₂H₄₃NO         | 338.34258  | 14.0865   | 1.1 x10⁷       | Total extract (leaves & stems)       |
|                    |                   |            |           |                | Petroleum ether fraction (leaves & stems) |
|                    |                   |            |           |                | Ethyl acetate fraction (leaves & stems) |
|                    |                   |            |           |                | Aqueous fraction (leaves & stems)    |
|                    |                   |            |           |                | Total extract (roots)               |
| 8β-(Angeloyloxy)-1β-hydroxyarbusculin B | C₂₀H₂₆O₅ | 345.16991  | 8.1676    | 9.8 x10⁷       | Total extract (leaves & stems)       |
|                    |                   |            |           |                | Petroleum ether fraction (leaves & stems) |
| Unidentified       | —                 | 348.30155  | 7.0980    | 6.2 x10⁷       | Ethyl acetate fraction (leaves & stems) |
|                    |                   |            |           |                | Aqueous fraction (leaves & stems)    |
| Unidentified       | —                 | 353.17305  | 9.4894    | 1.2 x10⁷       | Ethyl acetate fraction (leaves & stems) |
|                    |                   |            |           |                | Aqueous fraction (leaves & stems)    |
| Unidentified       | —                 | 364.29672  | 5.1790    | 1.5 x10⁷       | Ethyl acetate fraction (leaves & stems) |
|                    |                   |            |           |                | Aqueous fraction (leaves & stems)    |
| Unidentified       | —                 | 413.26697  | 13.7744   | 3.0 x10⁷       | Total extract (leaves & stems)       |
| Detected Compounds                  | Molecular Formula | m/z             | R<sub>t</sub> (min) | Peak intensity | Extract / fraction                        |
|------------------------------------|-------------------|-----------------|--------------------|----------------|------------------------------------------|
| Umbelliferone                      | C<sub>9</sub>H<sub>6</sub>O<sub>3</sub> | 163.03988       | 2.3036             | 9.4 x 10<sup>5</sup> | Total extract                           |
| Unidentified                       | —                 | 163.03999       | 3.5816             | 6.6 x 10<sup>5</sup> | Total extract                           |
| Unidentified                       | —                 | 222.09232       | 8.9535             | 31.2 x 10<sup>5</sup> | Ethyl acetate fraction                  |
| Unidentified                       | —                 | 224.14053       | 2.4815             | 6.5 x 10<sup>5</sup> | Ethyl acetate fraction                  |
| Unidentified                       | —                 | 226.1563        | 3.5038             | 10.8 x 10<sup>5</sup> | Ethyl acetate fraction                  |
| Undeca-2E,4E-diene-8,10-diynoic acid piperidine | C<sub>16</sub>H<sub>19</sub>NO | 242.15106       | 1.7740             | 1.9 x 10<sup>5</sup> | Ethyl acetate fraction                  |
| Unidentified                       | —                 | 307.12119       | 4.1940             | 8.0 x 10<sup>5</sup> | Ethyl acetate fraction                  |
| Cirsimaritin                       | C<sub>17</sub>H<sub>14</sub>O<sub>6</sub> | 313.07092       | 6.1632             | 4.2 x 10<sup>5</sup> | Ethyl acetate fraction                  |
| 13Z-docosenamide                   | C<sub>22</sub>H<sub>43</sub>NO | 338.34258       | 14.0865            | 1.0 x 10<sup>7</sup> | Ethyl acetate fraction                  |
| Unidentified                       | —                 | 369.168         | 8.1705             | 3.9 x 10<sup>5</sup> | Aqueous fraction                        |
| Unidentified                       | —                 | 413.26697       | 13.7744            | 3.4 x 10<sup>7</sup> | Total extract                           |
| 3,5-di-O-caffeoylquinic acid       | C<sub>25</sub>H<sub>24</sub>O<sub>12</sub> | 515.11959       | 3.5832             | 2.7 x 10<sup>8</sup> | Ethyl acetate fraction                  |
| 3,5-di-O-caffeoylquinic acid methyl ester | C<sub>26</sub>H<sub>26</sub>O<sub>12</sub> | 529.13464       | 4.3452             | 5.8 x 10<sup>5</sup> | Ethyl acetate fraction                  |

**Table S2.** Identification of chemical composition of *Gazania rigens* by LC/MS.
**Table S3.** The antimalarial activity of the total ethanol extracts and different fractions of *Z. elegans* and *G. rigens* (primary phase assay).

| Group | % inhibition |
|-------|--------------|
| Chloroquine (0.079µg/mL) | 100          |
| Total extract of *Z. elegans* (leaves & stems) (15.87µg/mL) | 6            |
| Petroleum ether fraction of *Z. elegans* (leaves & stems) (15.87µg/mL) | 39           |
| Ethyl acetate fraction of *Z. elegans* (leaves & stems) (15.87µg/mL) | 63           |
| Aqueous fraction of *Z. elegans* (leaves & stems) (15.87 µg/mL) | 11           |
| Total extract of *Z. elegans* (roots) (15.87µg/mL) | 25           |
| Total extract of *G. rigens* (15.87µg /mL) | 4            |
| Petroleum ether fraction of *G. rigens* (15.87µg /mL) | 18           |
| Ethyl acetate fraction of *G. rigens* (15.87µg /mL) | 28           |
| Aqueous fraction of *G. rigens* (15.87 µg/mL) | 14           |

**Table S4.** The antimalarial activity of the ethyl acetate fraction of *Z. elegans* (secondary phase assay) IC$_{50}$ µg/mL.

| Group | P. falciparum $D_6$ | P. falciparum $W_2$ | VERO cells |
|-------|------------------|------------------|-------------|
|       | $IC_{50}$  | SI    | $IC_{50}$ | SI    | $IC_{50}$ |             |
| Chloroquine (0.238-0.026 µg/mL) | > 0.026 | > 9  | < 0.026 | > 9  | > 0.238 |             |
| Ethyl acetate fraction of *Z. elegans* (leaves & stems) (47.6-5.29 µg/mL) | 21.03 | 2.3  | 13.72 | 3.5  | > 47.6 |             |

**Table S5.** The antileishmanial and antitrypanosomal activities of the total ethanol extracts and different fractions of *Z. elegans* and *G. rigens* (primary phase assay).

| Group | % inhibition |
|-------|--------------|
| L_donovani_Pinh | L_donovani_AMA ST_Pinh | L_donovani_AMASTTH P_Pinh | T_brucei_Pinh |
| Amphotericin B (2 µg/mL) | 100  | 96  | 97  | -  |
| α-Difluoromethylornithine (20 µg/mL) | -    | -   | -   | 100 |
| Total extract of *Z. elegans* (leaves & stems) (20 µg/mL) | 1    | 1   | 19  | 4   |
| Petroleum ether of *Z. elegans* (leaves & stems) fraction (20 µg/mL) | 2    | 0   | 20  | 7   |
| Ethyl acetate fraction of *Z. elegans* (leaves & stems) (20 µg/mL) | 5    | 0   | 18  | 5   |
| Aqueous fraction of *Z. elegans* (leaves & stems) | 7    | 3   | 20  | 10  |
| Extract Type                                      | Activity 1 | Activity 2 | Activity 3 | Activity 4 |
|--------------------------------------------------|------------|------------|------------|------------|
| Total extract of *Z. elegans* (roots) (20 µg/mL) | 6          | 0          | 17         | 6          |
| Total extract of *G. rigens* (20 µg/mL)          | 6          | 1          | 17         | 7          |
| Petroleum ether of *G. rigens* (20 µg/mL)        | 1          | 0          | 4          | 0          |
| Ethyl acetate fraction of *G. rigens* (20 µg/mL) | 5          | 0          | 13         | 1          |
| Aqueous fraction of *G. rigens* (20 µg/mL)       | 6          | 0          | 19         | 5          |