ACCSCIENCE PUBLISHING

Cancer Plus

ORIGINAL RESEARCH ARTICLE

Evaluating anticancer effects of geraniin supplementation in a syngeneic mouse model of breast cancer: Identification of differentially regulated plasma proteins

Supplementary File

A. Immunoassay on i-Ome discovery protein microarray

A1. Serum dilution

All serum samples were thawed for 30 min in a shaking incubator at 20°C. After the samples were completely thawed, each sample was vortexed three times vigorously and spun down for 3 min at 13,000 rpm. Following that, 22.5 µL of serum sample was added into 4.5 mL of serum assay buffer (SAB) containing 0.1% (v/v) Triton, 0.1% bovine serum albumin (BSA) in phosphate-buffered saline at 20°C and mixed homogeneously. The tube was tilted during aspiration to avoid lipid bilayer carryover. The serum dilution process was performed in a class II biosafety cabinet.

A2. Biomarker assay

The KREX array-based serological assay is quantitative across five orders of magnitude, with detection limits in the pg/mL range. This assay entails serial dilution studies to be performed on tested serum samples before autoantibody assay on immunome array to ensure both assay and samples meet the accepted threshold, as outlined in quality control. First, the array was removed from the storage buffer using forceps, placed in the slide box and rack containing 200 mL cold SAB, and shaken on an orbital shaker at 50 rpm for 5 min. Then, the slide was placed, array side up, in a slide hybridization chamber with individual sera, which had been diluted earlier. All slides were scanned using the barcode scanner into the relevant batch record and incubated on a horizontal shaker at 50 rpm for 2 h at 20°C.

A3. Array washing after serum binding

The protein microarray slide was then rinsed twice in individual "pap jars" with 30 mL SAB, followed by 200 mL of SAB in the slide-staining box for 20 min on the shaker at 50 rpm at room temperature. All slides were transferred sequentially and in the same orientation.

A4. Incubation with Cy3-anti-IgG

After incubation, the slide was dipped in 200 mL of SAB, three times for 5 min at 50 rpm at room temperature. Excess buffer was removed by immersing the slide in 200 mL of pure water for a few minutes. Slides were then dried for 2 min at room temperature and then stored at room temperature until scanning (preferably the same day). Hybridization signals were measured with a microarray laser scanner (Agilent Scanner; SureScan, USA) at 10 µm resolution. Fluorescence intensities were detected and quantified according to the manufacturer's instructions, whereby each spot was plotted using Agilent Feature Extraction software.

A5. Bioinformatics analysis

A5.1. Image analysis: Raw data extraction

The aim of image analysis was to evaluate the amount of autoantibody present in the serum sample by measuring the median intensities of all the pixels within each probed spot. A raw.tiff format image file was generated for each slide.

Automatic extraction and quantification of each spot on the array were performed using GenePix Pro 7 software (Molecular Devices, USA), which outputs the statistics for each probed spot on the array. This includes the mean and median of the pixel intensities within a spot along with its local background. A GenePix Array List (.GAL) file for the array was generated to aid with image analysis. This file contains information on all probed spots and their positions on the array. Following data extraction, a GenePix Results (.GPR) file was generated for each slide, containing information for each spot; Protein ID, protein name, and foreground and background intensities. In the datasheet generated from the experiment, both foreground and background intensities of each spot are represented as relative fluorescence units.

A5.2. Data handling and pre-processing

For each slide, proteins and control probes were spotted in quadruplicate (four arrays on each slide). The following steps were performed to verify the quality of the protein array data before proceeding with data analysis. First, the calculation of net intensities for each spot was determined by subtracting background signal intensities from the foreground signal intensities of each spot. For each spot, the background signal intensity was calculated using a circular region with three times the diameter of the spot, centered on the spot. Next, the calculation of the percentage of the coefficient of the variant (CV %) was performed to determine the variations between the replica spots on each slide (Equation SI):

$$CV\% = \frac{Standard\,deviation}{Mean} \times 100\% \qquad \text{Equation SI}$$

Next, we normalized the intensity using composite normalization, which includes quantile-based and total intensity-based modules. This method assumes that different samples share a common underlying distribution of their control probes while considering the potential existence of flagged spots within them. The immunome array uses Cy3-labeled biotinylated BSA (Cy3-BSA) replicates as the positive control spots across slides. Hence, it is considered a housekeeping probe for the normalization of signal intensities for any given study. The quantile module adopts a previously described algorithm[1]. This reorganization enables the detection and handling of outliers or flagged spots in any of the 20 Cy3BSA control probes. A total intensity-based module was then implemented to obtain a scaling factor for each sample. This method assumes that post-normalization, the positive controls should have a common total intensity value across all samples.

A5.3. Batch normalization

Since the samples were assayed at two different batches, batch normalization was performed before data analysis. Following data processing, ComBat normalization was performed on the normalized fluorescent intensity values to adjust for possible batch effects from both batches [4]. These ComBat-normalized fluorescent intensities across 1,600+ autoantibodies were used as inputs for model generation.

A5.4. Data analysis and biomarker identification

The identification of biomarkers was done using the penetrance fold change method as described in (Sumera et al., 2020)[1].

Based on the penetrance fold change described in Sumera *et al*., 2020[1], putative biomarkers were identified and ranked according to the i-penetrance fold-change >2.0; ii-penetrance frequency case 10% and penetrance frequency differential 10%.

B. Figures

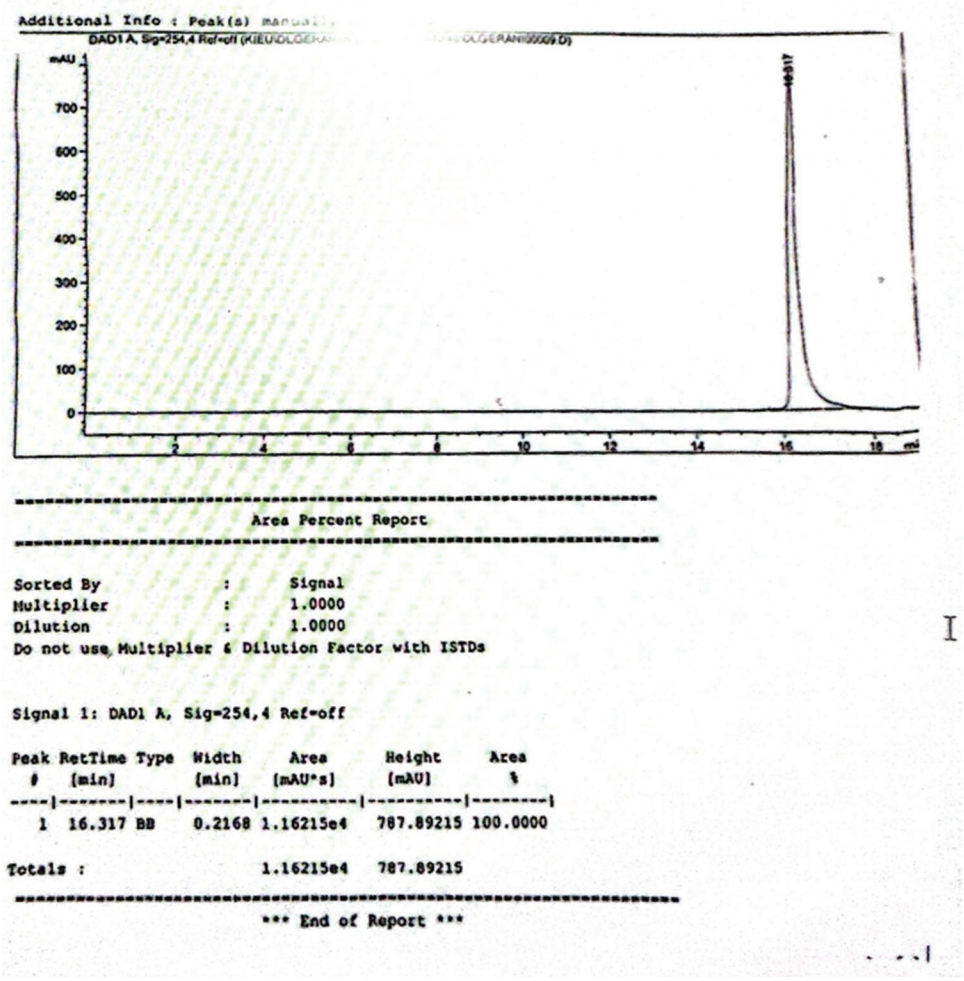

Figure S1. Purity of geraniin as determined by high-performance liquid chromatography

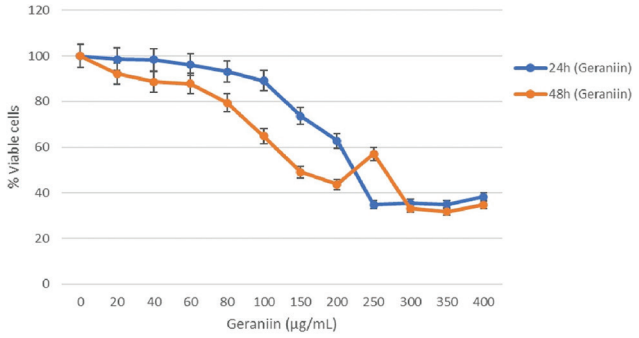

Figure S2. Cell-based studies. The 4T1 murine mammary cancer cells were treated with different concentrations of geraniin. Cell viability after 24 and 48 h of exposure was determined using the water-soluble tetrazolium salt proliferation assay (n = 3).

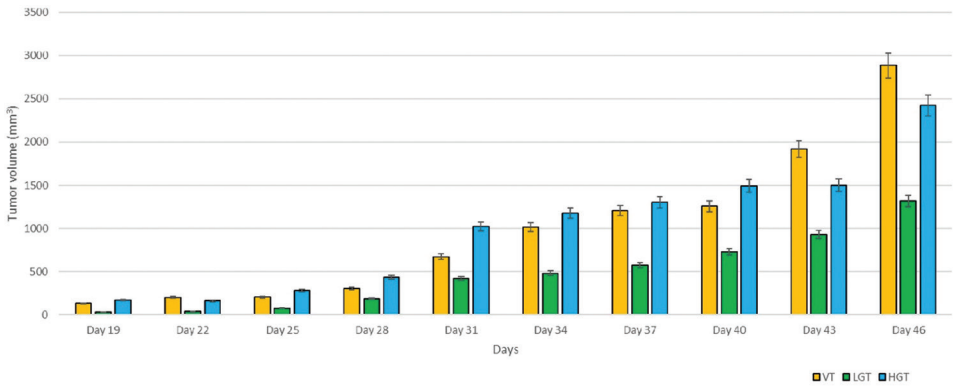

Figure S3. Tumor volume with low and high doses of geraniin. Distribution of tumor volume (mm^3) of three treatment groups of mice inoculated with tumor and supplemented with vehicle (VT; yellow), low-dose geraniin (LGT; green), and high-dose geraniin (HGT; blue) at different time points (days 19, 22, 25, 28, 31, 34, 37, 40, 43, and 46). The tumor volume (mm^3) of each group (n = 3) is presented as the mean ± standard deviation. Based on follow-up time points, the mean volume of the three treatment groups is significantly different (p<0.05) at all time points, except on day 37.

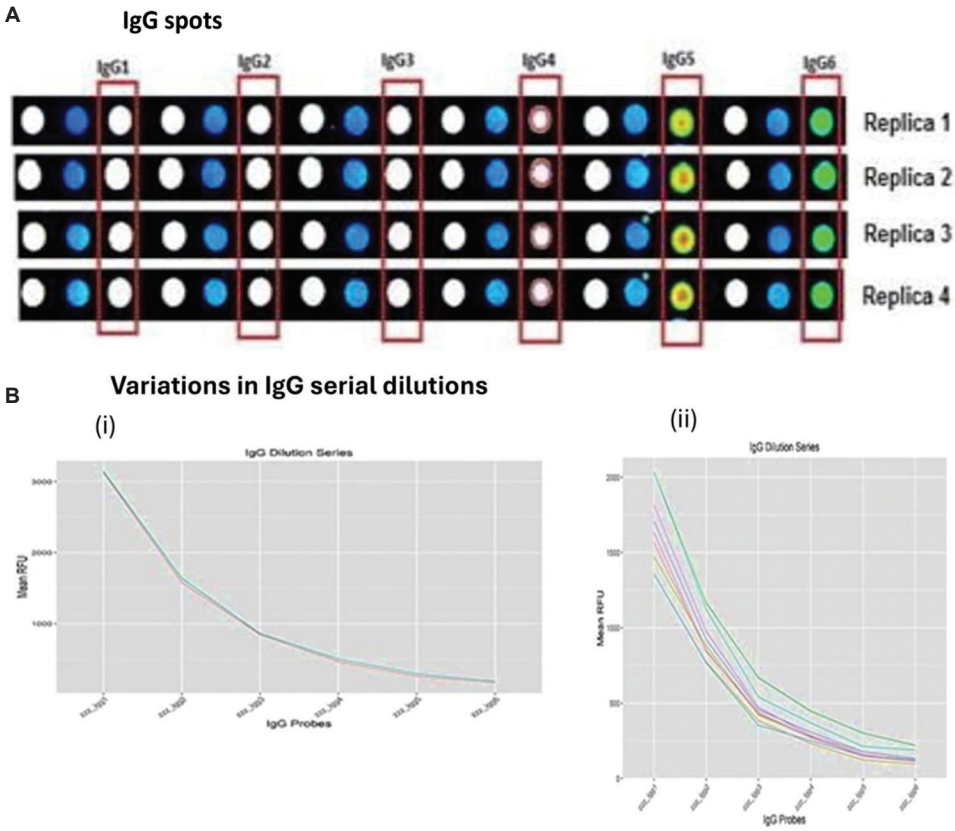

Figure S4. IgG dilution curve across six experimental animals and reactivity profile of IgG subtypes. (A) Shows an array of IgG spots for different experimental animals (IgG1 to IgG6) across four replicates (Replica 1 to 4). The intensity of the spots indicates antibody binding or signal detection. (B) IgG dilution series showing the relationship between various IgG probe concentrations and the corresponding mean relative fluorescence units. Each colored line likely represents data from one of the six experimental animals. Abbreviation: IgG: Immunoglobulin G.

Reference

1. Sumera A, Anuar ND, Radhakrishnan AK, *et al*. A novel method to identify autoantibodies against putative target proteins in serum from beta-thalassemia major: A pilot study. *Biomedicines*. 2020;8(5):97.

doi: 10.3390/BIOMEDICINES8050097