








ORIGINAL RESEARCH ARTICLE

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

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Abstract

Geraniin, an ellagitannin present in many seeds, nuts, fruits, and plants, has anticancer, antioxidant, antiviral, antimicrobial, antimutagenic, cardiovascular protective, and hypoglycemic properties. Geraniin was tested for its anticancer and plasma protein-modifying properties in a syngeneic mouse model of breast cancer (BC). A mouse mammary cancer cell line (4T1) was injected into the mammary pad of female BALB/c mice to produce BC. Daily oral gavage of geraniin (0.5 mg) or soy oil was given to animals having visible tumors. For 6 – 7 weeks, tumor growth was evaluated. At postmortem, cancer tissue was removed for histopathology, and the plasma was analyzed using a commercial protein array platform. Geraniin supplementation reduced tumor growth and liver metastasis ($p < 0.05$) and altered 20 plasma proteins, including Tropomyosin 3 (TPM3), catenin beta-1 (CTNNB1), hemopoietic lineage cell-specific protein 1 (HCLS1), and Serine/threonine-protein kinase 10 (STK10). Six biomarkers (RAD23 homolog B, HCLS1, CTNNB1, A type of type II restriction enzyme, TPM3, and STK10) were higher in geraniin-treated samples than in control samples, regardless of tumor induction. Monitoring plasma protein expression in a BC model indicated tumor progression, metastasis, and potential diagnostic or therapeutic biomarkers. The 4T1 cell line, an exceptionally invasive mammary cancer model, accurately replicates human triple-negative BC, making it valuable for investigating metastatic behavior and treatment. Plasma protein dynamics in this model may identify tumor aggressiveness regulators and therapeutic targets. Geraniin possesses antitumor and anti-metastasis characteristics and could be developed to treat BC. The autoantibody response toward these antigens and fluctuation in the response could suggest a potential marker or a predictive marker toward treatment with geraniin.

Keywords: Geraniin; Breast cancer; Mouse model; Protein array; Biomarker

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Citation: Hui HJ, Xin CNZ, Anuar ND, *et al.* Evaluating anticancer effects of geraniin supplementation in a syngeneic mouse model of breast cancer: Identification of differentially regulated plasma proteins. *Cancer Plus*. 2025;7(1):83-95.
doi: 10.36922/cp.7115

Received: December 19, 2024

Revised: February 26, 2025

Accepted: March 20, 2025

Published online: April 15, 2025

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1. Introduction

Breast cancer (BC) is the most common cancer in women from both developed and underdeveloped nations. The World Health Organization in 2020 reported that there were 2.3 million women diagnosed with BC, and there were around 685,000 deaths globally due to BC. As of the end of 2020, there were 7.8 million women alive who were diagnosed with BC in the past 5 years, making it the world's most prevalent cancer.^{1,2} In the United States, there were 264,121 new BC cases in 2019.³ According to the Malaysian National Cancer Registry report for 2017 – 2021, BC made up 19% of cancers reported in females, followed by colorectal cancer (13.5%).⁴ Although BC is ranked as the fifth most common cause of death in women, there has been some improvement in BC survival rates.^{5,6}

Cancer diagnosis and its treatment can have lasting physical and psychological repercussions on the lives and well-being of BC patients. Current chemotherapeutic drugs used in the treatment of BC include Tamoxifen,^{7,8} Herceptin,^{7,9,10} and Paclitaxel.^{7,10,11} Although these chemotherapeutic drugs are effective in treating BC and can decrease the death rate in BC patients, these drugs have been linked to side effects, such as endometrial proliferation, endometrial hyperplasia, endometrial cancer, and uterine sarcomas,¹² thromboembolic diseases,¹² and in some patients, it can result in the development of drug resistance with prolonged use.^{13,14} A decline in quality of life was observed not only after initial therapy but also persisted as a concern in long-term survival.¹⁵ The financial burden of care for a BC patient is considerable. The anticipated incidence rates of BC are expected to escalate, thereby leading to an increase in associated expenses.¹⁶ Consequently, it is essential to devise alternative treatment strategies to enhance patient adherence and quality of life and alleviate the financial burden on BC patients. In addition, improving disease prognosis is a primary objective.

Chemoprevention and other treatment modalities are currently sought to improve bioavailability and efficacy and reduce the cost of care for cancer patients. Many of these conventional therapeutic strategies are largely ineffective for metastatic cancers, and some of the treatment methods have various side effects, such as the development of drug resistance. In addition, this disease can recur. Hence, there is a need to identify alternative treatment modalities, including identifying and using natural bioactive compounds with anticancer activities that can delay or reverse carcinogenesis, and pose lesser side effects.

In this study, we explored using a natural bioactive compound, geraniin, an ellagitannin. Geraniin was first isolated from *Geranium thunbergii* Sieb. et Zucc., a

tannin-rich medicinal herb popularly used as a remedy for intestinal disorders in Japan.¹⁷ Ellagitannins occur naturally in certain fruits, such as raspberries, strawberries, and blackcurrants¹⁸; pomegranates and grapes¹⁹; and nuts.^{20,21} Geraniin has been identified as a constituent in extracts from many plants and is commonly used in traditional medicine preparations in Asia.²² Plants rich in ellagitannins are widely used for medicinal purposes due to their various health benefits,²³ such as antioxidant,²⁴ radioprotective,²⁵ antimicrobial,^{26,27} anti-inflammatory,^{28,29} cell cycle arrest,^{30,31} and apoptosis induction.^{30,31} In addition, geraniin was reported to possess anticancer effects in various human cancer cell lines, such as human glioma cells,³⁰ BC cells,³¹ colon cancer cells,^{32,33} ovarian cancer cells,³⁴ lung cancer cells,³⁵ and osteosarcoma cells.³⁶ To date, the anticancer effects of geraniin supplementation have not been evaluated using a syngeneic animal model of BC.

In this paper, we present the anticancer effects following daily supplementation of geraniin in a syngeneic mouse model of BC and the differential expression of proteins in the plasma isolated from these animals.^{37,38} The 4T1 murine mammary cancer cells used to induce BC in BALB/c mice were triple-negative BC (TNBC) cells, which are clinically difficult to treat and highly metastatic.^{39,40} BC was induced in the animal by injecting the 4T1 mammary cancer cells into the mammary pad of female BALB/c mice to induce cancer.³⁸

2. Materials and methods

2.1. Animals

Five-week-old female BALB/c mice were purchased from an accredited animal supplier (Chenur, Malaysia). The mice were housed at the animal holding facility (AHF) of the International Medical University (IMU; Malaysia). The mice were housed in ventilated cages, with three per cage in the AHF. The bedding for the cages was purchased from a commercial source and was changed once every 5 days. The mice were supplied with reverse osmosis water and standard food pellets, which were available *ad libitum*.

2.2. Test compounds

Pure geraniin (Figure S1) was purchased from a commercial source, i.e., Nanjing Manhay Medical Technology Co., Ltd. (China). Soy oil (Soya Lite, Malaysia) was the vehicle used to prepare geraniin for oral supplementation.

2.3. Tumor cell lines and culture conditions

The 4T1 murine mammary cancer cell line (ATCC: CRL-2539) was obtained from the cell culture archive of the IMU research laboratory. Injection of the 4T1 cells into

the mammary pad can induce BC in BALB/c mice, which resembles human stage IV BC, in terms of tumor growth and the spread of cancer cells.⁴¹

2.4. Mouse model of BC

The experimental model used was a syngeneic mouse model of BC described previously.³⁸ Five-week-old female BALB/c mice were allowed to acclimatize in the IMU AHF for 1 week. Following this, the mice were randomly assigned to tumor-induced and non-tumor groups. The mice in the tumor-induced group received a single injection (100 µL) of 4T1 cells (1×10^4 cells/mL) into their right mammary fat pad.³⁸ The mice in the non-tumor group received a similar injection but with no cells. The tumor-inoculated mice were monitored daily for signs of tumor growth.^{36,38} When the tumor was palpable (around day 12), the mice were randomly assigned into vehicle or treatment groups (Table 1). The mice in groups A and C were fed once a day with 50 µL of vehicle (soy oil) by oral gavage. In comparison, the mice in groups B and D were fed 50 µL of 0.5 mg of geraniin in the vehicle by oral gavage daily until the animals were sacrificed on day 46. This study used soy oil as the vehicle as it does not contain geraniin. The geraniin supplement was prepared fresh every day.

Tumor size was measured once every 3 days, where two perpendicular diameters were obtained using a digital caliper (length [L] and width [W]), which were used to calculate the tumor volume³⁶:

$$\text{Tumor volume (V)} = 0.52 \times L^2 \times W \quad (\text{I})$$

The tumor-induced mice in the vehicle-fed and geraniin-fed groups were monitored daily for signs of distress to minimize animal suffering. On day 46, the animals in both groups were humanely euthanized when the mice in the vehicle-fed group started displaying signs of distress due to tumors, such as reduced mobility or reduced eating. None of the animals were euthanized before day 46 due to worsening conditions. The animals were euthanized by placing them in a tank containing diethyl ether when signs of distress or a decline in health conditions were seen. This study was approved by the Joint Committee for Research and Ethics of IMU and complied with the university's and international guidelines on the use of animals in research (BMS I/2018[01] and BMS I/2018[14]).

2.5. Histopathological analysis

At autopsy, the breast tumor, liver, and lungs were harvested and fixed in 10% buffered formaldehyde solution for 24 h and processed for histopathological analysis. Thin sections (4 µM) were prepared and stained with hematoxylin and eosin and mounted with Listerine-phthalate-xylene. Following this, the slides were viewed under a light microscope to look for signs of metastasis and necrosis.

2.6. Protein expression

The Sengenics protein microarray was used for a high throughput quantification of autoantibodies in plasma samples from 11 mice (Table 2). The serum from each experimental animal was subjected to an autoantibody screening assay using the Sengenics Immunome Array

Table 1. Experimental groups

Group	Number of mice	Tumor ^a inoculated	Daily oral gavage ^{**}
A	6	No	Vehicle
B	6	No	Geraniin (0.5 mg)
C	6	Yes	Vehicle
D	6	Yes	Geraniin (0.5 mg)

Note: Vehicle refers to soybean oil; ^ainjected with 4T1 cells in mammary pad; ^{**}once the tumor is palpable.

Table 2. Annotations of total cases and controls

Sample ID	Group	Annotated ID for analysis	Group name
Case 026229	Control 2 (no tumor, no treatment)	TNEG-CTRL 026229	Control
Case 026230	Control 2 (no tumor, no treatment)	TNEG-CTRL 026230	
Case 024709	Control 1 (tumor, no treatment)	TPOS-CTRL 024709	Tumor alone
Case 024720	Control 1 (tumor, no treatment)	TPOS-CTRL 024720	
Case 024719	Control 1 (tumor, no treatment)	TPOS-CTRL 024719	
Case 024718	Control (no tumor, fed with 0.5 mg geraniin daily)	TNEG 024718	Geraniin
Case 024717	Control (no tumor, fed with 0.5 mg geraniin daily)	TNEG 024717	
Case 024683	Control (no tumor, fed with 0.5 mg geraniin daily)	TNEG 024683	
Case 024682	Test (tumor, fed with 0.5 mg geraniin daily)	TPOS 024682	Tumor+ Geraniin
Case 024687	Test (tumor, fed with 0.5 mg geraniin daily)	TPOS 024687	
Case 024686	Test (tumor, fed with 0.5 mg geraniin daily)	TPOS 024686	

(Sengenics Corporation, Malaysia). The detailed sample annotation is presented in Table 2. Serum samples were subjected to autoantibody assay using the Sengenics protein microarray, where bound immunoglobulin G (IgGs) were detected and quantified as described previously.⁴² Details of the steps used in the Sengenics protein microarray are provided in the Supplementary File (A. Immunoassay on i-Ome discovery protein microarray).

2.7. Statistical analysis

Statistical analysis was carried out for all the results obtained using Statistical Package for the Social Sciences (SPSS) software (SPSS Statistics Version 25; IBM Corporation, USA). The mean and standard deviation of tumor size and weight were collected from all mice from the treatment and control groups, which consisted of six mice per group (Table 1). Assuming that the data are normally distributed, the analysis of variance test in the SPSS software was used to compare differences among the treatment groups. The significance level was set at $p < 0.05$. Data presented in texts and figures were represented as mean \pm standard deviation.

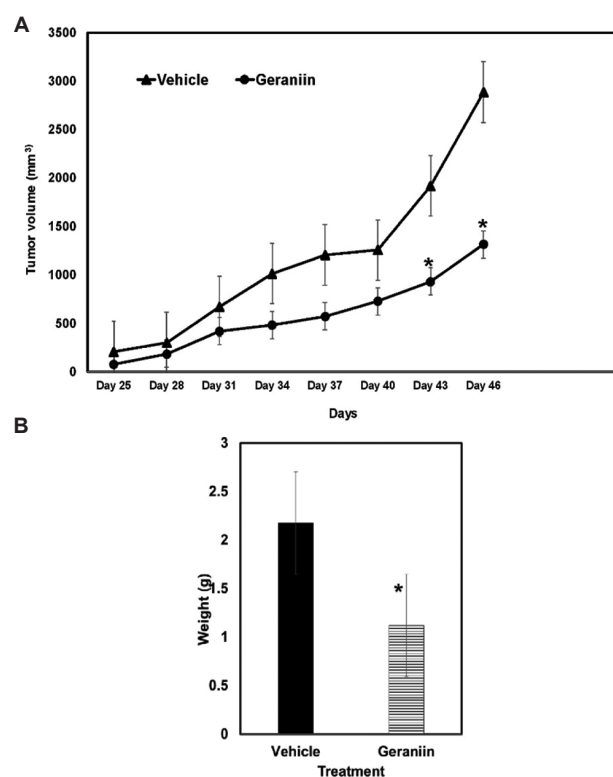


Figure 1. Tumor volume and tumor weight of mice. (A) Tumor volume measured every 3 days using a digital caliper. (B) Tumor weight measured at autopsy. Each data point represents the mean \pm standard deviation of six mice ($n = 6$) per group. * $p < 0.05$.

3. Results

3.1. Effects of geraniin on proliferation of murine breast cancer cells

Geraniin had anti-proliferative effects ($p < 0.01$) on the 4T1 murine breast cancer cells at the concentrations tested. In addition, the inhibition observed was dose- and time- dependent (Figure S2). The half maximal inhibitory concentration (IC₅₀) of geraniin on the 4T1 cells at two time-points (24 and 48 hours) were determined from the cell viability plots (Figure S2).

3.2. Antitumor effects of geraniin

Tumor-induced animals fed daily with geraniin displayed a reduction ($p < 0.05$) in tumor growth compared to mice fed with the vehicle (Figure 1A). At autopsy, the tumor from each animal was excised and weighed. The average weight of tumors isolated from mice fed daily with geraniin was lower ($p < 0.05$) than those fed daily with the vehicle (Figure 1B and S3).

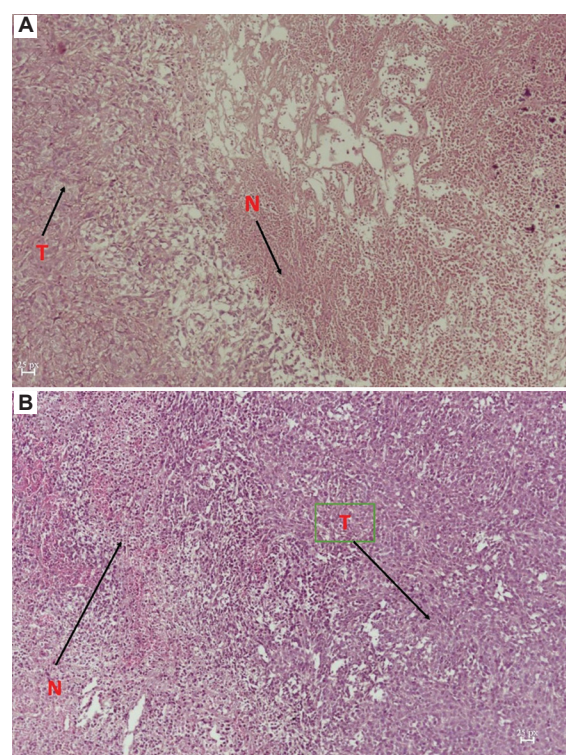


Figure 2. Photomicrographs of H&E-stained primary tumor excised from mice fed daily with (A) vehicle (soy oil) or (B) 0.5 mg geraniin by oral gavage. Both tumor sections feature areas with extensive necrosis. Magnification: 100 \times . Scale bars: 25px. Abbreviations: H&E: Hematoxylin and eosin; N: Necrosis; T: Tumor.

3.3. Histopathology of primary tumor and metastasis

The primary tumors excised from animals that were fed with either the vehicle or geraniin. Geraniin-fed animals displayed extensive areas of necrosis within the range of 25 – 80% (Figure 2). Furthermore, primary tumors from both groups had pleomorphic tumor cells with large nuclei, prominent nucleoli, vesicular chromatin, and <10 mitoses/high power fields (hpf), and <10% of tubule formation

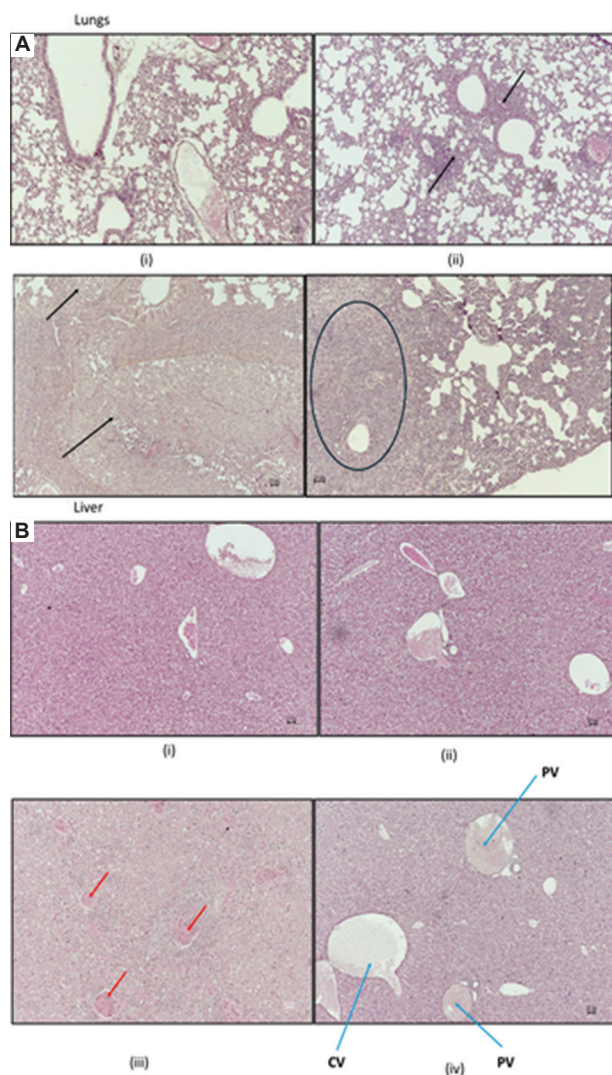


Figure 3. Photomicrographs of H&E-stained (A) lung and (B) liver tissue sections from (i and ii) normal mice fed daily with (i) vehicle or (ii) 0.5 mg geraniin, and from (iii and iv) tumor-induced mice fed with (iii) vehicle or (iv) 0.5 mg geraniin. Black arrows indicate the presence of lymphovascular emboli in the lungs (A, ii, and iii), while the black circle indicates the presence of metastasis (A, iv). Red arrows indicate areas with extramedullary hemopoiesis (B, iii), while blue arrows indicate congestion of central veins (CV) or portal veins (PV). Magnification: 100 \times . Scale bars: 25 μ m.

Abbreviation: H&E: Hematoxylin and eosin.

in the tumor was observed (Figure 2). The tumor cells were noted to be moderately differentiated. Lung sections from the non-tumor-induced mice fed with the vehicle displayed normal lung parenchyma (Figure 3A, i), while the lung sections from mice fed with geraniin displayed mild to moderate peri-bronchial and perivascular chronic inflammatory infiltrates (Figure 3A, ii). In tumor-induced mice, the lung sections of mice fed with vehicle displayed lymphovascular emboli in the lung (Figure 3A, iii), while the lung sections of mice fed with geraniin indicated the presence of metastasis (Figure 3A, iv). Liver sections from non-tumor-induced mice fed with vehicle or geraniin displayed normal liver parenchyma (Figure 3B, i, and ii). In tumor-induced mice, the liver sections from mice fed with vehicle displayed extensive extramedullary hemopoiesis in the liver (Figure 3B, iii). In contrast, the liver sections from mice fed with geraniin exhibited congestion of central and portal veins (Figure 3B, iv).

3.4. Protein array

3.4.1. Internal control within the standard threshold

The samples were assayed in two separate batches. Nine samples were assayed in the first batch, and two negative control samples (no treatment, no tumor) were assayed in the subsequent batch. All samples passed the quality control; a signal variation within the accepted threshold. The coefficient of variation (CV; %) of the IgG controls was 5.1%. The signal of all proteins across samples and arrays in the two assay batches had an average CV of 6.4%. The IgG spots served as a positive control for array spotting and were fabricated in dilution series to assess the binding capacity of Cy3-conjugated secondary antibody, anti-IgG. The experimental ratios for the dilution series of IgGs are displayed in Equation II, where “x” represents the initial concentration. The images of the IgG spots on the immunome array are provided in Figure S4.

$$\text{IgG1: IgG2: IgG3: IgG4: IgG5: IgG6} \quad (\text{II})$$

$$x: 0.5x: 0.25x: 0.125x: 0.0625x: 0.03125x$$

Based on the signal intensity of IgG controls, secondary incubation was carried out with serial dilution steps starting from IgG1 to IgG6. Variation studies of our IgG serial dilution to the experimental (ideal) IgG serial dilution can be seen in Figure 4B. Nonetheless, the CV between each sample's dilution and the experimental dilution was within the standard threshold (i.e., below 15%). The mean for the first and second assays is 9.71% and 6.27%, respectively.

3.4.2. Putative biomarker for BC

An antigen is considered a biomarker if it fulfills the following two conditions: (i) penetrance frequency ≥ 2 amongst the

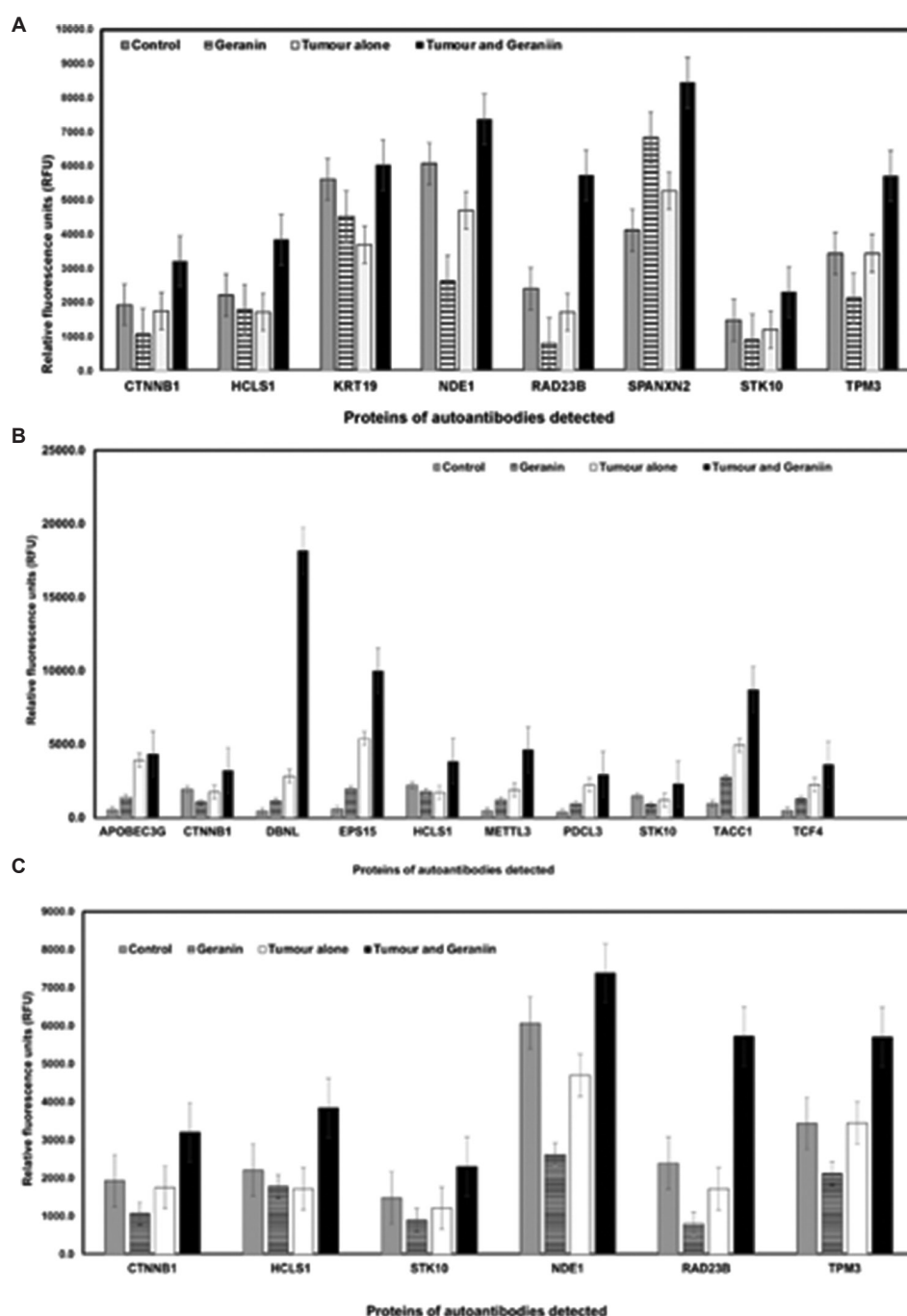


Figure 4. Biomarkers of autoantibodies that were identified using the immunome array. (A) The top eight autoantibodies that were elevated ($p < 0.05$) in plasma from tumor-induced mice fed daily with 0.5 mg/mL geraniin (Tumor + Geraniin) compared to tumor-induced mice fed daily with vehicle (Tumor alone), and the expression of these proteins in the plasma of non-tumor-induced mice fed daily with vehicle (Control) or 0.5 mg/mL geraniin ($n = 3$). (B) The main biomarkers related to anticancer effects and signaling processes that may affect host immune responses were elevated ($p < 0.05$) in plasma from tumor-induced mice fed with geraniin compared to tumor-induced mice fed with the vehicle (Control) ($n = 3$). (C) The top six biomarkers related to anticancer effects and signaling processes that may affect host immune responses were elevated ($p < 0.05$) in plasma from tumor-induced mice fed with geraniin compared to tumor-induced mice fed with the vehicle (Control) ($n = 3$).

Abbreviations: APOBEC3G: Apolipoprotein B mRNA editing enzyme; CTNNB1: Catenin beta 1; DBNL: Drebrin-like protein; EPS15: Epidermal growth factor receptor substrate 15; HCLS1: Hematopoietic lineage cell-specific protein; KRT19: Keratin, type I cytoskeletal 19; METTL3: N6-adenosine-methyltransferase 70 kDa subunit; NDE1: A type of type II restriction enzyme; PDCL3: PhosducinLike 3; RAD23B: UV excision repair protein RAD23 homolog B; SDCCAG8: Serologically defined colon cancer antigen 8; SPANXN2: Sperm protein associated with the nucleus on the X chromosome N2; STK10: Serine/threonine-protein kinase 10; TACC1: Transforming acidic coiled-coil-containing protein 1; TCF4: Transcription factor 4; TPM3: Tropomyosin 3.

cases; and (ii) elevation fold change (or penetrance fold change) relative to control samples ≥ 2.0 -fold. Through this novel approach, we have shortlisted several elevated biomarkers that are specific to each treatment set-up in reference to the no-treatment (control) group and the different treatment groups (Table 3). In the case of treated (with tumor) versus treated (no tumor), we identified 614 antigens as potential biomarkers (Table 3). Of these, six biomarkers (RAD23 homolog B [RAD23B], hemopoietic lineage cell-specific protein 1 [HCLS1], catenin beta-1 [CTNNB1], A type of type II restriction enzyme [NDE1], Tropomyosin 3 [TPM3], and Serine/threonine-protein kinase 10 [STK10]) overlapped with the aforementioned group. In the case of treated (with tumor) vs. no treatment (no tumor), three biomarkers (RAD23B, Sperm protein associated with the nucleus on the X chromosome N2 [SPANXN2], and TPM3) were identified (Table 3). Conversely, in the no treatment (with tumor) versus no treatment (no tumor) group, GALK1 was identified as a candidate biomarker (Table 3). Eight biomarkers (RAD23B, HCLS1, STK10, CTNNB1, TPM3, NDE1, Keratin, Type I cytoskeletal 19 [KRT19], and SPANXN2) with very high relative fluorescence units (RFU) were significantly elevated ($p < 0.05$) in plasma from tumor-induced mice fed with geraniin (Tumor + Geraniin) compared to tumor-induced mice fed with vehicle (Tumor alone) (Figure 4A). However, some of these biomarkers were not specifically related to anticancer or signaling events that might be related to the anticancer or immune response to cancer. Hence, we analyzed the RFU of other biomarkers that were identified in the analysis; that is, seven new biomarkers (Apolipoprotein B mRNA editing enzyme, Epidermal growth factor receptor substrate 15 [EPS15], Drebrin-

like protein, N6-adenosine-methyltransferase 70 kDa subunit, PhosducinLike 3, transforming acidic coiled-coil-containing protein 1, and TCF4), in addition to the three (CTNNB1, HCLS1, and STK10) that were identified in the first analysis (Figure 4B). Six of the biomarkers (RAD23B, HCLS1, CTNNB1, NDE1, TPM3, and STK10) were found to be consistently elevated in the geraniin-treated samples irrespective of tumor-induction compared to control samples (no tumor, no treatment) (Figure 4C). On batch normalization, the average signal of each of the antigens was calculated and plotted (Figure 5). Treated samples (no

Table 3. Summary of biomarkers ($n=626$) identified in various experimental setups

Experimental setup	Number of biomarkers	Overlapping biomarkers
Treated (with tumor) versus no treatment (with tumor)	8	RAD23B, HCLS1, STK10, CTNNB1, TPM3, NDE1, KRT19, SPANXN2
Treated (with tumor) versus treated (no tumor)	614	RAD23B, HCLS1, CTNNB1, NDE1, TPM3, STK10
Treated (with tumor) versus no treatment (no tumor)	3	RAD23B, SPANXN2, TPM3
No treatment (with tumor) versus no treatment (no tumor)	1	GALK1

Abbreviations: SPANXN2: Sperm protein associated with the nucleus on the X chromosome N2; TPM3: Tropomyosin 3; RAD23B: RAD23 homolog B; CTNNB1: Catenin beta 1; HCLS1: Hematopoietic lineage cell-specific protein; STK10: Serine/threonine-protein kinase 10; NDE1: A type of type II restriction enzyme; KRT19: Keratin, type I cytoskeletal 19.

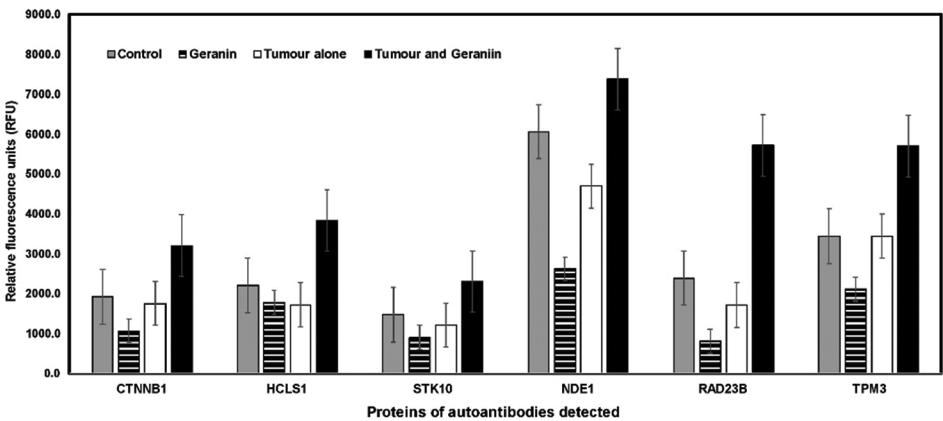


Figure 5. A comparison of the six common autoantigens identified across the two studies. The average normalized RFU of each antigen from all samples ($n = 3$) in each analysis was calculated and plotted. The groups are classified as follows: Control: Non-tumor-induced mice fed daily with the vehicle; Geraniin: Non-tumor-induced mice fed daily with geraniin; Tumour alone: Tumor-induced mice fed daily with the vehicle; Tumour + Geraniin: Tumor-induced mice fed daily with 0.5 mg/mL geraniin.

Abbreviations: CTNNB1: Catenin beta 1; HCLS1: Hematopoietic lineage cell-specific protein; NDE1: A type of Type II restriction enzyme; RAD23B: UV excision repair protein RAD23 homolog B; STK10: Serine/threonine-protein kinase 10; TPM3: Tropomyosin 3.

tumor) were included in the experimental setup to serve as a negative control and to observe if treatment with geraniin induced a general autoantibody response. Indeed, there was a slight elevation in autoantibody response, observed from the baseline signal in the control samples (Figure 5).

4. Discussion

In tumor-induced mice fed daily with 0.5 mg geraniin, there was a marked reduction ($p < 0.05$) in tumor volume and size. In addition, the histopathological analysis revealed no signs of metastasis in the liver of geraniin-fed mice. There was a reduction in metastasis in the lung compared to tumor-induced mice fed with the vehicle. These findings suggest that geraniin is a suitable potential drug, as it has anticancer effects, which supports the findings from previous studies that reported geraniin inhibiting the proliferation of human lung adenocarcinoma,^{35,43} human BC,³¹ human glioma tumor,³⁰ and ovarian tumor³⁴ cell lines. To date, there is no data reporting on the anticancer effects of geraniin on a syngeneic mouse model of BC.

Several biomarkers appear to be differentially expressed in plasma geraniin-fed mice. A total of 19 biomarkers were found to be associated with BC and its subtypes (Table 4). In addition, various biomarkers, such as CTNNB1, KRT19, TPM3, EPS15, and TCF4, appear to be associated with several human cancers (Table 4). Of these biomarkers, CTNNB1, also known as Catenin beta 1, a protein with dual functions (i.e., involved in the regulation and coordination of cell-cell adhesion and gene), was reported to be present in many cancers (Table 4), which may indicate its role in mediating anticancer effects. The *CTNNB1* gene encodes the β -catenin protein. It is a component of the Wnt signaling pathway that has been demonstrated to significantly contribute to the development of certain malignancies.⁴⁴ β -catenin is essential for two critical developmental processes: (i) modulation of target gene expression and (ii) maintenance and establishment of cell-to-cell, cell-type-specific adhesion via the Wnt signaling pathway.⁴⁵ The Wnt/ β -catenin signaling pathway is essential for epithelial-mesenchymal transitions⁴⁶ and for regulating cell differentiation and proliferation, playing a significant role in biological homeostasis.⁴⁵

Mutations in the *CTNNB1* gene, which encodes β -catenin, are found in many malignancies.⁴⁵ These mutations induce modifications in the properties of the β -catenin protein, resulting in significant reprogramming of the nuclear transcriptional network.⁴⁵ A study indicated that the emergence of estrogen-dependent TNBC necessitates the presence of β -catenin.⁴⁷ This study demonstrated that β -catenin governs colony formation and cell motility *in vitro* and carcinogenesis *in vivo* in TNBC.⁴⁷

Table 4. Association of the top 20 biomarkers identified with breast cancer sub-types

Breast cancer sub-type	Number of associated antigens	Antigen (s) identified
Breast carcinoma	19	CTNNB1, EPS15, TPM3, DBNL, APPL1, RAD23B, TPM1, RAD23A, SDCCAG8, KRT19, TSGA10, METTL3, TACC1, TCF4, APOBEC3G, PAPSS2, STK10, HCLS1, NDE1
Medullary breast carcinoma	2	KRT19, CTNNB1
Lobular breast carcinoma	3	CTNNB1, KRT19, TPM3
Breast ductal adenocarcinoma	3	CTNNB1, EPS15, TPM3
Breast neoplasm	4	CTNNB1, EPS15, TPM3, KRT19
HER2-positive breast carcinoma	2	CTNNB1, TPM3
Inflammatory breast carcinoma	1	CTNNB1
Triple-negative breast cancer	3	CTNNB1, KRT19, TCF4
Invasive breast carcinoma	2	CTNNB1, KRT19
Breast adenocarcinoma	2	CTNNB1, KRT19
Mixed lobular and ductal breast carcinoma	1	CTNNB1
Metaplastic breast carcinoma	1	CTNNB1
Hereditary breast cancer	1	CTNNB1

Abbreviations: RAD23B: RAD23 homolog B; CTNNB1: Catenin beta 1; STK10: Serine/threonine-protein kinase 10; NDE1: A type of type II restriction enzyme; EPS15: Epidermal growth factor receptor substrate 15; DBNL: Drebrin-like protein; METTL3: N6-adenosine-methyltransferase 70 kDa subunit; TACC1: Transforming acidic coiled-coil-containing protein 1; APOBEC3G: Apolipoprotein B mRNA editing enzyme; KRT19: Keratin, type I cytoskeletal 19; SDCCAG8: Serologically defined colon cancer antigen 8; TPM3: Tropomyosin 3; HCLS1: Hematopoietic lineage cell-specific protein.

In this study, an increase of CTNNB1 was observed in tumor-induced mice fed with geraniin, which further supports geraniin's anticancer and antimetastatic effects. This work demonstrated that geraniin induces the enhanced production of autoantibodies targeting the CTNNB1 autoantigen, likely via the stimulation of the mitogen-activated protein kinase (MAPK) signaling system, which regulates the synthesis of *CTNNB1* gene products. Recently, it was reported that aberrant signaling

by CTNNB1 has been reported to be a potential therapeutic target in human BC.⁴⁸ when the identified biomarkers were used as input data in the Open Targets Platform (<https://www.targetvalidation.org/>).

The UV excision repair protein RAD23B is involved in nucleotide excision repair.^{49,50} This protein was upregulated in plasma from tumor-induced mice fed with geraniin, which suggests that geraniin may play a prominent role.⁵⁰ RAD23B has been proposed to potentially contribute to the advancement of BC and is further hypothesized to possess a tumor suppressor function in this context.⁵⁰ A significant proportion (>80%) of nuclear RAD23B was strongly correlated with histological BC Grades 1 and 2, characterized by modest mitotic activity, while elevated cytoplasmic RAD23B levels were significantly connected with histopathological BC grade 3.⁵⁰ The RAD23B protein has been documented to exhibit expression in primary breast carcinoma tissue⁵¹; nevertheless, most research on RAD23B has predominantly been conducted in yeast, leaving the function of its human homolog and possible involvement in human cancers largely unexplored.⁵²

There were increased levels of HCLS1 in the plasma of tumor-induced animals fed with geraniin compared to untreated or control animals. This protein has been reported to be a hidden player in migration, invasion, and tumor formation and was found to be overexpressed in ovarian carcinoma cells.⁵³ CTTN is a cytoskeletal protein whose overexpression enhances tumor aggressiveness by facilitating tumor migration, invasion, and metastasis. It is overexpressed in various solid tumors, including melanoma, ovarian, breast, and colorectal cancers.⁵³ A homolog of CTTN is HCLS1 (HS1/HCLS1), also known as LckBP1. The *HS1* gene was initially documented as being expressed solely in hematopoietic lineage cells, whereas CTTN is present in all cell types except these.⁵³ Despite a subsequent study indicating that HS1/HCLS1 is not confined to hematopoietic cells,⁵⁴ the function of HS1 in non-hematopoietic cells remains unclear.

TPM3 determines the functional capacity of actin filaments and compounds that target TPM3, which have been reported to have promising effects as anticancer agents both *in vivo* and *in vitro*.⁵⁵ The expression profile of TPM isoforms in a selection of cancer cell lines indicated that TPM 3.1 is among the most abundantly expressed isoforms.⁵⁶ TPM is a crucial component of actin filaments and a stabilizer that participates in most actin cytoskeletal processes.⁵⁶ The actin cytoskeleton is integral to various cancer cellular processes, including the maintenance of proliferation, evasion of replicative senescence, suppression of growth, and the initiation

of invasion and metastasis, characterized by significant alterations in actin cytoskeleton organization and the expression levels of specific actin-binding proteins.⁵⁶ TPM1 γ , TPM1 δ , and TPM2 β are high molecular weight Tpm's expressed in normal human breast epithelial cells, with their protein products incorporated into stress fibers.⁵⁷ Conversely, TPM1 ϵ and TPM4 τ are smaller molecular weight isoforms demonstrating expression in normal human breast epithelial cells.⁵⁷ Downregulation of TPM1 γ , TPM1 δ , and TPM2 β isoforms have been found in several malignant human breast cell lines and primary breast carcinomas.⁵⁷ In addition, TPM1 λ was elevated in numerous malignant cell lines relative to the normal cell lines.⁵⁷ This study reveals that TPM1 has a significant autoantigen-autoantibody binding signal intensity, indicating its potential as a BC biomarker to complement existing BC screening techniques.

A compelling study has revealed that geraniin demonstrates significant antiproliferative effects on Michigan cancer foundation-7 (MCF-7) human BC cells. The IC₅₀ values for these cells following 24, 48, and 72 h of geraniin administration were 42.32, 17.98, and 94 μ M, respectively. The research indicates that geraniin can impair the mitochondrial membrane potential and halt the S-phase of the cell cycle. Western blotting studies indicate that geraniin enhances the phosphorylation of the anti-apoptotic protein Bcl-2 and promotes the cleavage of the enzyme's poly (ADP-ribose) polymerase and caspase-3 in MCF-7 cells. The study demonstrates that geraniin activates p38 MAPK and promotes apoptosis in MCF-7 cells. The data substantiates the hypothesis that geraniin-induced apoptosis is facilitated by the activation of the ROS-mediated p38 MAPK pathway.^{58,59} Another study examined the antitumor effects of geraniin on the metabolism of human BC cells. The researchers examined the impact of geraniin on two distinct BC cell lines, MCF-7 and MDA-MB-231. The results demonstrated that geraniin exhibits an inhibitory effect against both MCF-7 and MDA-MB-231, as evidenced by the cytotoxicity assay.⁶⁰

Geraniin, isolated from geranium and in tannin form, exhibits antiproliferative properties and promotes apoptosis in colorectal and BC cells.³¹ Nonetheless, the applicability of geraniin to human cancer clinical trials is presently constrained. Therefore, more clinical trials are warranted to investigate the role of geraniin in cancer.⁵⁹

Geraniin has demonstrated significant potential as an anticancer drug; nonetheless, some crucial problems and limitations remain regarding its utilization in cancer therapy. The current literature primarily consists of preclinical studies, underscoring the necessity for

rigorously planned clinical trials to evaluate geraniin's safety, appropriate dose, and possible adverse effects in cancer patients. It is essential to acknowledge that geraniin's efficiency may vary considerably among different cancer types and stages, necessitating research on its usefulness against certain subtypes and metastatic conditions. Furthermore, investigating Geraniin's synergistic potential with other anticancer drugs or therapies may augment its efficacy and mitigate potential resistance mechanisms. The bioavailability of geraniin, its pharmacokinetics, and metabolites in humans is a crucial factor that necessitates thorough investigation, as it may substantially affect its therapeutic efficacy. Prolonged exposure to geraniin and possible resistance mechanisms must be examined to guarantee enduring therapeutic advantages. Determining the safety profile of geraniin in humans, especially with extended usage, is essential. Establishing the most efficacious treatment protocols, encompassing dosage schedules and methods of administration, is crucial to optimize geraniin's therapeutic efficacy. Ultimately, recognizing biomarkers or patient attributes indicative of geraniin's response offers the potential for individualized cancer treatment. To strengthen the discussion on future research directions, additional testing should focus on evaluating geraniin's pharmacokinetics, including absorption, distribution, metabolism, and excretion *in vivo*. Further studies using human-derived models, such as patient-derived organoids or xenograft models, could provide translational insights into its therapeutic potential. Clinical investigations assessing geraniin's bioavailability and efficacy in human trials would also be essential for its development as a viable therapeutic agent.

In summary, geraniin's evolution from a potential natural compound to a broadly applicable and safe anticancer agent requires the resolution of existing gaps and the pursuit of future perspectives to enable its transition from laboratory findings to clinical applications, ultimately benefiting cancer patients globally.

5. Conclusion

The results from this study demonstrate that geraniin has the potential to be developed as an anticancer agent. Future investigations should focus on assessing the pharmacokinetics of geraniin. Subsequent research utilizing human-derived models, such as patient-derived organoids or xenograft models, may yield translational insights into its therapeutic potential. Clinical studies evaluating the bioavailability and efficacy of geraniin in human subjects are crucial for its advancement as a potential medicinal agent. The antimetastatic property observed in this study provided more evidence for geraniin as a chemotherapeutic drug for BC. Furthermore, the autoantibody response

toward these antigens and fluctuation in the response could suggest a potential marker or a predictive marker toward treatment with geraniin.

Acknowledgments

The authors would like to thank IMU University (formerly known as IMU) for providing the two research grants that supported this study.

Funding

This study was supported by two research grants from IMU University (BMS I/2018[01] and BMS I/2018[14]) that supported the research activities of two 3rd-year undergraduate medical students.

Conflict of interest

Nur Diana Anuar, Nurul H Rutt, and Nurul Shielawati Mohamed Rosli are employees of the Sengenics Corporation company. This has not influenced the content of the manuscript.) No reference to the author's company is made, but it is declared for full transparency.

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Ethics approval and consent to participate

Ethical approval was obtained from the Joint Committee of Research and Ethics Committee, IMU (IMU-JC; BMS I/2018[01] and BMS I/2018[14]).

Consent for publication

Not applicable.

Availability of data

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request. All data generated or analyzed during this study are included in this published article and its supplementary file.

Further disclosure

Part of the findings of this paper was presented at the 19th World Congress of Basic and Clinical Pharmacology, Glasgow, United Kingdom on July 4, 2023.

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