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INVESTIGATION OF IMMUNOLOGICAL ADJUVANT ACTIVITIES OF *PANAX GIENSENG* SAPONINS IN MOUSE MODEL

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Abstract
The development of effective vaccines hinges upon the inclusion of an appropriate immunopotentiator or adjuvant to enhance both cellular and humoral immune responses. Adjuvants are compounds designed to augment vaccine efficacy, and there exists an imminent need for the creation of novel and improved adjuvants. Saponins, classified as amphipathic glycosides, have garnered significant attention due to their multifaceted biological activities and their ability to stimulate immune responses when paired with antigens like ovalbumin. In this investigation, *Panax ginseng* roots were purchased from Hyolim Agriculture Farming in North Korea, for saponin extraction. Methanol and n-butanol extraction protocol were employed for saponin extraction, and their profiles were meticulously characterized through Flash Chromatography (FC), TLC and HPLC. Two primary fractions, designated as PS-1 (*Panax ginseng*-1) and PS-2 (*Panax ginseng*-2), were isolated and subjected to evaluation as adjuvants in a mouse model, alongside ovalbumin. Over the course of 20 days, four groups of mouse were immunized with varying doses of PS-1 and PS-2, encompassing fractions such as PS-1±40, PS-1±60, PS-2±80, and PS-2±100. The adjuvant activity was subsequently compared with QuilA saponin (QuilA±100), while a sixth group of mouse immunized ovalbumin alone. Immunization responses were assessed at primary and secondary stages on the 10th and 20th days, respectively, by quantifying IgG, IgG4 subclass antibodies, and Interleukin-6 (IL-6) concentrations through ELISA. The findings revealed that increasing doses of *Panax ginseng* saponins led to heightened levels of IgG and IgG4 subclass antibodies in mouse serum, concurrently with the induction of IL-6 responses discerned from spleen cell cultures. Remarkably, the PS-2±100 saponin dose exhibited a substantial upsurge, elevating IgG antibody concentration by 96.26%, IgG subclass antibody concentration by 85.19%, and triggering a notable increase in IL-6 levels (246.67 pg/l on the 20th day), as evidenced by a 0.51 optical density (OD) value post-immunization. In conclusion, *Panax ginseng* saponins, particularly the PS-2±100 fraction, demonstrate potent adjuvant capabilities, significantly enhancing both humoral and cellular immune responses when administered alongside ovalbumin. This research contributes valuable

insights into the exploration of natural saponins as promising vaccine adjuvants, highlighting their substantial immunostimulatory potential and their prospective role in vaccine formulations.

Introduction

Recent advancements in both the pharmaceutical and cosmetic industries have witnessed a growing interest in the utilization of organic plants and their secondary metabolites, along with their derivatives, particularly as antimicrobials. Within the realm of effective vaccine development, the incorporation of a suitable adjuvant assumes a pivotal role in stimulating both humoral and cell-mediated immune responses. These adjuvants, often recognized as immunopotentiators, encompass secondary metabolites that are sourced from various organic plants. They play an indispensable role in vaccine formulations, substantially enhancing the efficiency of immunological processes [1] (Shah et al., 2017). This innovative approach capitalizes on the natural compounds found in organic plants and their derivatives, harnessing their potential to augment and optimize the immune response elicited by vaccines. Such endeavors represent a dynamic intersection between pharmaceutical and cosmetic research, promising exciting prospects for the future of immunization and skincare formulations.

In various regions, particularly across Asia, herbal medications have a rich historical tradition spanning centuries. These traditional remedies have proven to be efficacious in mitigating severe infections [Che et al., 2013]. However, the contemporary landscape of vaccine development underscores the imperative to engineer recombinant DNA vaccines, frequently augmented with compounds aimed at augmenting the efficacy of these second-generation vaccines. While many adjuvants have demonstrated substantial effectiveness, certain formulations are associated with adverse effects, including the formation of granulomas and the induction of pyrogenic responses post-injection. One commonly employed adjuvant, Complete Freund's Adjuvant (CFA), has displayed promise in eliciting robust cellular and humoral responses against diverse antigens. However, due to their associated adverse effects, several adjuvants are deemed unsuitable for human and veterinary vaccine applications. Consequently, there exists an urgent mandate to explore alternative immunopotentiators or adjuvants that are both safe and efficient in the formulation of vaccines (Chan et al., 2003) [2].

The historical utilization of herbal medicines finds its roots in ancient Egyptian civilization, with ginseng (*Panax ginseng*) standing out as one of the most extensively employed herbs across Asian territories [Kiefer and Pantuso, 2003]. Ginseng, renowned for its elevated concentration of active constituents and wide-ranging pharmacological effects, is emblematic of such herbal practices [Cheng et al., 2005]. Ginsenosides or panaxosides, representing natural steroid glycosides and triterpene saponins, constitute pivotal components within ginseng. Interestingly, ginseng has a historical association with pregnancy, albeit with uncertainties surrounding its *in vivo* impact on the developing fetus [2] (Chan et al., 2003).

Ginseng saponin encompasses numerous bioactive monomers, including primary polysaccharides, fatty acids, and mineral oils. Nevertheless, the preeminence of ginseng's pharmacological effects is predominantly attributed to triterpene saponins, colloquially known as ginsenosides [Shi et al., 2019]. The elucidation and classification of these saponins, initially characterized using thin-layer chromatography (TLC), bestowed upon them an alphanumeric nomenclature, exemplified by Rb1, Re, Rb2, Rc, and others [Shin et al., 2007]. Structurally, ginseng saponins feature a hydrophobic steroidal core, embellished with carbohydrate moieties tethered to specific positions. Their inherent hydrophobicity introduces challenges in terms of intestinal digestion, albeit with some degree of absorption ensuing subsequent to oral administration [8] (Wan et al., 2016).

Following oral ingestion, ginseng saponins undergo metabolism by intestinal flora, ultimately transforming into secondary glycosides, thereby amplifying their pharmacological potency [9]

(Wang et al., 2015). The physiological effects of ginseng extend across a spectrum of health-related facets, encompassing cardiovascular, anti-inflammatory, antiviral, and immunoregulatory domains [10] (Ben-Hur and Fulder, 1981) [11] (Attele et al., 1999). Their unique structural attributes facilitate interaction with cellular lipid bilayers, rendering them amenable to cellular entry and engagement with diverse cellular components, including receptors [12] (Yuan et al., 2010).

Prior research has delved into the amalgamation of ginseng saponins with aluminum hydroxide, serving as an adjuvant, to enhance antibody responses directed against bacterial antigens. Notably, ginseng saponin adjuvants have demonstrated the capability to induce both IgG1 and IgG2 antibodies, rendering them particularly valuable in vaccine formulations where the induction of an IgG2 response is of significance. Thus, the present study embarks upon an investigation into the hemolytic activities of varying doses of *Panax ginseng* saponins (PGS), alongside their potential to modulate cellular and humoral immune responses in BALB/c mice following immunization with ovalbumin (OVA) antigen.

Methodology

The present study was conducted with the aim of elucidating the immunological properties of *Panax ginseng* saponins in a mouse model. Various doses of saponins were extracted and subsequently verified through Flash chromatography, Thin-Layer Chromatography (TLC), and High-Performance Liquid Chromatography (HPLC). These extracted saponins were administered to six distinct groups of mice. Blood serum samples were subsequently obtained from these mice for the purpose of assessing their immune responses, specifically focusing on the levels of IgG, IgG4 subclass antibodies, and Interleukin-6 (IL-6). The quantification of these immunological parameters was carried out utilizing the Enzyme-Linked Immunosorbent Assay (ELISA) methodology.

Collection of plant (root) sample:

Panax ginseng root explants were procured from the Hyolim Agricultural Farming Association located in Goguryeo, North Korea. Taxonomic verification was carried out at the Royal Agricultural University in South Korea before the specimens were transported to Pakistan. Subsequently, the taxonomic identification of *P. ginseng* received formal approval from the Department of Botany at The University of Panjab, Pakistan. Following this taxonomic validation, the specimens were transferred to the Biotechnology laboratory at the University of Lahore for further research and study.

Sterilization of an ex-plants:

The sterilization procedure encompassed a tripartite process consisting of cleaning, washing, and stripping. The ginseng root specimens were initially subjected to a thorough washing procedure, involving rinsing with both tap water and subsequently autoclave-distilled water. Following this, the roots were slated for shadow drying. The primary objective behind the drying process was the complete removal of moisture from the plant roots, facilitating subsequent extraction procedures. Shadow drying was executed outdoors, in a shaded environment, where the ginseng roots were left undisturbed for a duration spanning a minimum of 3 to 4 weeks.

Extraction and confirmation of saponins from *Panax ginseng*:

Methanol used as the solvent in the extraction process of saponins from *P. ginseng* roots using a Soxhlet apparatus. The resulting extract was concentrated, followed by a subsequent round of extraction utilizing butanol: water saturation, as depicted in Figure 1. The butanol extract was subsequently subjected to drying and then mixed within a low-solvent phase, comprising a blend of chloroform, methanol, and water in a ratio of 64:26:10. The separation of saponins was accomplished through flash chromatography employing silica gel as the stationary phase.

Different saponin fractions were isolated and characterized based on their polarity. These fractions were eluted from the chromatographic column using a gradient of increasing chloroform polarity in conjunction with a stepwise adjustment in the concentration of H_2O and the solvent composition, specifically at ratios of 60:30:10, 64:26:10, 55:35:10, and 55:40:10 within the low-solvent phase. In total, approximately 13-14 ml of fractions were collected in accordance with the elution process. Each of these 14 eluted fractions underwent evaluation using Thin-Layer Chromatography (TLC) and HPLC.

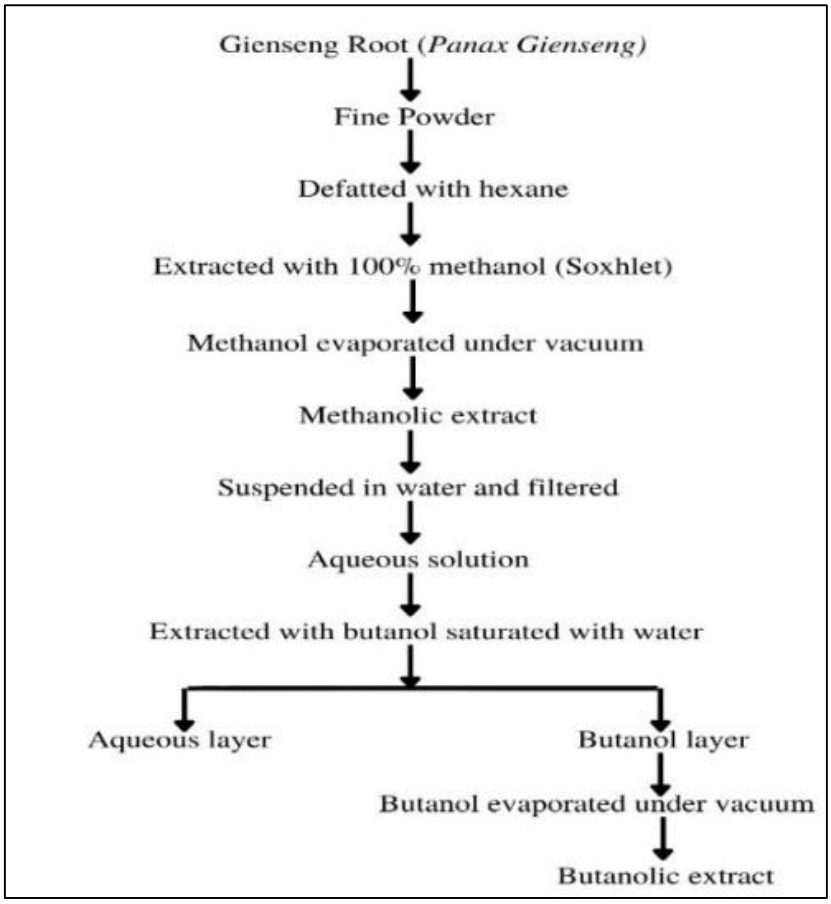

Figure 1: Extractions of saponins from the roots of *P. ginseng*

***P. ginseng* saponins as adjuvant activity in a mouse model:**

Animals used:

Sprague-Dual Albino mouse, aged between 6 to 8 weeks, were procured from the Animal Resources Center at the University of Lahore. The acquisition and subsequent scientific experimentation on these albino mouse received approval from the University of Lahore Animal Care Committee, adhering to the established guidelines and regulations set forth by the UOL Council of Animal Care.

Saponins toxicity test:

Sprague-Dual Albino mouse were employed as the experimental subjects to assess the toxicity of *Panax ginseng* saponin-1, *Panax ginseng* saponin-2, and the control group with Quil A. Subcutaneous injections of saponin solutions ranging from 40 to 100 µL in phosphate buffer saline were administered to five distinct groups of mouse. Mortality data for each group were recorded following a 96-hour observation period post-injection. Utilizing probit analysis, the dosage at which 50% of the subjects exhibited lethal effects (LD_{50}) due to saponin exposure was determined (Welkos and O'Brien, 1994).

Immunization:

Ovalbumin (OVA) (Sigma), served as the model antigen for the investigation of the adjuvant properties of Panax saponin-1, Panax saponin-2, and the control Quil A. Subcutaneous injections were administered, comprising 50 mg of OVA dissolved in phosphate buffer saline (PBS). These injections were administered to various groups of mouse either as OVA alone or in combination with 100 ml of PBS, along with *Panax ginseng* saponins or Quil A control. For *Panax ginseng* saponins, concentrations of 40 and 60 µL for PS-1, 80 and 100 µL for PS-2, and 100 µL for Quil A control were employed, each in conjunction with two different doses of *P. ginseng* saponins. The doses of PS-1 and PS-2 exhibited varying effects on mouse mortality, while the Quil A control displayed indications of toxicity in the mice. Cardiac punctures were performed on the 10^{th} and 20^{th} days post-immunization to collect serum samples from the mouse groups. The immunological responses, specifically IgG, IgG subclasses, and IL6, within these samples were subsequently analyzed utilizing the ELISA method. Further details regarding the immunization protocol are provided in Table 1.

Table No. 1. Immunization protocol in a mouse model

Saponin group No.	**Saponin doses(ul)**	**Experimental days**		
		0-day	**10^{th} -day**	**20^{th} -day**
PS 1	±40	1	2,3	3,4
PS 1	±60	1	2,3	3,4
PS 2	±80	1	2,3	3,4
PS 2	±100	1	2,3	3,4
Quil A	±100	1	2,3	3,4
OVA alone	±50	1	2,3	3,4

Legend: 1, Post-immunization; 2, Post- Post-immunization blood sample collection; 4, spleen cell.

Blood sample collection and storage:

Blood samples were collected for analysis on both the 10^{th} and 20^{th} days post-immunization, and these samples were collected in clotting vials. To isolate the blood serum, the samples were left to coagulate within serum separator tubes for a duration of 30 minutes. Subsequently, centrifugation was carried out at approximately 3000 rpm for a period of 10 minutes. The serum aliquot samples were then carefully preserved at either -20°C or -80°C until they were required for further experimentation involving ELISA. It is worth noting that repeated freeze-thaw cycles were avoided to maintain the integrity of the serum samples.

Immunological studies using ELISA:

The objective was to examine serum responses related to IgG, IgG4 subclasses, and IL6 concerning saponins, employing through ELISA method. In 96-well microtiter plates (Immulon Laboratories Inc., Chantilly, VA, USA), Ovalbumin at a concentration of 10 mg/mPBS was coated and incubated at 4°C for a duration of 18 hours. Subsequently, the microtiter plate wells underwent three cycles of thorough washing with PBS containing 0.05% Tween-20. Following this, the wells were subjected to incubation with PBS containing 1% bovine serum albumin (BSA) at 37°C for a period of 30 minutes. A further washing step was performed employing PBS-T. For the assessment of IgG, IgG subclasses, and IL6 responses, 100 ml aliquots of mouse serum, diluted at a ratio of 1:10, were utilized within these plates. These dilutions underwent incubation at 37°C for one hour. Subsequent to another PBS-T wash, the plates were exposed to biotinylated goat anti-mouse IgG and IgG subclasses, each diluted at a ratio of 1:1000, and incubated for an additional hour at 37°C. Following another round of washing with PBS-T, 100 ml of streptavidin alkaline phosphate (Gibco) conjugate was added and incubated at 37°C for one hour. The microtiter plates, containing PBS-T, were subjected to further washing, this time involving the addition of 100 ml alkaline phosphate substrate solution (comprising 1 mg/ml of p-nitrophenyl phosphate from 104 Phosphate substrate tablets, Sigma) to each well. This substrate solution was employed alongside a 1 M solution of

diethanolamine buffer, adjusted to pH 9.8. Finally, absorbance readings were obtained at 405 nm using an automated Varian UV-Vis spectrophotometer (Cary 50 Bio UV-Vis Spectrophotometer, Varian, Agilent Technologies, Santa Clara, California, USA).

Antigenic specific IL-6, response:

Splenic lymphocytes were suspended in RPMI 1640-FCS and introduced into every well of 96-well round-bottom cultured cell microtiter plates in 100 ml aliquots, with a cell concentration of 10^7 cells/ml (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). In duplicate cultures, each micro-well received 100 mg of OVA dissolved in 100 ml of RPMI 1640-FCS to stimulate the cells. Negative control samples consisted of cells cultivated solely in the growth medium. The plates were maintained at 37°C in a 5% CO_2 environment for 72 hours. Following incubation, the supernatants from the spleen cultures were collected and subjected to IL-6 analysis using an ELISA. The wells of 96-well microtiter plates were coated with 5 mg of anti-IL6 monoclonal antibodies (Arigo Biolaboratories, Hsinchu City 300 Taiwan) in 50 ml of PBS and incubated at 4°C for 18 hours. After washing the plates with PBS-T, culture broth dilutions, ranging from 1:2 to 1:128 in PBS-T, were added to the wells and incubated at 37°C for 2 hours. Subsequent to another PBS-T wash, 50 ml of biotinylated anti-IL-6 antibody (PharMingen), diluted at a ratio of 1:1000 in PBS-T, was added to each well and incubated at 37°C for 2 hours. Afterward, the plates were washed with PBS-T, and 50 ml of streptavidin-alkaline phosphate conjugate, diluted at a ratio of 1:1000 in PBS-T, was added to each well and incubated for one hour at 37°C. Following a PBS-T wash, 100 ml of alkaline phosphatase substrate solution was added to each micro-well. The absorbance of each well was measured at 405 nm using an automated spectrophotometer. Multiple samples from the culture supernatants were examined, and IL-6 levels were determined in pg/ml using a standard graph and synthetic murine IL-6. Data for each set of five mice were analyzed, and the IL-6 quantity in culture supernatants was presented as means ± SEM.

Mouse spleen tissue-sample preparation for IL6 responses:

Subsequently, the samples were loaded into the ELISA microtiter plate within the same batch. Prior to commencing the assay procedure, it is advisable to prepare all required chemicals. Standards and samples should be applied in duplicate to the Micro ELISA strip plate. Standards should be placed in the designated standard wells, and both reference and testing sample wells should be established. Approximately 50 µl of standards were added to the standard well, while testing samples with a sample dilution of approximately 40 µl were added to the testing sample well. The blank well remained empty. Each well received 100 µl of HRP-conjugate solution and was subsequently covered with an adhesive strip. The plate was then incubated at 37°C for a duration of 60 minutes.

After each well was aspirated and washed four more times to achieve a total of five washes, Wash Buffer (400 µL) was added to each well using a squirt bottle, manifold distributor, or automatic washer. Following the final wash, any remaining wash solutions were aspirated or decanted, and the plate was inverted and gently wiped clean with paper towels. Subsequently, 50 µL of chromogen solution A and 50 µL of chromogen solution B were added to each well. The plate was then incubated for 15 minutes at 37°C while being gently mixed. Care was taken to shield the plate from direct sunlight. Following incubation, a stop solution of approximately 50 µL was added to each well, resulting in a change in color from blue to yellow. To ensure thorough mixing in cases where the color change was not uniform or green, the plates were lightly tapped. Within 15 minutes, the optical density (O.D.) at 450 nm was measured using a microtiter Elisa reader.

Quantitative analysis of experimental samples was performed using a standard graph. The standard calibration curve was constructed by plotting the mean optical density (OD) at 450 nm for each of the six standard concentrations on the vertical (Y) axis against their corresponding concentrations on the horizontal (X) axis (Fig 2). The average OD value was calculated for each standard and sample after subtracting the mean value of the standard from all OD values. A statistical tool was employed to generate the standard curve (Figure 4.3.3). The OD value on the Y-axis was aligned with and extended to intersect the standard curve horizontally to determine the quantity of each sample. The

appropriate amount was then determined by drawing a vertical line to the X-axis at the point of intersection. It is essential to note that any deviations in operator technique, pipetting, washing, incubation conditions, or kit age may yield different results. The IL6 detection limit was established at 10-480 pg/ml, with a sensitivity of the test assessed at 1.0 pg/ml.

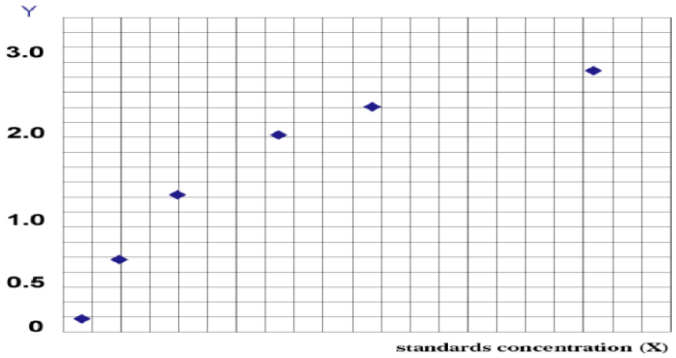

Figure 2. Standard curve to determine the concentration of IL in the samples.

Results

Isolation and extraction of *P. gienseng* saponin

The root of *Panax ginseng* exhibited a relatively elevated lipid content. To isolate the saponins from water-soluble carbohydrates and other polar constituents, the methanolic crude extract was suspended in water and subsequently extracted using saturated butanol-water. This butanolic extract yielded saponins at a concentration of 16.8%.

Detection of saponins fractions by using Flash Chromatography and TLC

In this study, a total of fourteen fractions were separated from the extract of *Panax ginseng*'s root using Flash Chromatography with silica gel, as illustrated in Figure 3. Four distinct fractions were of particular relevance for our investigation. The initial identification of saponins within these fractions was achieved through Thin Layer Chromatography (TLC). Silica gel separation was employed to distinguish between the various fractions, and this differentiation became evident when subjected to various reagents, resulting in a robust red band following acetic acid, sulfuric acid, methanol, and anisaldehyde reagent spraying [13] (Welkos and O'Brien, 1994).

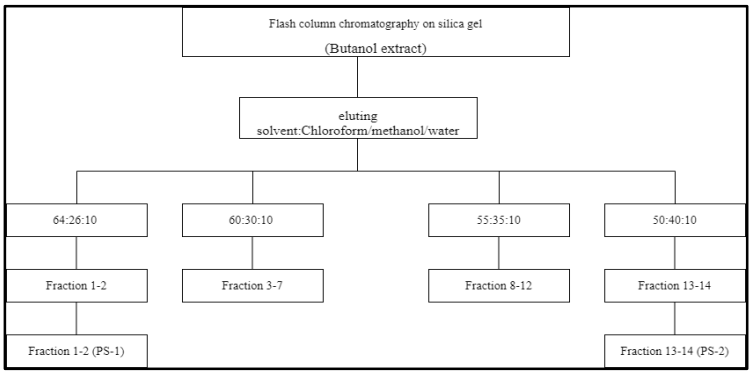

Figure 3. Different fractionations of *P. ginseng* saponin separation by using flash column chromatography

This study conducted Thin Layer Chromatography (TLC) analysis following the Wagner method [14] (Wagner and Bladt, 1996). Each fraction, approximately 25 µL in volume, was individually spotted onto a 60 F254 silica gel-coated aluminum plate (20x20-cm dimensions, 0.2-mm thickness, E. Merck D D; Darmstadt, Germany). The plate was then subjected to development using an Acetic acid: n-butanol: water developing solvent mixture (10:40:50). Subsequently, after air-drying, the plate was examined under UV light at 365nm (Figure 4). Notably, fractions PS1 and PS2 exhibited

two fluorescent spots on the silica gel under UV light, indicating the presence of saponins. The plate was further treated with a combination of acetic acid, sulfuric acid, and methanol (in a ratio of 85:10:5) and heated for 5 minutes at 100°C. This heating process facilitated the visualization of saponin fractions, which appeared brown in color. Fractions 1, 2, 13, and 14 of *P. ginseng* were selected for adjuvant immunological investigations in the mouse model and were designated as Panax saponin-1 and Panax saponin-2 (PS1 and PS2) accordingly (Fig. 5.2b). The effectiveness of *P. ginseng* saponin as an adjuvant was compared with control saponin, Quil-A, extracted from Q. saponaria (Croda Internationals Plc., Snaith, UK), in the mouse immunological studies.

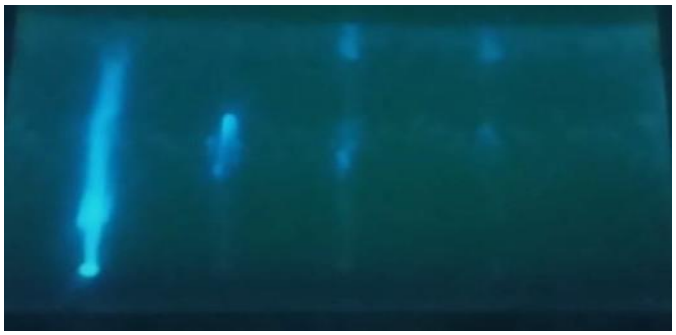

Figure 4. Saponins fractions under UV light at 366nm. PS-1 and PS-2 were seen clearly on the left side

Confirmation of *P. ginseng's* saponins by using HPLC

Fractions 1±14, which represent a combination of saponins, underwent analysis using High-Performance Liquid Chromatography (HPLC) employing a Beckman liquid chromatography system. Detection was carried out using a UV detector (Gold system, version 5.0) at a wavelength of 315 nm. The mobile phase consisted of 25% solution A, 10% ammonium acetate (C2H7NO2) buffer with a pH of 6.9, and 90% acetonitrile (C2H3N). Solution B comprised 90% ammonium acetate ($C_2H_7NO_2$) buffer with pH 6.9 and 10% acetonitrile. Distinct saponin peaks were identified at the specified 315 nm wavelength. Saponin fractions 1, 2, 13, and 14 were specifically chosen for the immunological adjuvant study, and their respective peaks are depicted in Figure 5.

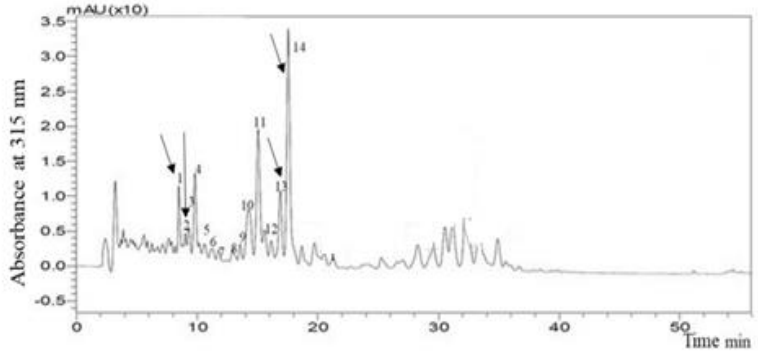

Figure 5: Typical HPLC chromatograms of saponin peaks at 315 absorbance/time (min) from *P. ginseng* (a) PS-adjuvant immunological investigations of saponin fractions extracted from *P. Ginseng* named as, 1, 2 (Panax saponin-1) and 13, 14 (Panax saponin-2). Quil A was used as control.

Saponins toxicity in a mouse model

The lethal effects of different saponin doses, including Panax saponin-1 and Panax saponin-2, were investigated through subcutaneous administration in a mouse model. The results revealed that the Quil A saponin (used as a control) exhibited higher lethality compared to P. ginseng saponins. Specifically, Panax saponin-1 and Panax saponin-2 demonstrated lethal effects at doses of 100 and 180 µL, respectively. In contrast, Quil A exhibited an LD_{50} of approximately 100 µL.

Immunological responses of *P. ginseng* saponins adjuvant in a mouse model

In this study, both control and experimental groups consisted of five mice each. The results indicated that in the control group of mice that received ovalbumin alone, the mixture of OVA with saponins significantly elevated anti-ovalbumin IgG levels on the 20th day post-immunization compared to the levels observed on the 10th day post-immunization. Notably, on the 10th day post-immunization, there was no discernible activity of IgG, IgG subclasses, and anti-OVA. Furthermore, higher doses of Panax saponin-1 (60 µL) and Panax saponin-2 (100 µL) induced elevated levels of IgG antibodies when compared to Quil A (100 µL) in the mouse model.

The detection of IgG antibody levels post-immunization with saponins was investigated in groups of five mice. Blood samples were collected to analyze the increased IgG antibody concentrations following saponin doses using the ELISA method. The immunological assays revealed a higher concentration of IgG antibodies post-immunization with increased saponin doses compared to the 10th day, as presented in Table 2. The results demonstrated that higher saponin doses led to an increase in IgG antibody levels in mice. As shown in Table and Figure 5.6, the serum displayed a higher concentration of anti-OVA when mice were administered a 60 µL dose of saponin on the 10th day. On the 20th day, IgG levels increased to 331.9 mg/dl with 60 µL of PS1 compared to the 10th day, which showed 297.0 mg/dl.

Table 2: Differences in IgG antibody responses on days 10th and 20th in the mouse model

Saponins doses group (µL)	**Serum IgG Anti-OVA (OD 405)**	
	Day-10th	**Day-20th**
PS-1±40	150.9	196.3
PS-1±60	297.0	331.9
PS-2±80	380.0	402.4
PS-2±100	490.0	509.0
QuilA±100	146.6	300.9
OVA alone ±	76.3	86.6

Legend: Serum samples diluted1:10. Averages of five-mouse groups with 2SEM.P<0.03, P<0.01, P<0.001 vs. control group getting no saponins.

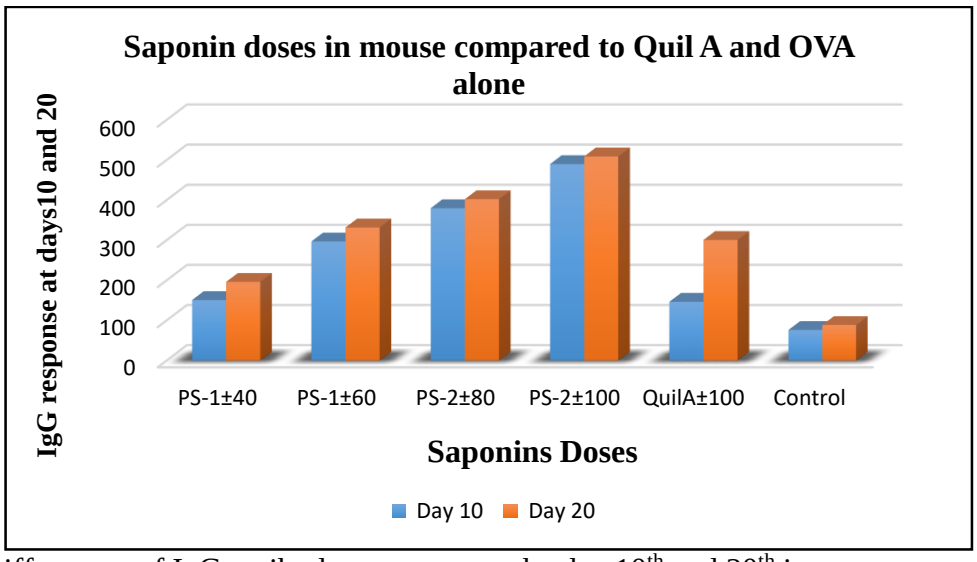

Figure 6. Differences of IgG antibody responses at the day 10th and 20th in a mouse model. Legend: Control=OVA-alone

The booster dose of ovalbumin combined with saponins was administered to five groups of mice, resulting in a significant increase in the concentration of IgG anti-OVA on the 20th day post-immunization compared to the control group. On the 10th day post-immunization, there was no apparent impact of ginseng saponins. However, by day 20, the higher doses of Panax saponin-1 and

Panax saponin-2 induced IgG anti-OVA levels comparable to those induced by Quil A. Table 2 and the figure 6 depict the administration of different doses of P. ginseng saponins in the groups of mice, leading to the production of IgG antibodies in the mice's blood. Specifically, the PS-1±40 µL dose induced an IgG antibody concentration of 233.9 mg/dl after the 10th day post-immunization and 276.3 mg/dl after the 20th day post-immunization. The PS-1±60 µL dose induced 297.0 mg/dl IgG antibody concentration after ten days post-immunization and 331.9 mg/dl IgG antibody concentration after 20 days post-immunization. In comparison, the PS-2±80 µL dose induced 403.0 mg/dl IgG antibody concentration after ten days post-immunization and 434.4 mg/dl IgG antibody concentration after 20 days post-immunization. The other dose of PS-2±100 µL induced 511.0 mg/dl IgG antibody concentration after ten days post-immunization and increased it to 537.0 mg/dl IgG antibody concentration after 20 days post-immunization. When compared to the control group receiving OVA alone, it was evident that on the 10th day post-immunization, IgG antibody concentration was 76.3 µL, while it increased to 86.6 µL on the 20th day post-immunization.

Responses of IgG4 antibody subclass by using immunological assays

Blood samples were obtained from five groups of mice both after post-immunization and post-immunization with saponins. The ELISA method was employed to assess the immunological responses of IgG4 subclasses. The serum from mice was analyzed for IgG4 subclass anti-OVA responses on both the 10th and 20th days post and post-immunization. The results revealed elevated IgG4 subclass responses when higher doses of Panax saponin-1 (60 µL) and Panax saponin-2 (100 µL) were compared to the control group receiving Quil A (100 µL) and the mice injected with ovalbumin antigen alone. This is presented in Table 3 and Figure 7.

Table No.3: Differences of IgG4 subclasses antibodies responses on days 10th and 20th in a mouse model

Types of saponins with different dose groups (µL)	**Serum IgG4 subclasses Anti-OVA (OD 405)**	
	Day-10th	**Day-20th**
PS-1±40	39.5	52.1
PS-1±60	81.7	98.3
PS-2±80	69.3	81.9
PS-2±100	125.4	147.2
QuilA±100	19.2	37.4
Control ± 50 (OVA)	10.2	17.9

Legend: Serum samples diluted1:10. Averages of five-mouse groups with 2SEM. P<0.02, P<0.04, P<0.001 vs. control group getting no saponins.

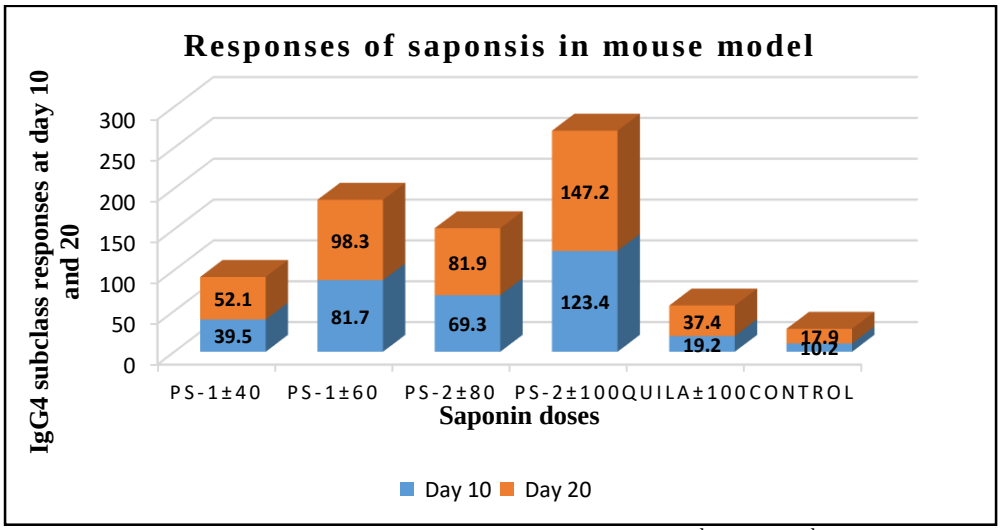

Figure 7. Responses of IgG4 antibody subclass at days 10th and 20th in a mouse model

The serum indicating IgG4 subclass and anti-ovalbumin responses post-immunization were documented in Table 2. Higher doses were associated with significantly increased IgG4 subclass levels as the days progressed. Panax saponin-1 (60 µL) and Panax saponin-2 (100 µL), when compared to the control saponin Quil A (100 µL) and the group injected with ovalbumin antigen alone, exhibited this trend, as shown in Table & Figure 5.7. The administration of different doses of P. ginseng saponins to five groups of mice resulted in increased IgG4 subclass antibodies.
Specifically, the PS-1±40 dose induced an IgG4 subclass antibody concentration of 39.5 µL after 10 days of primary immunization, and this increased to 52.1 µL after 20 days of secondary immunization. Similarly, the PS-1±60 dose induced 81.7 µL of IgG4 subclass antibody concentration after 10 days of primary immunization and 98.3 µL after 20 days of secondary immunization. For the PS-2±80 dose, it induced 69.3 µL of IgG4 subclass antibody concentration after 10 days of primary immunization and 81.9 µL after 20 days of secondary immunization. Lastly, the PS-2±100 dose caused 125.4 µL of IgG4 subclass antibody concentration after 10 days of primary immunization and 147.2 µL after 20 days of secondary immunization. In comparison, the QuillA±100 (Quillaja saponaria Molina) saponin dose induced 19.2 µL of IgG4 subclass antibody concentration after 10 days of primary immunization and 37.4 µL after 20 days of secondary immunization. When compared to the control group, where only OVA was administered, 10.2 µL of IgG4 subclass antibody concentration was detected after 10 days of primary immunization, which increased to 17.9 µL after 20 days of secondary immunization.

Antigenic-specific IL-6 response in mice model

On the 20th day post-saponin administration, the spleens of the mice were extracted for the assessment of IL-6 responses using the ELISA method. Blood serum IL-6 and anti-ovalbumin responses were evaluated, particularly in the groups of mice that received higher doses of Panax saponin-1 (60 µL) and Panax saponin-2 (100 µL), in comparison to the group that received control saponin Quil A (100 µL) and the group injected with ovalbumin antigen alone. The IL-6 concentration data is presented in Table 4 and Figure 8.

Table 4. IL-6 (Pg/l) response with OVA-specific antibody in a mouse at day 20th (post-immunization)

Sr. No.	Saponins group doses (µL)	IL-6 Concentration (Pg/l)
1.	PS-1±40	165.67
2.	PS-1±60	188.33
3.	PS-2±80	230.00
4.	PS-2±100	246.67
5.	QuilA±100	116.54
6.	Control±50 (OVA-alone)	61.12

Legend: PS-1 and PS-2=Experimental group; Quil A and OVA alone=control group.

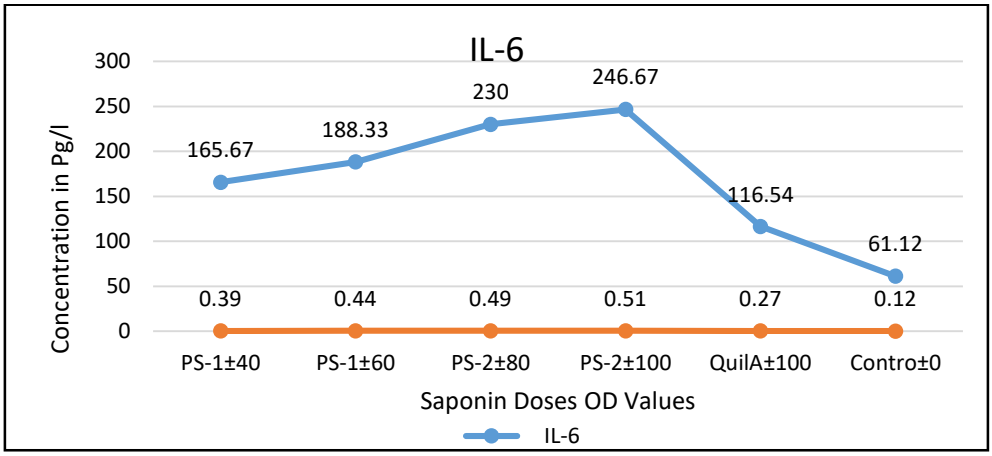

Figure. 8: Immunological responses of IL-6 response with OVA-specific antibody in a mouse model at day 20th post-immunization

Immunizing mice with OVA-specific saponins resulted in a significant increase in IL-6 levels on the 20th day post-immunization when compared to the control group of mice. The graphical representation of the results presented in Table 4 and Figure 8 illustrates that different doses of saponin led to elevated IL-6 concentrations. Specifically, the PS-1±40 µL dose induced an IL-6 concentration of 165.67 Pg/l on the 20th day post-immunization, with a detectable OD value of 0.39. The PS-1±60 µL dose increased the IL-6 concentration to 188.33 Pg/l on the 20th day post-immunization, with a detectable OD of 0.44. Other saponin doses, including PS-2±80 µL and PS-2±100 µL, resulted in IL-6 concentrations of 230.00 Pg/l and 246.67 Pg/l on the 20th day, respectively, with detectable OD values of 0.49 and 0.51. In comparison, the control saponin QuillA±100 (*Quillaja saponaria* Molina) increased the IL-6 concentration to 116.54 Pg/l post-immunization, with a detectable OD of 0.27. These results were also compared to the control group of mice administered with OVA alone, which showed an IL-6 concentration increase to 61.12 Pg/l with a 0.12 OD value. The impact of *P. ginseng* saponins and Quill A saponins on mouse spleen cells' release of interleukin-6 (IL6) in response to OVA antigen, in comparison to the ovalbumin antigen in the phosphate buffer saline control group, demonstrated that higher doses of P. ginseng saponin (Panax saponin-1, $p<0.03$; Panax saponin-2, $p<0.02$) and saponin Quill A ($p<0.04$) significantly enhanced IL-6 secretion in the mouse blood. IL-6 was thus detected in varying quantities at different OD levels.

Comparison of IgG antibody concentration between days 10th and 20th after administration of different doses of saponins

In this research, spectrophotometric analysis was employed to assess IgG antibody concentration between days 10 and 20. The findings revealed a notable increase in overall IgG antibody concentration on the 20th day, as depicted in Table 5. The most substantial percentage change was observed in response to the PS-2±100 saponin dose, indicating a remarkable 96.26% increase in IgG antibody concentration. In summary, the percentage results demonstrate a direct correlation between the increment in *P. ginseng* saponin dose and the elevation in IgG antibody concentration.

Table No.5: Comparison of IgG antibody concentration between 10 to 20 days of immunization of *P. ginseng* saponins.

Mouse No.	Saponin Doses (µL)	IgG Antibody Concentration (mg/dl)		Percentage change in IgG antibody concentration (%)
		10^{th} day	20^{th} day	
1	PS-1±40	150.9	196.3	76.86
2	PS-1±60	297.0	331.9	89.48
3	PS-2±80	380.0	402.4	94.43
4	PS-2±100	490.0	509.0	96.26
5	QuilA±100	146.6	300.9	48.72

Comparison of IgG4 antibody subclasses concentration between 10^{th} to 20^{th} days after administration of different doses of saponins

Spectrophotometric analysis was employed to assess the concentration of total IgG4 subclass antibodies between days 10 and 20. The results revealed a significant increase in the overall concentration of IgG4 subclass antibodies following saponin administration, as illustrated in Table 6. The most substantial percentage change, amounting to 85.19%, was observed in response to the PS-2±100 saponin dose. In summary, the overall percentage findings demonstrate a clear relationship between the elevated doses of *Panax ginseng* saponin and the increased concentration of IgG4 subclass antibodies.

Table No. 6: Comparison of IgG4 subclass antibody concentration between 10 to 20 days after administration of different doses of saponins**.**

Mouse No.	Saponin Dose (µL)	IgG Antibody Concentration (mg/dl)		Percentage change in IgG4 subclasses antibodies concentration. (%)
		Ten day	20 day	
1	PS-1±40	39.5	52.1	75.81
2	PS-1±60	81.7	98.3	83.11
3	PS-2±80	69.3	81.9	84.61
4	PS-2±100	125.4	147.2	85.19
5	QuilA±100	10.2	17.9	48.72

Comparison of IgG and IgG4 subclasses antibodies concentration against different doses of saponin in a mouse model.

The most notable variation was detected through a one-way ANOVA analysis conducted to evaluate the relationship between saponin doses and IgG, as well as IgG subclass antibodies. The obtained p-value of $P<0.0118$ signifies a higher probability rate and underscores the significant distinctions between the control group and the various experimental saponin doses.

Table 7: Overall comparison of IgG and IgG subclasses antibodies concentration against different doses of saponins in a mouse model

Source	Some of squares (SS)	Degrees of freedom (Df)	Mean square (MS)	F statistic	p-value
Saponin doses	112,631.1238	5	37,543.7079	72.5144	0.0118
error	2,070.9650	4	517.7413		
total	114,702.0888	7			

Legend: Significant ($P<0.05$); highly significant ($P<0.01$): NS = Non-significant ($P>0.05$); SD = Standard deviation; SE = Standard error

Discussion

The study explored the immunological effects of saponins extracted from *Panax ginseng* roots. These roots were meticulously processed, sterilized, dried, and finely ground into a powder. Saponins were subsequently extracted from these roots using methanol/n-butanol, a process in line with prior research by Wan et al. (2016). Several fractions were obtained and identified through various techniques such as Flash Chromatography, Thin Layer Chromatography (TLC), and High-Performance Chromatography (HPLC). Specifically, four saponin fractions, denoted as 1, 2, 13, and 14, were selected for their potent hemolytic activity and dominant saponin peaks. These fractions were designated as PS-1 (*Panax ginseng*-1, comprising fractions 1 and 2) and PS-2 (*Panax ginseng*-2, comprising fractions 13 and 14). The adjuvant potential of *P. ginseng* saponins was compared to QuilA, a well-established adjuvant extracted from *Q. saponaria* Molina.

The subsequent phase of this study involved assessing immunological responses in a mouse model. A total of six groups of mice were used, each receiving different treatments: four groups received *Panax ginseng* saponins (PS-1 and PS-2) at varying doses, one group received the control QuilA saponin (QuilA), and the final group was injected with ovalbumin alone. The effects of primary immunization were evaluated on the 10th day, while secondary immunization's impact was assessed on the 20th day. Blood samples, specifically serum, were collected after primary and secondary immunizations to analyze antibody concentrations, including IgG and IgG4 subclass. Interleukin-6 (IL-6) concentrations were also measured using mouse serum and the ELISA method.

The study findings indicated that increasing the concentration of both PS-1 and PS-2 saponins led to elevated levels of IgG, IgG4 subclass antibodies, and IL-6. Notably, the PS-2±100 saponin dose exhibited the most significant increase, with a 96.26% rise in IgG antibody concentration. Adjuvants

and other immunostimulatory substances have garnered substantial interest due to their capacity to enhance immune responses when used with weak antigens in soluble and subunit vaccines. This research aligns with prior studies that have explored the immunostimulatory properties of saponins extracted from various sources, including Panax ginseng.

Furthermore, the study observed that *P. ginseng* saponins, specifically PS-1 and PS-2, did not induce harm or distress when administered to mice, unlike QuilA, which exhibited harmful effects. The investigation suggested that adjuvant activity was present in both *Q. saponaria* (QuilA) and *P. ginseng* saponins. However, since a mixture of *P. ginseng* saponins was used in this study, further research is needed to pinpoint the specific structural components responsible for the adjuvant activity. In summary, this study contributes to our understanding of the immunological effects of saponins extracted from *Panax ginseng*, shedding light on their potential as adjuvants to enhance immune responses. These findings align with the broader research on immunostimulatory compounds and their role in vaccine development.

Conclusion

These findings demonstrate that adjuvant activity was not observed with *Q. saponaria* saponins (Quil A control), while saponins extracted from *P. ginseng* exhibited significant hemolytic activity in the mouse model. This study highlights the remarkable efficiency of *Panax ginseng* saponins as adjuvants. In this study, six groups of mice were subjected to various saponin treatments, including control groups receiving Quil A or ovalbumin (OVA) alone. It was observed that higher doses of Panax ginseng saponins resulted in increased titers of IgG and IgG4 subclass antibodies, along with elevated IL-6 concentrations in mouse serum. Notably, the most substantial increases were observed in the group administered PS-2±100 μL saponins, with a 96.26% rise in IgG, an 85.19% increase in IgG4 subclass antibody concentration, and an IL-6 concentration of 246.67 Pg/l on the 20th day post-immunization. These results underscore the potential of *Panax ginseng* saponins as highly effective adjuvants.

Authors Contribution

KG: Conceived the idea, performed the whole experimental study and prepared the first draft; AN: perform experimental work, SM: helped in statistical analysis, IA: helped in giving the shape of the final draft.

References

1. Shah, Ruchi R, Kimberly J. Hassett, and Luis A. Brito. "Overview of vaccine adjuvants: Introduction, history, and current status." *Vaccine Adjuvants: Methods and Protocols*, vol. 1, no. 13, (2017).
2. Chen, Chun Tao, Zhi Jun Wang, Moses Sing Sum Chow, and Christopher Wai Kei Lam. "Herb-herb combination for therapeutic enhancement and advancement: theory, practice, and future perspectives." *Molecules*, vol. 18, no. 5, pp 5125-5141, 2013.
3. Kiefer, David S, and Traci Pantuso. "Panax ginseng." *American family physician*, vol. 68, no. 8, pp. 1539-1542, (2003).
4. Cheng, Yong, Li.hong Shen, and Jun.tian Zhang. "Anti-amnestic and anti-aging effects of ginsenoside Rg1 and Rb1 and its mechanism of action." *ACTA pharmacologica sinica*, vol. 26, no. 2, pp. 143-149, 2015.
5. Chan, L. Y., P. Y. Chiu, and T. K. Lau. "An in-vitro study of ginsenoside Rb1-induced teratogenicity using a whole rat embryo culture model." *Human Reproduction*, vol. 18, no. 10, pp. 2166-2168, 2003.
6. El Ashmaoui, H. M., and S. M. Girgis. "Evaluation of the potential mutagenic effects of ginseng on maternally treated post-implanted mouse foetuses." *The Egyptian Journal of Hospital Medicine*, vol. 13, no. 1, pp. 57-65, (2003).

7. Shi, Ze.Yu, Jin.Zhang Zeng, and Alice Sze Tsai Wong. "Chemical structures and pharmacological profiles of ginseng saponins." *Molecules,* vol. 24, no. 13, pp. 2443, (2019).
8. Shin, Dong Jin, Myung Ha Yoon, Hyung Gon Lee, Woong Mo Kim, Byung Yun Park, Yeo Ok Kim, Lan Ji Huang, and Jin Hua Cui. "The effect of treatment with intrathecal ginsenosides in a rat model of postoperative pain." *The Korean Journal of Pain,* vol. 20, no. 2, pp. 100-105, 2007.
9. Lü, Jian.Ming, Jun Jiang, Md Saha Jamaluddin, Zhengdong Liang, Qizhi Yao, and Changyi Chen. "Ginsenoside Rb1 blocks ritonavir-induced oxidative stress and eNOS downregulation through activation of estrogen receptor-beta and upregulation of SOD in human endothelial cells." *International Journal of Molecular Sciences,* vol. 20, no. 2, pp. 294, 2019.
10. Shin, Kyung.Chul, and Deok.Kun Oh. "Classification of glycosidases that hydrolyze the specific positions and types of sugar moieties in ginsenosides." *Critical reviews in biotechnology,* vol. 36, no. 6, pp. 1036-1049, 2016.
11. Wan, Jin.Yi, Chong.Zhi Wang, Zhi Liu, Qi.Hui Zhang, Mark W. Musch, Marc Bissonnette, Eugene B. Chang, Ping Li, Lian.Wen Qi, and Chun.Su Yuan. "Determination of American ginseng saponins and their metabolites in human plasma, urine and feces samples by liquid chromatography coupled with quadrupole time-of-flight mass spectrometry." *Journal of Chromatography B,* no. 1015, pp. 62-73, 2016.
12. Ben.Hur, Ehud, and Stephen Fulder. "Effect of Panax ginseng saponins and Eleutherococcus senticosus on survival of cultured mammalian cells after ionizing radiation." *The American Journal of Chinese Medicine,* vol. 9, no. 01, pp. 48-56, 1981.
13. Attele, Anoja S., Ji An Wu, and Chun.Su Yuan. "Ginseng pharmacology: multiple constituents and multiple actions." *Biochemical pharmacology,* vol. 58, no. 11, pp. 1685-1693, 1999.
14. Yuan, Chun.Su, Chong.Zhi Wang, Sheila M. Wicks, and Lian.Wen Qi. "Chemical and pharmacological studies of saponins with a focus on American ginseng." *Journal of ginseng research,* vol. 34, no. 3, pp. 160, 2010.
15. Welkos, Susan, and Alison O'Brien. "[2] Determination of median lethal and infectious doses in animal model systems." *Methods in enzymology,* vol. 235, pp. 29-39, 1994.
16. Bladt, Sabine. *Plant Drug Analysis: A thin layer chromatography atlas*. Springer Science & Business Media, 2009.
17. Stavrianidi, Andrey, Igor Rodin, Arkady Braun, Elena Stekolshchikova, and Oleg Shpigun. "Single.run HPLC/ESI-LITMS profiling of ginsenosides in plant extracts and ginseng based products." *Biomedical Chromatography,* vol. 29, no. 6, pp. 853-859, 2015.
18. van Setten, Dirk C., and Gerrit van de Werken. "Molecular structures of saponins from Quillaja saponaria Molina." *Saponins used in traditional and modern medicine,* pp. 185-193, 1996.
19. Liu, Xiaojie, Samantha Ahlgren, Henrie AAJ Korthout, Luis F. Salomé.Abarca, Lina M. Bayona, Robert Verpoorte, and Young Hae Choi. "Broad range chemical profiling of natural deep eutectic solvent extracts using a high performance thin layer chromatography–based method." *Journal of Chromatography A,* vol. 1532, pp.198-207, 2018.
20. Eppstein, Deborah A., Noelene E. Byars, and Anthony C. Allison. "New adjuvants for vaccines containing purified protein antigens." *Advanced Drug Delivery Reviews,* vol. 4, no. 2, pp. 233-253,1989.
21. Hwang, Su.Hyun, Myoung.Sook Shin, Taek Joon Yoon, and Kwang.Soon Shin. "Immunoadjuvant activity in mice of polysaccharides isolated from the leaves of Panax ginseng CA Meyer." *International journal of biological macromolecules,* vol. 107, pp. 2695-2700, 2018.
22. Qiu, Tianxin, Pengfei Gu, Adelijiang Wusiman, Haiyu Ni, Shuwen Xu, Yue Zhang, Tianyu Zhu et al. "Immunoenhancement effects of chitosan-modified ginseng stem-leaf saponins-encapsulated cubosomes as an ajuvant." *Colloids and Surfaces B: Biointerfaces,* vol. 204, pp. 111799, 2021.
23. Zhuo, Xunhui, Hongchao Sun, Suhua Wang, Xiaolu Guo, Haojie Ding, Yi Yang, Ying Shan, and Aifang Du. "Ginseng stem-and-leaf saponin (GSLS)-Enhanced protective immune

responses induced by Toxoplasma gondii heat shocked protein 70 (HSP70) against toxoplasmosis in mice." *Journal of Parasitology,* vol. 103, no. 1, pp. 111-117, 2017.

24. Wang, Yong, Xuemei Cui, Lijia Yuan, Babar Maqbool, Wei Xu, Shanshan He, Ran Guan, and Songhua Hu. "A solution with ginseng saponins and selenium as vaccine diluent to increase Th1/Th2 immune responses in mice." *Journal of Immunology Research,* vol. 2020, pp. 1-13, 2020.
25. Mosmann, T.R., Schumacher, J.H., Street, N.F., Budd, R., O'garra, A., Fong, T.A.T., Bind, M.W., Moore, K.W.M., Sher, A. and Fiorentino, D.F. Diversity of cytokine synthesis and function of mouse CD4+ T cells. *Immunological reviews*, vol. 123, no. 1, pp.209-229, 1991.
26. Huang, Yiqun, Yong Zou, Luhui Lin, and Ruiji Zheng. "Ginsenoside Rg1 activates dendritic cells and acts as a vaccine adjuvant inducing protective cellular responses against lymphomas." *DNA and Cell Biology,* vol. 36, no. 12, pp. 1168-1177, 2017.
27. Lee, Yun.Kyung, Kyung.Hoon Choi, Hae.Soo Kwak, and Yoon Hyuk Chang. "The preventive effects of nanopowdered red ginseng on collagen-induced arthritic mice." *International Journal of Food Sciences and Nutrition*, vol. 69, no. 3, pp. 308-317, 2018.
28. Ratan, Zubair Ahmed, Mohammad Faisal Haidere, Yo Han Hong, Sang Hee Park, Jeong.Oog Lee, Jongsung Lee, and Jae Youl Cho. "Pharmacological potential of ginseng and its major component ginsenosides." *Journal of ginseng research,* vol. 45, no. 2, pp. 199-210, 2021.
29. Wang, Lei, Xiankui Li, Yi-Min Song, Bin Wang, Fu-Rui Zhang, Rui Yang, Hua.Qi Wang, and Guo.Jun Zhang. "Ginsenoside Rg3 sensitizes human non-small cell lung cancer cells to γ-radiation by targeting the nuclear factor-κB pathway." *Molecular Medicine Reports*, vol. 12, no. 1, pp. 609-614, 2015.