

Aqueous Soybean Extract Reduces the Blood Lymphocyte Fraction in Immunized Mice, Independently of Its Stigmasterol Content

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ABSTRACT

Background: Immunosuppressant therapy remains central to the management of autoimmune disease, motivating the search for accessible plant-derived immunomodulators; soybean (*Glycine max*) is rich in phytosterols, and stigmasterol has been proposed as a lymphocyte-suppressing constituent.

Aims: To test whether the activity of a whole aqueous soybean extract on the circulating lymphocyte fraction is attributable to its stigmasterol content, by comparing the extract directly with purified stigmasterol.

Methods: Aqueous maceration yielded 450 g of extract from 2.5 kg of soybean, and stigmasterol was estimated by HPLC. Male BALB/c mice (n = 3 per group) were immunized with 1% sheep red blood cells (SRBC) on days 0 and 10 and treated orally from day 10 to 24 with extract (EK) or purified stigmasterol (STG) at 10, 50, or 100 mg per animal per day; lymphocyte counts were made from blood smears on days 9, 18, and 24.

Results: HPLC placed extract stigmasterol at or below the limit of quantification, indicating that matched nominal doses of extract and purified compound delivered very different amounts of stigmasterol. At day 24, all six treated groups showed a lower lymphocyte fraction than the immunized untreated control (K+, 43.0 ± 1.7 ; one-way ANOVA $F(7,16) = 83.8$, $p < 0.0001$, $\omega^2 = 0.96$), and a split-plot analysis gave a significant group $\times$ time interaction ($F(14,32) = 25.1$, $p < 0.0001$). The non-immunized control did not differ from K+. The extract produced a dose-dependent reduction (EK100, 22.1 ± 3.1); stigmasterol also lowered lymphocytes but, being solubility-limited and of unverified delivered dose, could not be read as a dose-response.

Conclusion: Because the activity was recovered by aqueous extraction whereas stigmasterol is practically water-insoluble, stigmasterol is unlikely to be the constituent responsible; other water-soluble soy constituents are more probable mediators. A key limitation is that the delivered dose in the purified-stigmasterol arm could not be verified owing to its poor aqueous solubility. As the outcome was the circulating lymphocyte fraction rather than an SRBC-specific antibody titre, the findings do not by themselves establish suppression of the antigen-specific response.

Keywords: *Glycine max*; Stigmasterol; Lymphocyte; Sheep red blood cells; Immunomodulation.

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1. Introduction

Autoimmune disease, in which the immune system loses tolerance to self and attacks healthy tissue, affects an estimated 3–5% of the global population and includes more than eighty conditions such as systemic lupus erythematosus, rheumatoid arthritis, and multiple sclerosis (Davidson & Diamond, 2001; Hayter & Cook, 2012). Because most autoimmune conditions have no definitive cure, management relies on immunosuppressant therapy that dampens the lymphocyte responses driving tissue injury (Whitacre, 2001; Rifa'i et al., 2008). Conventional immunosuppressants are limited by cost, cumulative toxicity, and non-selective activity, sustaining interest in plant-derived compounds as safer, more accessible immunomodulators.

Lymphocytes are the principal effectors of adaptive immunity and a rational pharmacological target: reducing their proliferation and differentiation lessens antigen-specific immunity, whether protective or, in autoimmunity, pathogenic (Rifa'i et al., 2008; Fransson et al., 2012). Lymphocyte responsiveness can be provoked with a defined antigen and quantified, giving a tractable screening model. Sheep red blood cells (SRBC) are widely used because they are readily obtained, antigenically strong, and elicit a reproducible T-dependent response without the risks of live microbial antigens (Wang et al., 2000; Ladics, 2007).

SRBC was selected as the immunogen because it is inexpensive, readily obtained, strongly antigenic, and elicits a reproducible T-dependent response without the biosafety requirements of live microbial antigens, which makes it a standard vehicle for screening candidate immunomodulators (Wang et al., 2000; Ladics, 2007). The primary outcome, however, was the blood lymphocyte fraction rather than the antigen-specific antibody response that is the conventional endpoint of this model (Ladics, 2007). The lymphocyte fraction was chosen because it can be obtained serially from the same animal by tail-vein sampling, giving a longitudinal readout within a small cohort, and because it responds to the proliferative expansion that immunization provokes. It is nevertheless a blunt instrument: it does not distinguish T from B lineages, it reflects a proportion rather than an absolute cell number, and it is influenced by factors unrelated to antigen-specific immunity including stress, corticosteroid tone and haemodilution. Antigen-specific antibody titres and plaque-forming-cell assays, which would have addressed the humoral response directly, were not undertaken, as serological endpoints were outside the scope of the approved protocol and blood was collected into EDTA for hematological analysis rather than processed to serum. The consequence is that the present design can test whether a treatment alters the circulating lymphocyte fraction, but cannot establish suppression of the SRBC-specific response; that distinction is carried through the interpretation below.

Among plant constituents proposed to possess such activity, phytosterols have attracted particular attention. Stigmasterol, a 3 β -hydroxy Δ 5 phytosterol and a major component of the soybean (*Glycine max*) sterol fraction, has been reported to restrain lymphocyte activation in several systems (Kaur et al., 2011; Aboobucker & Suza, 2019), attenuating concanavalin-A-stimulated T-cell proliferation and cytokine production (Le et al., 2017). Earlier studies of ethanolic *Polyscias obtusa* and *Elephantopus scaber* leaf extracts likewise attributed immunosuppressive effects partly to stigmasterol (Roffico & Djati, 2014; Asfi & Djati, 2014). These reports, however, inferred a role for stigmasterol from extract composition rather than testing the compound directly, leaving open whether it is the operative constituent or merely a marker.

Soybean is an attractive vehicle for this question: it is inexpensive, abundant, and already consumed as a functional food, yet its pharmacological effects at defined doses remain poorly characterized (Krisnawati, 2017; Liu, 2012). Because stigmasterol is strongly lipophilic, its recovery depends on solvent, with polar organic systems yielding far more than water (Slavin & Yu, 2012); an aqueous extract is therefore expected to be very poor in stigmasterol, potentially below the analytical limit of quantification. Because the immunosuppressive activity of stigmasterol-containing extracts has so far been inferred from extract composition rather than demonstrated by testing the isolated compound against the whole material, experimental evidence bearing directly on the identity of the active constituent is lacking. A water-based extract was chosen deliberately: unlike ethanolic or other organic-solvent preparations, an aqueous extract leaves no residual solvent that could itself perturb immune function and confound an immunological readout, and it reflects the way soybean is consumed as food. Comparing this whole aqueous extract, which is expected to be essentially devoid of extractable stigmasterol, with the purified compound at matched nominal doses provides a test of attribution by exclusion: if the extract remains

active while containing negligible stigmasterol, then stigmasterol is unlikely to account for that activity. The inference is one of exclusion rather than identification, it can argue against stigmasterol as the operative constituent without establishing which constituent is responsible. We therefore tested whether the activity of a whole aqueous soybean extract on the circulating lymphocyte fraction is attributable to its stigmasterol content, estimating extract stigmasterol by HPLC and comparing the extract directly with purified stigmasterol at matched nominal oral doses (10, 50, and 100 mg per animal per day) in SRBC-immunized BALB/c mice, using the blood lymphocyte differential as the primary readout. We hypothesized that soybean extract would reduce the lymphocyte response dose-dependently and that contrasting a stigmasterol-poor extract with the purified compound would reveal whether the effect depends on stigmasterol or on other soybean constituents.

2. Methods

Plant Material and Extract Preparation

Soybean (*Glycine max* var. Anjasromo) seeds were purchased as a single lot from Pasar Demangan, Jl. Affandi, Demangan, Gondokusuman, Yogyakarta, Indonesia; batch identity was not recorded and no voucher specimen was deposited. Seeds were processed immediately without an intervening storage period. They were sorted by flotation (retaining filled seeds that sank in water), washed under running water, air-dried, oven-dried at 40 °C, milled, and passed through a 40-mesh sieve; moisture content of the milled powder was not determined. Powder (2.5 kg) was macerated in distilled water at an overall material-to-solvent ratio of 1:5 (w/v), the 12.5 L total being divided over three successive 24-h macerations of approximately 4.2 L each (1:1.67 w/v per cycle), with filtration through cloth between cycles and fresh solvent added to the residue each time. Combined filtrates were concentrated in a vacuum rotary evaporator at 70 °C and dried at 40 °C in a Petri dish to a brown paste (450 g), a yield of 18.0% w/w. The paste was stored at 4 °C and was held approximately six to ten weeks before first administration; its stability over this interval was not assessed. Before dosing, the paste was suspended in sterile distilled water and filtered through qualitative paper until no residue remained, to ensure a homogeneous liquid preparation; the removed residue was not weighed, so administered extract doses are reported as nominal. Preparations were made at 20, 100 and 200 mg/mL for the 10, 50 and 100 mg/animal/day doses respectively (30, 150 and 300 mg in 1.5 mL per cage of three animals), administered as 0.5 mL by oral gavage. An overview of the experimental workflow is shown in Figure 1.

HPLC Estimation of Stigmasterol

Stigmasterol was quantified under contract by the Laboratory of Chemistry, Faculty of Science and Mathematics, Universitas Kristen Satya Wacana, Salatiga (report No. 03/06/2021/LAB KIM/FSM/UKSW, 11 June 2021) on a KNAUER Smartline system with a Eurospher 100-5 RP C-18 column (150 × 4.6 mm) at 30 °C, methanol:water (99:1, v/v) at 1 mL/min and UV/VIS detection at 254 nm, against purified stigmasterol (Wellnature Biotech Co., Ltd, Shaanxi, China) as external standard. Calibration gave $y = 109.3x + 7808.3$ with $r^2 = 0.999$, LOD 0.058 µg/mL and LOQ 0.192 µg/mL; accuracy and precision were not evaluated. Because phytosterols absorb maximally near 205–210 nm, detection at 254 nm gives limited sensitivity, and validated methods use low-UV detection (Careri et al., 2001). The method therefore supports only a qualitative statement, that stigmasterol was present at or below trace level, and no numerical stigmasterol value is used to support the conclusions.

Preparation of 1% SRBC Antigen

Fresh sheep blood (PT Gajah Mungkur Indotama, Jakarta) was centrifuged (3000 rpm, 10 min) and the plasma discarded. Packed cells were washed at least three times in phosphate-buffered saline (PBS, pH 7.2) and diluted to a 1% (v/v) working suspension.

Animals and Ethical Approval

Male BALB/c mice (6 weeks, 25–35 g; Tikus Lovers Jogja, Bantul) were acclimatized for 7 days and housed three per cage with sawdust bedding and ad libitum feed and water. Procedures were approved by the Health Research

Ethics Committee, Faculty of Medicine, Universitas Kristen Duta Wacana (Ethical Clearance No. 1313/C.16/FK/2021, 29 June 2021) and complied with institutional and national guidelines for the humane care and use of laboratory animals.

Experimental Design and Treatments

Twenty-four mice were randomly assigned to eight groups of three. Allocation sequences were produced with a computer random-number generator by an investigator not involved in outcome assessment, and cage cards were coded so that the assessor who later counted the blood smears remained blinded to group. Replication was limited to three per group by resource and ethical constraints (below the Federer minimum of four for eight treatments). Groups were a non-immunized control (K-), an immunized untreated control (K+), and six immunized groups given soybean extract (EK) or purified stigmasterol (STG) at 10, 50, or 100 mg per animal per day. The three doses were chosen to span a wide range bracketing amounts used in earlier rodent studies of soybean and phytosterol preparations and to allow a dose-response to be detected. Doses were fixed per animal rather than body-weight-adjusted; across 25–35 g this corresponds to ≈ 285 –400 mg/kg (lowest) and $\approx 2,860$ –4,000 mg/kg (highest). All groups except K- were immunized by intraperitoneal injection of 0.1 mL of 1% SRBC on days 0 and 10 (booster); K- received sterile water on the same schedule. Extract or stigmasterol was dissolved in sterile distilled water with stirring; stigmasterol suspensions were filtered through qualitative paper to remove coarse particles. The delivered STG dose was not assayed and, given the compound's low aqueous solubility, may have been sub-nominal. Treatments were given by oral gavage (0.5 mL) once daily from day 10 to day 24. On day 24 mice were euthanized by chloroform-overdose inhalation. This method was the facility's standard practice at the time and was included in the protocol approved by the Health Research Ethics Committee, Faculty of Medicine, Universitas Kristen Duta Wacana (Ethical Clearance No. 1313/C.16/FK/2021, 29 June 2021). Chloroform is not among the methods endorsed by the current AVMA Guidelines for the Euthanasia of Animals, which cite operator-exposure hazard and inconsistent depth of anaesthesia; this is a deviation from present best practice and is acknowledged as such. All animal procedures were carried out after the approval date, extraction and HPLC analysis having been completed in May and June 2021. Future studies should use an approved method such as carbon dioxide with a confirmatory secondary step.

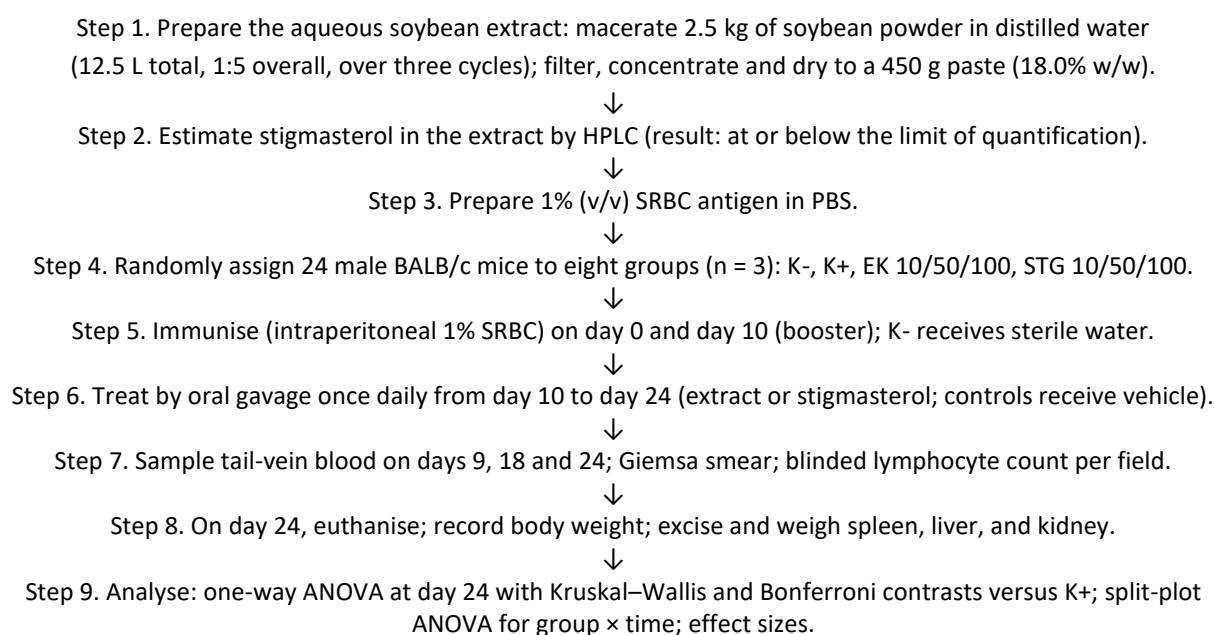


Figure 1. Overview of the experimental workflow

Lymphocyte Counts (Primary Outcome)

Peripheral blood was sampled from the tail vein on days 9, 18 and 24, smeared, methanol-fixed (5 min), Giemsa-stained (30 min), rinsed and air-dried. Lymphocytes were identified by standard Giemsa morphological criteria. For each animal, three separate microscopic fields were enumerated and the counts averaged to give one value per animal per time point; all slides were coded before reading and a single assessor, blinded to group, scored every slide, removing inter-observer variation. Neutrophils were counted separately and not within the same 100-cell field, so lymphocyte and neutrophil counts do not share a denominator; per-animal values summed above 100 in 15 of 72 records, confirming this. These data are therefore not a conventional 100-cell leukocyte differential. The primary outcome is the lymphocyte proportion per counted field, a semi-quantitative index of the circulating lymphocyte fraction.

Morphometric, Organ Measurements and Histopathology

Body weight was recorded on days 0 and 24. After euthanasia the spleen, liver, and kidney were excised and weighed, and organ indices expressed relative to terminal body weight. At necropsy on day 24 the liver and kidney were excised, fixed in 10% neutral buffered formalin, processed routinely to paraffin, sectioned at 5 μ m and stained with haematoxylin and eosin. Sections were examined by light microscopy (Olympus CX23) and photographed through the eyepiece with a digital camera at 10 $\times$ objective magnification, giving a field diameter of approximately 2000 μ m. One field per section was recorded. Images were subsequently coded, randomized and assessed blinded to treatment group, using a conventional semi-quantitative severity scale (0 = none, 1 = minimal, 2 = mild, 3 = moderate, 4 = marked) applied to hepatocellular vacuolation, degeneration or necrosis, lobular and portal inflammation and sinusoidal dilatation in liver, and to tubular degeneration or necrosis, tubular dilatation, protein casts, glomerular change, interstitial inflammation and vascular congestion in kidney. Liver sections were available for all eight groups; kidney sections were available for five treated groups only (EK10, EK50, STG10, STG50, STG100) and not for either control group or for EK100. Because the assessment rested on a single field per section at limited magnification, several endpoints could not be graded, and the analysis is reported as qualitative supporting material rather than as a formal histopathological evaluation.

Statistical Analysis

The individual mouse was the experimental unit ($n = 3$), represented by the mean of its triplicate field counts; values are mean $\pm$ SD. All analyses used unrounded field means; values in the tables are rounded for presentation only. Group differences at day 24 were tested by one-way ANOVA and confirmed by Kruskal–Wallis, with pre-planned Bonferroni-corrected contrasts of each group against K+. Because each animal was sampled on days 9, 18 and 24, the longitudinal analysis used split-plot ANOVA with group as the between-animal factor and day as the within-animal factor, and the group $\times$ time interaction as the test of differential change; within-group paired t-tests comparing day 9 with day 24 are reported as supporting information only. Effect sizes were omega-squared (ω^2) for the model and Hedges' g for contrasts. Significance was set at $p < 0.05$. One measurement occasion was excluded before analysis: the field-2 readings for all three STG100 animals at day 9 (see Table 2 footnote). No other data were excluded. Because SDs from $n = 3$ are imprecise, reported effect sizes indicate direction and approximate magnitude only, with wide confidence limits. Reporting followed the ARRIVE 2.0 guidelines (Percie du Sert et al., 2020), with deviations ($n = 3$; chloroform euthanasia) noted below.

3. Results

Stigmasterol Content of the Aqueous Extract

No stigmasterol peak was detected in a methanol-prepared extract. For the aqueous extract the contract laboratory reported 0.023 mg/g; because the sample response fell below the calibration intercept ($y = 109.3x + 7808.3$; LOD 0.058 μ g/mL, LOQ 0.192 μ g/mL) and the method was not validated for accuracy or precision, this figure is treated here as an approximate upper bound rather than a measured concentration. The absence of a peak in the methanol extract, where stigmasterol should be more readily recovered, indicates under-detection

by the assay rather than true absence from the seed, and reinforces that the method supports only a qualitative statement: the aqueous extract contained stigmasterol at or below trace level. On chemical and analytical grounds the extract contains little stigmasterol, so matched nominal doses of extract and purified compound delivered very different amounts and are best read as distinct interventions.

Reduction of the Blood Lymphocyte Fraction at Day 24

Immunization established the expected reference: K+ sustained a high lymphocyte fraction (43.0 ± 1.7), whereas all six treated groups fell below it (Table 1). The group effect was robust (one-way ANOVA $F(7,16) = 83.8$, $p < 0.0001$, $\omega^2 = 0.96$) and remained significant by Kruskal–Wallis ($H = 21.9$, $p = 0.003$). After Bonferroni correction, all six treated groups differed from K+: STG10 (18.7 ± 1.2), STG50 (21.6 ± 1.5), EK100 (22.1 ± 3.1), EK50 (26.7 ± 0.3), EK10 (28.4 ± 3.7) and STG100 (34.0 ± 0.7); all Bonferroni-corrected $p \leq 0.023$. The non-immunized control did not differ significantly from K+ (47.1 ± 0.8 ; $p = 0.13$), indicating that immunization alone produced no detectable change in the circulating lymphocyte fraction at this time point.

Table 1. Soybean extract and stigmasterol reduce the blood lymphocyte fraction at day 24.

Group	Treatment	Lymphocytes (mean $\pm$ SD, n = 3)	Bonferroni p vs K+	Hedges g
K–	Non-immunised control	47.1 ± 0.8	0.132 (ns)	+2.5
K+	Immunised, untreated	43.0 ± 1.7	—	—
EK10	Extract, 10 mg/animal/day	28.4 ± 3.7	0.023 *	–4.1
EK50	Extract, 50 mg/animal/day	26.7 ± 0.3	0.0005 *	–10.9
EK100	Extract, 100 mg/animal/day	22.1 ± 3.1	0.0035 *	–6.7
STG10	Stigmasterol, 10 mg/animal/day	18.7 ± 1.2	0.0002 *	–13.4
STG50	Stigmasterol, 50 mg/animal/day	21.6 ± 1.5	0.0006 *	–10.7
STG100	Stigmasterol, 100 mg/animal/day	34.0 ± 0.7	0.0068 *	–5.7

Values are the lymphocyte proportion per counted field (mean of three fields per animal), n = 3 animals per group. One-way ANOVA $F(7,16) = 83.8$, $p < 0.0001$, $\omega^2 = 0.96$; Kruskal–Wallis $H = 21.9$, $p = 0.003$. Pre-planned contrasts versus K+ are Bonferroni-corrected across the seven comparisons; * $p < 0.05$. All six treated groups differed from K+; the non-immunized control did not.

The Reduction Develops During the Treatment Window

Because each animal was sampled three times, group differences over time were tested by split-plot analysis of variance with group as the between-animal factor and day as the within-animal factor. This gave a significant group $\times$ time interaction, $F(14,32) = 25.1$, $p < 0.0001$ (group $F(7,16) = 106.1$, $p < 0.0001$; day $F(2,32) = 172.8$, $p < 0.0001$), establishing that the trajectory of the lymphocyte fraction differed between groups rather than changing uniformly with time (Table 2). Treated groups declined after dosing began on day 10, whereas K+ remained essentially unchanged and K– rose by 9.8 percentage points. Supporting within-group comparisons of day 9 with day 24 were significant in five of six treated groups (paired t-tests, $p \leq 0.021$) but not in STG100 (–8.8 percentage points, $p = 0.095$) once the invalid day-9 readings were excluded; these paired tests cannot themselves establish that change over time differed between groups, and are reported as secondary to the interaction test.

Table 2. The reduction develops during the treatment window.

Group	Day 9	Day 18	Day 24
K–	37.3 ± 1.2	39.3 ± 5.6	47.1 ± 0.8
K+	46.9 ± 0.4	51.4 ± 1.3	43.0 ± 1.7
EK10	46.1 ± 0.8	33.2 ± 1.2	28.4 ± 3.7
EK50	45.2 ± 0.7	31.2 ± 1.0	26.7 ± 0.3
EK100	49.8 ± 0.2	29.0 ± 1.5	22.1 ± 3.1
STG10	42.0 ± 0.6	39.0 ± 4.2	18.7 ± 1.2
STG50	38.0 ± 0.0	21.6 ± 3.5	21.6 ± 1.5
STG100	42.8 ± 4.8	23.3 ± 2.7	34.0 ± 0.7

Comparison of Extract and Purified Stigmasterol

Soybean extract reduced the lymphocyte fraction dose-dependently (28.4 → 26.7 → 22.1 for EK10 → EK50 → EK100). Purified stigmasterol also lowered lymphocytes but without a graded pattern (18.7, 21.6, 34.0 for STG10, STG50, STG100); because it is practically water-insoluble and suspensions were filtered before dosing, the delivered dose is unknown and cannot be read as a dose–response (Table 3). When the two arms are placed side by side, they bear directly on whether stigmasterol accounts for the extract’s activity. The whole extract contained stigmasterol only at or below trace level, yet it produced a graded reduction reaching about 21 percentage points below K+ at the highest dose (EK100, 22.1 vs K+, 43.0), within a highly significant overall model (one-way ANOVA $F(7,16) = 83.8$, $p < 0.0001$, $\omega^2 = 0.96$) in which every extract dose differed from K+ (Bonferroni-corrected contrasts, $p \leq 0.023$). Because the extract produced this effect while containing stigmasterol at or below trace level, the chemical and biological findings together argue against stigmasterol as the constituent responsible for the extract’s activity. The evidence is exclusionary: it does not identify the active constituent, and it does not exclude a contribution from stigmasterol at concentrations below the detection limit of this assay.

Reduction = K+ mean (43.0) minus group mean at day 24. Extract shows a graded reduction across nominal dose. Purified stigmasterol (STG) is shown at nominal dose only: it is practically water-insoluble, and suspensions were filtered before dosing, so the delivered dose is unknown, and these values cannot be read as a dose–response. Extract doses are also nominal, as the extract was filtered before dosing and the removed residue was not weighed.

Table 3. Soybean extract produces a dose-dependent effect; stigmasterol delivery is unverified.

Group	Nominal dose (mg/animal/day)	Day-24 reduction vs K+ (percentage points)
EK10	10	14.6
EK50	50	16.3
EK100	100	20.9
STG10	10	24.3
STG50	50	21.4
STG100	100	9.0

Neutrophil Differential (Secondary)

Day-24 neutrophil counts also differed among groups (ANOVA $F(7,16) = 80.0$, $p < 0.0001$); however, per-animal neutrophil and lymphocyte values summed to more than 100 in 15 of 72 records, indicating they were not scored against a single 100-cell count. Neutrophil data are therefore reported as secondary and exploratory, with no contrasts performed and no mechanistic interpretation drawn (Table 4).

Table 4. Neutrophil differential (secondary, exploratory).

Group	Neutrophils (mean ± SD, n = 3)
K–	75.7 ± 0.6
K+	42.7 ± 3.5
EK10	46.0 ± 5.3
EK50	43.0 ± 2.6
EK100	17.7 ± 4.6
STG10	22.7 ± 2.5
STG50	31.3 ± 1.2
STG100	31.3 ± 4.5

Morphometric Outcomes

All immunized groups gained body weight over the 14-day treatment period, whereas K– lost weight; the highest-dose groups gained most (EK100, +6.3 g; STG100, +5.3 g) (Table 5). ANOVA showed no significant treatment effect on spleen, kidney or liver weights (all $p \geq 0.26$) and relative organ weights were comparable across groups. In the blinded histological assessment, no gradable lesion was identified in the sections available (Figure 2 and 3); minimal vascular congestion was recorded in three of seven assessable kidney sections, distributed across EK10,

STG10 and STG100 without relation to dose and with collection-related blood retention an equally plausible cause. Hepatocellular and tubular degeneration or necrosis, and glomerular change, could not be graded in any section at the available resolution, and no kidney section was available for either control group. The histological findings therefore neither support nor exclude organ toxicity.

Table 5. Morphometric and organ-weight outcomes by group.

Group	Description	Body-wt change (g)	Spleen (g)	Spleen (% BW)	Kidney (g)	Kidney (% BW)	Liver (g)	Liver (% BW)
K-	Non-immunized control	-4.67	0.25 ± 0.07	0.81	0.71 ± 0.08	2.27	2.49 ± 0.25	8.02
K+	Immunized, untreated	4	0.33 ± 0.06	0.85	0.66 ± 0.14	1.68	2.59 ± 0.50	6.58
EK10	Extract 10 mg	4	0.27 ± 0.06	0.69	0.74 ± 0.16	1.92	2.88 ± 0.75	7.46
EK50	Extract 50 mg	1.33	0.33 ± 0.12	0.99	0.63 ± 0.05	1.88	2.39 ± 0.30	7.11
EK100	Extract 100 mg	6.33	0.33 ± 0.07	0.88	0.69 ± 0.08	1.86	2.51 ± 0.27	6.73
STG10	Stigmasterol 10 mg	4.33	0.49 ± 0.06	1.49	0.69 ± 0.06	2.10	3.11 ± 0.48	9.51
STG50	Stigmasterol 50 mg	2	0.30 ± 0.20	1.02	0.59 ± 0.17	1.98	2.59 ± 1.44	8.74
STG100	Stigmasterol 100 mg	5.33	0.35 ± 0.05	0.92	0.62 ± 0.07	1.64	2.44 ± 0.60	6.43

Mean ± SD, n = 3 (K- organs n = 2). Body-weight change over days 0–24; organ weights and relative organ weights (organ ÷ final body weight × 100). Notes. Values are group means ± standard deviation. Relative organ weight = organ weight ÷ mean final body weight × 100. All immunized groups gained body weight; only the non-immunized control (K-) lost weight, with no organ enlargement indicative of overt toxicity. One-way ANOVA (8 groups): spleen $F(7,15) = 1.46$, $p = 0.26$; kidney $F(7,15) = 0.61$, $p = 0.74$; liver $F(7,15) = 0.36$, $p = 0.91$.

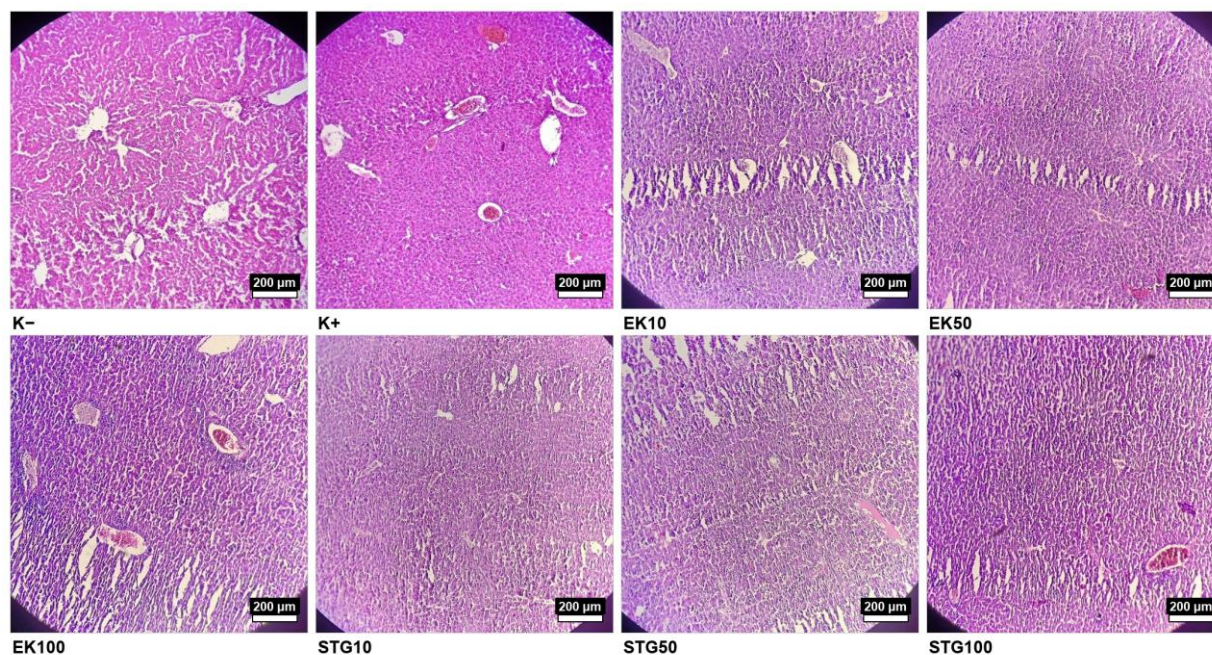


Figure 2. Liver histology (H&E) by treatment group. Representative liver sections, haematoxylin and eosin, one field per section, photographed at 10× objective magnification (Olympus CX23; field diameter approximately 2000 µm). All eight groups are represented. Scale bar 200 µm. Repeated cleaving and directional stretching in several fields reflect section preparation and should not be interpreted as sinusoidal dilatation or necrosis.

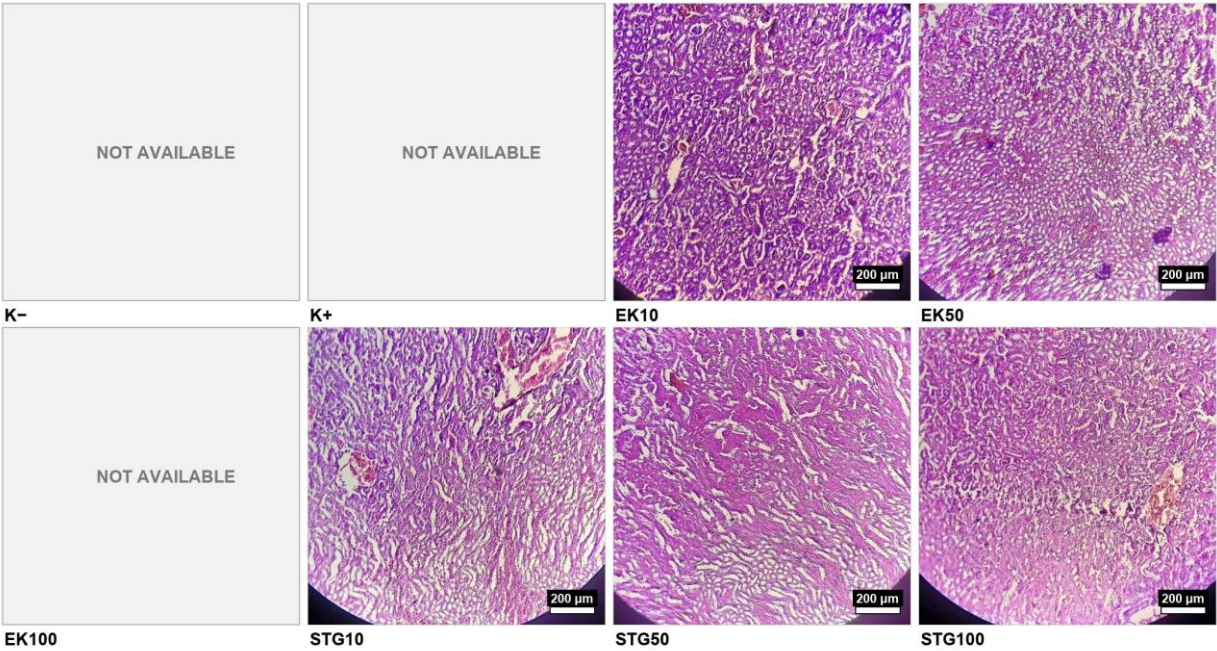


Figure 3. Kidney histology (H&E) by treatment group. Representative kidney sections, haematoxylin and eosin, one field per section, photographed at 10× objective magnification (Olympus CX23; field diameter approximately 2000 μm). Scale bar 200 μm. Sections were unavailable for the non-immunized control (K–), the immunized untreated control (K+) and EK100; these positions are shown as blank panels. Because no control kidney section was available, the panel provides no baseline against which treated sections can be judged.

Table 6. Blinded histological assessment of liver and kidney sections.

(a) Liver (n = 14 sections)										
Group	Image	Assessable	Vacuolation/ lipid	Degeneration/ necrosis	Lobular inflammation	Portal inflammation	Sinusoidal dilatation	Overall impression	Confidence (1–5)	
K–	HIS-016	Y	NA	NA	0	NA	NA	NA	1	
K+	HIS-002	Y	0	NA	0	0	0	normal	3	
EK10	HIS-011	Y	0	NA	0	NA	NA	NA	2	
EK10	HIS-013	Y	0	NA	0	NA	NA	NA	2	
EK50	HIS-008	Y	0	NA	0	NA	NA	NA	2	
EK50	HIS-017	Y	0	NA	0	NA	NA	NA	2	
EK100	HIS-004	Y	0	NA	0	NA	NA	NA	2	
EK100	HIS-019	Y	0	NA	0	NA	NA	NA	2	
STG10	HIS-018	Y	0	NA	0	NA	0	normal	2	
STG10	HIS-023	Y	0	NA	0	NA	0	normal	2	
STG50	HIS-005	Y	0	NA	0	NA	NA	NA	2	
STG50	HIS-006	Y	0	NA	0	NA	NA	NA	2	
STG100	HIS-009	Y	0	NA	0	NA	NA	NA	2	
STG100	HIS-022	Y	0	NA	0	NA	NA	NA	2	
(b) Kidney (n = 9 sections)										
Group	Image	Assessable	Tubular degeneration /necrosis	Tubular dilatation	Protein casts	Glomerular change	Interstitial inflammation	Vascular congestion	Overall impression	Confidence (1–5)
EK10	HIS-003	Y	NA	0	0	NA	0	0	normal	2
EK10	HIS-007	Y	NA	0	0	NA	0	1	minimal change	2
EK50	HIS-012	Y	NA	0	0	NA	0	0	normal	2
EK50	HIS-021	N	NA	NA	NA	NA	NA	NA	NA	1
STG10	HIS-020	Y	NA	NA	0	NA	0	1	minimal change	2
STG50	HIS-001	Y	NA	NA	0	NA	NA	0	NA	1
STG50	HIS-015	Y	NA	NA	NA	NA	NA	0	NA	1
STG100	HIS-010	N	NA	NA	NA	NA	NA	NA	NA	1
STG100	HIS-014	Y	NA	NA	0	NA	0	1	minimal change	2

Images from Figures 2 and 3 were cropped to remove group labels, resampled to a common size, randomly ordered and assigned codes, then scored by assessor blinded to treatment group; the code was broken only after scoring was complete. Severity scale: 0 = none, 1 = minimal, 2 = mild, 3 = moderate, 4 = marked. NA = not assessable owing to image quality, artifact, or absent or insufficient target structures; NA is not zero, and a score of 0 means no convincing finding in the interpretable portion of that single field rather than exclusion of subtle injury. Two kidney sections (EK50, STG100) were not assessable at all. Degeneration or necrosis could not be graded in any of the 23 sections, and glomerular change could not be graded in any of the 9 kidney sections. Confidence did not exceed 3 of 5 for any image. The only positive findings were minimal (grade 1) vascular congestion in three kidney sections (EK10, STG10, STG100); these showed no relation to dose, were contradicted within EK10 by a score of 0 in its other section and are equally consistent with collection-related blood retention. No control kidney section was available for comparison. The assessment therefore neither supports nor excludes organ toxicity.

4. Discussion

This study finds that an aqueous soybean extract dose-dependently reduced the circulating lymphocyte fraction of SRBC-immunized mice (Table 1), specifically during the treatment window (Table 2). Because the activity survived aqueous maceration and filtration, stigmasterol, which is practically water-insoluble, is unlikely to be the constituent responsible. The activity was recovered into an aqueous preparation, which constrains what the responsible constituent can be: aqueous maceration preferentially extracts polar constituents and recovers lipophilic sterols poorly, so a preparation made this way is expected to be depleted of stigmasterol while retaining water-soluble material. This does not establish that the active compound is itself freely water-soluble, the extract is a complex mixture, and a sparingly soluble constituent could in principle be carried by other components, but it does make a lipophilic sterol an unlikely candidate for the activity observed, and it decouples biological activity from stigmasterol content in this preparation. Consistent with this, HPLC indicated only a sub-quantifiable stigmasterol level (supporting evidence only, given the sub-optimal 254-nm method). The study cannot positively define stigmasterol's role, since extract stigmasterol was not reliably quantified and the purified-stigmasterol arm delivered an unknown, solubility-limited dose; following the earlier reports that attributed immunosuppressive effects of stigmasterol-containing extracts to that compound (Roffico & Djati, 2014; Asfi & Djati, 2014).

The immunological readout warrants caution. SRBC is a T-dependent antigen that mainly elicits a humoral, B-cell response, whereas the primary outcome was a total lymphocyte count per field that does not distinguish T from B lineages and is influenced by factors unrelated to antigen-specific immunity, including stress, corticosteroid tone, and haemodilution. The standard SRBC endpoint is the antigen-specific antibody response (Ladics, 2007), which was not measured. The study can therefore conclude only that the extract reduces the circulating lymphocyte fraction, not that it suppresses the SRBC-specific response (which requires antibody-titre or plaque-forming-cell assays). A further indication of the readout's limits is that the non-immunized and immunized untreated controls did not differ significantly at day 24 (47.1 ± 0.8 versus 43.0 ± 1.7 ; $p = 0.13$), so immunization alone produced no detectable change in the lymphocyte fraction in this cohort. The outcome measure was therefore able to register the treatment effect but not the immunization itself, which further limits its interpretation as a measure of antigen-specific immunity.

Purified stigmasterol also lowered the lymphocyte fraction, consistent with reports that stigmasterol and related phytosterols restrain mitogen-stimulated T-cell proliferation and cytokine release (Le et al., 2017); however, because the compound is practically water-insoluble and suspensions were filtered before dosing, its delivered dose is unknown and this arm cannot be read as a dose-response. Soybean is a rich source of candidate immunomodulators with documented effects on lymphocyte activation and inflammatory signalling (Liu, 2012; Yuan et al., 2019). Among these, the isoflavones genistein and daidzein are readily water-dispersible and have been reported to inhibit lymphocyte proliferation and to shift T-helper cytokine balance, while soyasaponins can dampen lymphocyte activation and macrophage inflammatory signalling; both classes are efficiently recovered by aqueous extraction, and both have reported immunosuppressive activity (Sakai & Kogiso, 2008; Sun et al., 2014). None was measured or tested in the present study; they represent plausible candidates that warrant direct testing in future work, and are not offered as an explanation of the present result. The previous studies inferred a role for stigmasterol from its presence in active extracts; the present comparison tests that inference experimentally and does not support it. The design supports an argument by exclusion rather than a positive attribution: the delivered stigmasterol dose was not verified, extract stigmasterol was not reliably quantified, and both preparations were filtered before dosing, so doses are nominal in both arms.

Because doses were fixed per animal, the highest (100 mg) corresponds to $\approx 2,860\text{--}4,000$ mg/kg, which is high and raises the possibility of non-specific effects. Available indicators gave no positive signal of toxicity: high-dose groups gained weight and organ weights showed no treatment effect (all $p \geq 0.26$; Table 5). These observations are reassuring but limited. No haematological, biochemical or functional safety endpoints were measured, histological assessment was qualitative and incomplete, and no formal toxicological evaluation was undertaken. The study therefore does not constitute a safety assessment, and the absence of a detected effect at these doses should not be read as evidence of safety. Sustained weight gain argues against a severe stress response, but glucocorticoid-mediated lymphopenia cannot be excluded, as corticosterone was not measured and would also scale with dose. Future studies should use lower, body-weight-normalized doses and measure corticosterone alongside antibody titres.

A strength is the side-by-side comparison of the whole extract with its purified candidate constituent at matched nominal doses under blinded counting, which allowed the extract's activity to be dissociated from stigmaterol. Several limitations also apply. The sample was small ($n = 3$ per group), below the Federer minimum, limiting power; although the day-24 effect was robust under both analyses, replication in larger cohorts is needed. The HPLC method was not optimized for phytosterols and only partially validated, so the stigmaterol estimate is uncertain and the conclusion does not rest on it; stigmaterol was undetected even in a methanol extract, indicating assay under-detection rather than true absence (Careri et al., 2001). The delivered stigmaterol dose was unverified. Counting was not standardized to a single 100-cell field, so the neutrophil data are exploratory only, and the automated haematology run was excluded owing to prolonged storage. Euthanasia used chloroform, the facility's approved method at the time but not endorsed by current AVMA guidelines; future studies should use an approved method such as CO₂ with a confirmatory step.

Building on these findings, it is recommended that future work should: (i) validate an HPLC method specifically for phytosterols, using low-UV detection (205–210 nm) with full assessment of accuracy and precision, to quantify stigmaterol reliably in soybean matrices; (ii) verify the actual delivered stigmaterol dose, for example by using a validated solubilising vehicle and assaying the administered preparation, so that a genuine dose-response can be tested; (iii) isolate and test individual water-soluble soybean constituents, notably genistein, daidzein, and soyasaponins, through bioassay-guided fractionation to identify the active compound(s); and (iv) incorporate additional immunological endpoints, including cytokine profiling, SRBC-specific antibody titres, and lymphocyte immunophenotyping by flow cytometry, to confirm the mechanism and define the immune compartment affected.

5. Conclusion

Within these limits, the study provides preliminary *in vivo* evidence that aqueous soybean extract dose-dependently reduces the circulating lymphocyte fraction in immunized mice. The findings do not support the hypothesis that stigmaterol is responsible for this effect: the hypothesis of a dose-dependent extract activity was supported, whereas the hypothesis attributing that activity to stigmaterol was not, because the effect persisted in an extract containing negligible stigmaterol and resides in a water-soluble fraction. Attention is therefore redirected to other soybean constituents. Future work combining bioassay-guided fractionation, antigen-specific serology, and lymphocyte immunophenotyping in adequately powered cohorts should identify the responsible compounds and define the affected immune compartment, clarifying whether soybean preparations merit development as accessible natural immunomodulators.

Conflict of Interest

The author declares no conflict of interest.

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