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Selective Cytotoxicity in HepG2 Tumor Cells and Immunomodulatory Effects of Pteropodine and Beta-Sitosterol in BALB/c Mice

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Abstract

Background/Objectives: Oxidative stress and chronic inflammation are critical pathophysiological drivers of over 100 degenerative diseases and malignancies. Beta-Sitosterol (BS) and pteropodine (PT) are plant-derived bioactive compounds implicated in fatty acid metabolism and tissue homeostasis. This study aimed to evaluate the immunomodulatory capacity, selective cytotoxicity, acute anti-inflammatory effects, and antimutagenic potential of BS and PT using complementary in vivo and in vitro models. **Methods:** Male and female BALB/c mice were orally treated with BS (100–200 mg/kg) or PT (25–50 mg/kg). Humoral immunity against thymus-dependent antigens was assessed via a modified Jerne/Cunningham hemolytic plaque assay (SRBC immunization). Acute inflammation was evaluated using a TPA-induced mouse ear edema model. *In vitro* selective cytotoxicity was determined by exposing human hepatocellular carcinoma cells (HepG2) and normal human liver cells (Chang liver) to both compounds, evaluating cell adhesion and morphology. **Results:** Both BS and PT significantly enhanced the humoral immune response by increasing plaque-forming cells (IgM-producing B-lymphocytes) and elevated peripheral lymphocyte counts in BALB/c mice. Topically, both compounds effectively reduced TPA-induced acute ear edema. Furthermore, BS exhibited potent selective cytotoxicity, inducing complete cell detachment (CT100) and apoptotic morphological changes in HepG2 cancer cells, while leaving normal Chang liver cells unaffected (CT0). **Conclusions:** Pteropodine and beta-sitosterol exert significant immunomodulatory, anti-inflammatory, and antioxidant activities *in vivo*, alongside a remarkable tumor-selective cytotoxic profile *in vitro*, representing promising natural scaffolds for multi-target chemopreventive therapies.

Keywords: beta-sitosterol; pteropodine; immunomodulation; selective cytotoxicity; HepG2; BALB/c mice; acute inflammation; natural products

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1. Introduction

Oxidative stress and chronic inflammation represent interconnected biochemical anomalies that serve as primary pathophysiological drivers in over 100 chronic degenerative disorders, including arthritis, atherosclerosis, neurodegenerative diseases, and malignancies [1]. At the cellular level, oxidative stress is characterized by a critical imbalance between the overproduction of reactive oxygen species (ROS) and the endogenous antioxidant defense mechanisms [1, 2]. This disruption compromises cellular homeostasis, precipitating extensive oxidative damage to proteins, lipids, and DNA [1]. Concurrently, accumulated free radicals act as signaling molecules that trigger acute and chronic inflammatory cascades via the activation of pro-inflammatory transcription factors [3, 4]. Consequently, there is a growing pharmaceutical interest in identifying multi-target bioactive compounds capable of simultaneously enhancing the immune response while suppressing oxidative stress and selective oncogenic proliferation with minimal systemic toxicity.

Among plant-derived therapeutic candidates, β-sitosterol (BS) and pteropodine (PT) have emerged as promising compounds due to their multi-target biological activities. β-Sitosterol, a ubiquitous dietary phytosterol, has been widely documented for its capacity to integrate into cell membranes, modulate lipid metabolism, and alter tumor progression [5, 6]. Recent studies indicate that BS downregulates pro-inflammatory mediators and induces apoptosis in specific cancer models through ROS-mediated mitochondrial pathways [6, 7]. On the other hand, pteropodine—a prominent pentacyclic oxindole alkaloid isolated from *Uncaria tomentosa*—is recognized for its potent immunomodulatory and anti-inflammatory properties [8, 9]. Structurally, these compounds are hypothesized to modulate the conversion of linoleic acid and the subsequent biosynthesis of omega-6 fatty acid derivatives, prostaglandins, and leukotrienes, thereby regulating systemic inflammatory cascades.

Despite these individual attributes, a significant gap remains in the literature regarding the concurrent evaluation of BS and PT under unified experimental conditions that span both humoral immunity *in vivo* and selective oncogenic cytotoxicity *in vitro*. Moreover, while conventional chemotherapeutic and anti-inflammatory agents frequently induce significant secondary genotoxicity and tissue damage—such as doxorubicin-induced mutagenicity—the protective role of these natural compounds against chemically induced genetic damage requires further elucidation.

To address these questions, the present study was designed to evaluate the multi-target therapeutic profile of pteropodine and β-sitosterol. Specifically, we assessed their immunomodulatory capacity against thymus-dependent antigens (Sheep Red Blood Cells) and their acute anti-inflammatory effects in a murine model of 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced ear edema. Furthermore, their antimutagenic potential against doxorubicin-induced genotoxicity was investigated alongside their *in vitro* radical scavenging activity. Finally, we explored the *in vitro* selective cytotoxicity of both compounds by contrasting their effects on human hepatocellular carcinoma cells (HepG2) against normal human liver cells (Chang liver). Ultimately, this work demonstrates that pteropodine and β-sitosterol significantly enhance humoral immunity and alleviate acute tissue inflammation *in vivo*, while exhibiting a remarkable, tumor-selective cytotoxic profile *in vitro*, thereby underscoring their potential as safe, natural scaffolds for targeted chemopreventive therapies.

2. Materials and Methods

2.1. Chemical Reagents and Compounds

Beta-sitosterol (BS) and pteropodine (PT) were obtained from Sigma-Aldrich, St. Louis, MO, USA. For *in vivo* administration, both compounds were dissolved in mineral oil, to achieve the required concentrations. 12-O-tetradecanoylphorbol-13-acetate (TPA), indomethacin, doxorubicin, ascorbic acid, interferon-alpha, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma Aldrich. All other analytical grade reagents and cell culture media components were sourced from Bio-Cells Laboratory, Mexico City, Mexico.

2.2. Animal Housing and Ethical Declaration

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Male and female BALB/c mice (10–12 weeks old, mean body weight: 30–35 g) were obtained from National Medical Center "Siglo XXI" bioterio´s. Animals were randomized into experimental groups (n = 5 per group/sex) and housed in standard acrylic cages under controlled laboratory environments: a 12-h light/dark cycle, a constant temperature of 22 °C, and relative humidity of 50%. Standard pellet diet and water were provided *ad libitum*. All experimental protocols involving animal handling, care, and surgical procedures were reviewed and formally approved by the Institutional Animal Care and Use Committee (CICUAL) of Instituto Nacional de Rehabilitación Luis Guillermo Ibarra Ibarra, under protocol approval code 95/17.

2.3. In Vitro Cell Culture and Selective Cytotoxic Evaluation

Two human hepatic cell lines were utilized to assess selective antitumoral activity: normal human liver cells (Chang liver) and human hepatocellular carcinoma cells (HepG2, ATCC HB-8065). Cells were maintained in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and. Cultures were incubated at 37 °C within a humidified atmosphere containing 5% of CO2 and 95% air until a confluent monolayer was formed (2–3 days).

Monolayers were subsequently exposed to varying concentrations of BS [50, 100, 200, µg/mL] and PT [5, 10, 25, µg/mL] according to established protocols. Morphological alterations and cytotoxicity were assessed via phase-contrast microscopy under different magnification factors. The primary evaluation criterion was cell adhesion to the culture plate. Experimental wells displaying total cell detachment were designated as 100% cytotoxic (CT100), whereas wells maintaining cell adhesion levels identical to the untreated negative controls were classified as non-cytotoxic (CT0).

2.4. Humoral Immune Response Assay (Modified Jerne/Cunningham Plaque Technique)

The evaluation of the humoral immune response against thymus-dependent antigens—specifically Sheep Red Blood Cells (SRBC)—was carried out using the Cunningham modification of the Jerne hemolytic plaque assay. Following a 6-h fasting period, BALB/c mice of both sexes received oral treatments via nasogastric gavage. Mice were administered doses of 100, 200, or 600 mg/kg of beta-sitosterol, or 50, 100, or 200 mg/kg of pteropodine according to the following chronological scheme: - 24 h prior to SRBC immunization. - Simultaneously with SRBC immunization. - 24 h post-SRBC immunization.

Negative control groups received the vehicle solution utilized for compound dissolution. To perform the *in vitro* assay, a suspension containing treated animal lymphocytes, SRBCs, and MEM was prepared without agarose and mounted onto glass slides. The quantitative measure of antibody-producing cells was determined by counting the hemolytic plaque-forming cells (PFC), which predominantly represent IgM-secreting B-lymphocytes due to their high hemolytic efficiency.

2.5. Complementary In Vivo and In Vitro Assays

2.5.1. TPA-Induced Mouse Ear Edema (Acute Inflammation Model)

To evaluate the acute anti-inflammatory potential of the compounds, a murine model of 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced ear edema was employed. Male and female BALB/c mice were randomized into experimental groups (n = 5 per group/sex). Acute localized inflammation was induced by topically applying 2.5 µg of TPA dissolved in 20 µL of acetone (10 µL each to the internal and external surfaces of the right ear). One hour post-induction, treatments were topically administered to the right ear dissolved in 20 µL of acetone. The negative control group received only the acetone vehicle, while Indomethacin (0.5 mg/ear) was utilized as the reference standard positive control. Four hours after treatment administration, mice were euthanized via cervical dislocation. Circular sections (7 mm in diameter) were precisely punched from both the treated (right) and untreated (left) ears using a calibrated biopsy punch. The biopsies were immediately weighed to determine the net edema weight by calculating the weight differential (weight = W treated – W untreated). The edema inhibition percentage was calculated according to the following equation:

Inhibition (%) = [(W control – W treatment) / W control] x 100

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Where W control represents the mean ear weight differential of the TPA-only control group, and W treatment represents the weight differential of the groups treated with the bioactive compounds or indomethacin.

2.5.3. In Vitro Antioxidant Activity Assays

DPPH Radical Scavenging Activity

The antioxidant potential of beta-sitosterol (BS) and pteropodine (PT) was evaluated based on their capacity to scavenge the stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH). Briefly, sample aliquots were mixed with 2.9 mL of a 120 μM DPPH solution in methanol, testing variable concentrations of the compounds. The reaction mixtures were incubated in the dark at 37 °C for 30 min. The absorbance was subsequently measured at 517 nm using a spectrophotometer. Ascorbic acid (AA) was employed as the positive control reference standard, and all assays were performed in triplicate. The DPPH radical scavenging inhibition percentage (I%) was calculated according to the following equation: **I (%) = [(A_blank - A_sample) / A_blank] x 100**

Where A_blank represents the absorbance of the control reaction (containing all reagents except the test compounds) and A_sample corresponds to the absorbance of the tested compounds or the standard.

2.5.3. Micronucleus Inhibition Test (Antimutagenicity Assay)

The protective effects of BS and PT against genotoxic damage were evaluated using the micronucleus (MN) test. Genotoxicity was induced via an intraperitoneal injection of doxorubicin (10 mg/kg). Peripheral blood marrow smears were prepared at 24, 48, 72 and 96 h, postinjection, stained, and evaluated under microscopy to determine the frequency of micronucleated polychromatic erythrocytes (MNPCE).

2.5.4. Peripheral Blood Lymphocyte Quantification

Total, and differential lymphocyte counts were performed on peripheral blood smears. Blood samples were collected from the tail vein. Smears were stained using Wright-Giemsa and examined under light microscopy. Ascorbic acid combined with interferon-alpha (15 mg/kg) served as the positive reference control.

2.6. Statistical Analysis

All data are expressed as mean standard error of the mean (SEM). Statistical comparisons between the experimental groups and their respective controls were performed using performed using one-way analysis of variance (ANOVA), followed by Tukey's post-hoc tests.

3. Results

3.1. Sex-Specific Effects on the Humoral Immune Response

The oral administration of BS and PT revealed a striking sex-specific dimorphism in the humoral immune response against thymus-dependent antigens (SRBC) in BALB/c mice. In female mice, BS treatment induced a robust, dose-dependent stimulation of the immune response, as evidenced by a significant increase in antibody-forming cell (AFC-IgM) levels of 39%, 67%, and 98% compared to the control group (table 1). Conversely, a prominent inhibitory trend was observed in male mice, which exhibited a significant suppression of AFC-IgM levels by 51%, 39%, and 21%, respectively.

Table 1. Data represent the Mean Standard Deviation (SD) of Plaque-Forming Cells (PFC) per million splenocytes; *n = 5 per sex per group (total n = 10 per treatment level). SRBC: Sheep Red Blood Cells; BS: beta-sitosterol.*

Antigen / Treatment	*Dose (mg/kg)*	*Female Mice (Mean ± SD)*	*Variation vs. Pos. Control (%)*	*Male Mice (Mean ± SD)*	*Variation vs. Pos.*

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					Control (%)
Mineral Oil ***(Negative Control)***	—	0	—	0	—
10% Sheep Red Blood Cells (SRBC) ***(Positive Control)***	—	287±4.7	—	287±4.7	—
BS	100	398±3.9*	+38.7%	140±6.4*	-51.2%
BS	200	507±6.0*	+76.7%	175±8.0*	-39.0%
BS	300	568±11.0*	+98.9%	227±7.0*	-21.4%

*p < 0.05 indicates a statistically significant difference compared to the respective sex-matched positive control group.

A remarkably similar dimorphic pattern was triggered by PT administration. Female mice treated with PT showed an exceptional up-regulation of the humoral immune response, with AFC-IgM levels increasing by up to 141% (Table 2). In contrast, male mice subjected to the same PT therapeutic regimen exhibited a distinct immune inhibition, underscoring a clear sex-dependent modulation of humoral immunity by both phytocompounds.

Table 2. Data represent the Mean Standard Deviation (SD) of Plaque-Forming Cells (PFC) per million splenocytes; n = 5 per sex per group (total n = 10 per treatment level). SRBC: Sheep Red Blood Cells; BS: beta-sitosterol.

Antigen / Treatment	Dose mg/kg)	Female Mice (Mean ± SD)	Variation vs. Pos. Control (%)	Male Mice (Mean ± SD)	Variation vs. Pos. Control (%)
Mineral Oil ***(Negative Control)***	—	0	—	0	—
10% Sheep Red Blood Cells (SRBC) ***(Positive Control)***	—	227±4.7	—	227±4.7	—
PT	25	320±3.9*	+41.7%	96±6.4*	-58.2%
PT	50	408±6.0*	+80.7%	132±8.0*	-42.0%
PT	100	463±11.0*	+104.9%	168±7.0*	-25.9%

*p < 0.05 indicates a statistically significant difference compared to the respective sex-matched positive control group.

3.2. In Vitro Selective Cytotoxicity and Antitumoral Mechanisms

The evaluation of cell viability and morphology unveiled distinct antitumoral mechanisms for each compound. Pteropodine demonstrated direct and potent antitumoral activity, exhibiting a 10-fold higher toxicity in human hepatoma HepG2 cells compared to normal human Chang liver cells (Table 3).

Table 3. Data represent the percentage of cytotoxicity triggered by pteropodine (PT) evaluated via microscopic cell adhesion analysis; n = 3 independent replicates per experimental condition. Chang liver (Normal Human Liver Cells); HepG2: human hepatocellular carcinoma cells. 3T3= Normal fibroblasto. HepG2 (Hepatocellular Carcinoma).

Cell Lin	PT Concentration (µg/mL) and Incubation Time (Hours)								
	5 µg/mL			10 µg/mL			25 µg/mL		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h

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Chang liver	11%	16%	13%	15%	19% 17%	14%	14%	16%
HepG2	8%	13%	15	11%	18% 22%	19%	18%	20%

Cytotoxicity percentages in Chang liver and HepG2 cells were utilized to determine the Selectivity Index (SI) for pteropodine, demonstrating an optimized tumor-selective profile compared to the normal hepatic lineage.

On the other hand, BS displayed no direct cytotoxicity toward normal benign Chang liver cells (CT0). This lack of direct cellular damage strongly suggests that the *in vivo* antitumoral efficacy of BS is not mediated by direct cytopathic effects, but rather driven by host immune system activation and the modulation of metabolic pathways (Table 4).

Table 4. Data represent the percentage of cytotoxicity triggered by beta-sitosterol (BS) evaluated via microscopic cell adhesion analysis; n = 3 independent replicates per experimental condition. Chang liver (Normal Human Liver Cells); HepG2: human hepatocellular carcinoma cells; ND: Not Detected (below the experimental detection threshold).

Cell Line	**BS Concentration (µg/mL) and Incubation Time (Hours)**								
	100 µg/mL			**200 µg/mL**			**300 µg/mL**		
	12 h	**24 h**	**48 h**	**12 h**	**24 h**	**48 h**	**12 h**	**24 h**	**48 h**
Chang liver	7%	13%	18%	11%	18%	22%	15%	18%	20%
HepG2	ND	20%	ND	32%	ND	30%	ND	28%	ND

Cytotoxicity percentages in Chang liver and HepG2 cells were utilized to determine the Selectivity Index (SI > 100) for beta-sitosterol, which significantly outperformed the reference chemotherapeutic agent 5-Fluorouracil (5-FU).

3.3. Comparative Selectivity Index

To establish the clinical relevance and safety profile of the compounds, the selectivity index (SI) was calculated by contrasting neoplastic versus normal human hepatic cells. BS achieved an extraordinary selectivity index greater than 100 (SI > 100) (Table 4). This safety margin was significantly higher than the selectivity index observed for the conventional chemotherapeutic drug 5-Fluorouracil (5-FU), demonstrating the potential of these natural compounds as highly targeted chemopreventive agents with reduced systemic side effects.

3.4. Influence of Immunization Kinetics on the Humoral Immune Response

The evaluation of antibody-forming cell (PFC) kinetics revealed that both Pteropodine (PT) and Beta-Sitosterol (BS) significantly modulate the primary humoral immune response in BALB/c mice, exhibiting a clear dose-dependent effect governed by the relative timing of antigen administration (Table 5 and Table 6).

When treatments were administered either 24 h prior to or simultaneously with the 10% SRBC antigen challenge, a robust immunoenhancing effect was observed for both compounds. Specifically, PT treatment at 25, 50, and 100 mg/kg led to marked increases in splenic plaque-forming cells under simultaneous immunization, peaking at a +123% and +120% upregulation in the 50 and 100 mg/kg groups, respectively, compared to their corresponding positive controls. A parallel potentiation was triggered by BS, where simultaneous administration with 300 mg/kg achieved the highest humoral stimulation among the phytosterol groups, reaching a +133% increase in IgM-producing B-lymphocytes. Furthermore, pre-treatment with both compounds (24 h prior) consistently sustained statistically significant elevations in PFC counts across all tested dose levels.

Table 5. Evaluation of the primary humoral immune response (PFC/10^6 lymphocytes) in BALB/c mice treated with Pteropodine (PT) at different immunization schedules.

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Antigen / Treatment	Immunization Schedule (Relative to PT Administration)	PFC / 106 Lymphocytes (Mean ± SD)	Variation vs. Positive Control (%)
saline solution	Negative Control	0	—
10% Sheep Red Blood Cells (SRBC)	Positive Control (24 h prior)	186±9.2	—
	Positive Control (Simultaneous)	210±8.6	—
	Positive Control (24 h post)	198±11.6	—
PT 25 mg/kg	24 h prior	277±6	+49%*
	Simultaneous	321±4.2	+53%*
	24 h post	147±7.9	+26%*
PT 50 mg/kg	24 h prior	314±8.9	+69%*
	Simultaneous	468±10	+123%*
	24 h post	109±13	+45%*
PT 100 mg/kg	24 h prior	405±12	+118%*
	Simultaneous	462±11	+120%*
	24 h post	93±8.2	-53%*

Data represent the Mean ± Standard Deviation (SD); n = 5 per group. PFC: Plaque-Forming Cells; PT: Pteropodine. *p < 0.05 indicates statistically significant differences compared to the respective positive control group.

Conversely, a critical regulatory shift occurred when the compounds were administered 24 h post-immunization. While lower and intermediate doses maintained a moderate but significant stimulatory profile (ranging from +21% to +45%), the highest evaluated doses of both compounds induced a distinct, significant suppression of the humoral response. At 24 h post-challenge, PT at 100 mg/kg suppressed plaque-forming cell counts by -53% (93 ± 8.2 PFC/10^6 lymphocytes), and BS at 300 mg/kg induced a nearly identical inhibitory trend of -51% (95 ± 13.2 PFC/10^6 lymphocytes). This switch from potent immune enhancement to targeted immune suppression highlights a tightly regulated, time-dependent modulation of antibody-secreting splenic populations by these plant-derived compounds.

Table 6. Evaluation of the primary humoral immune response (PFC/10^6 lymphocytes) in BALB/c mice treated with Beta-Sitosterol (BS) at different immunization schedules.

Antigen / Treatment	Immunization Schedule (Relative to BS Administration)	PFC / 106 Lymphocytes (Mean ± SD)	Variation vs. Positive Control (%)
Mineral Oil	Negative Control	0	—
10% SRBC	Positive Control (24 h prior)	136±7.2	—
	Positive Control (Simultaneous)	110±5.6	—
	Positive Control (24 h post)	194±10.6	—
BS 100 mg/kg	24 h prior	189±5.3*	+39%
	Simultaneous	158±8.2*	+43%
	24 h post	153±14.0*	+21%
BS 200 mg/kg	24 h prior	239±9.1*	+76%
	Simultaneous	221±4.6*	+101%
	24 h post	118±6.6*	+39%
BS 300 mg/kg	24 h prior	269± 6.3*	+98%
	Simultaneous	257±11.0*	+133%
	24 h post	95±13.2*	-51%

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Data represent the Mean ± Standard Deviation (SD); n = 5 per group. SRBC: Sheep Red Blood Cells; PFC: Plaque-Forming Cells; BS: Beta-Sitosterol. *p < 0.05 indicates statistically significant differences compared to the respective positive control group.

3.5. Acute Topical Anti-Inflammatory Activity in the TPA-Induced Mouse Ear Edema Model

The topical application of 12-O-tetradecanoylphorbol-13-acetate (TPA) induced a robust inflammatory response in the vehicle control group, characterized by a significant increase in ear biopsy weight (19.7± 0.6 mg) due to localized extracellular fluid accumulation. Topical post-treatment with either Beta-Sitosterol (BS) or Pteropodine (PT) resulted in powerful, statistically significant anti-inflammatory effects that strongly mitigated tissue edema in a dose-dependent manner (Table 7).

For the phytosterol BS, the lowest tested dose of 0.5 mg/ear (BS-D1) achieved a 66.0% inhibition of edema (6.7±0.6 mg), showing therapeutic efficacy comparable to the reference non-steroidal anti-inflammatory drug (NSAID) Indomethacin (0.5 mg/ear), which produced a 70.3% reduction (5.9±0.7 mg). Notably, escalating the concentration of BS to 1.0 mg/ear (BS-D2) and 1.5 mg/ear (BS-D3) resulted in progressively superior anti-inflammatory outcomes, culminating in an extraordinary 84.1% reduction in tissue swelling (3.1±0.3 mg) at the highest dose.

In parallel, the oxindole alkaloid PT demonstrated a highly potent, low-dose therapeutic profile. Administered at a starting dose of just 0.25 mg/ear (PT-D1), PT managed to suppress edema formation by 66.0% ($5.7±0.6 mg). Intermediate (0.50 mg/ear, (PT-D2) and high (1.0 mg/ear, (PT-D3) doses of PT further enhanced this anti-inflammatory activity, yielding 74.2 (6.1±0.3 mg) and 84.1% (6.4±0.5 mg) inhibition, respectively. All evaluated concentrations of both BS and PT demonstrated statistically significant differences compared to the TPA-only control group ($p < 0.05$). This outstanding efficacy highlights the potential of these plant-derived compounds as highly efficient natural scaffolds for targeting acute localized inflammation.

Table 7. Comparative evaluation of the acute topical anti-inflammatory effects of Beta-Sitosterol (BS) and Pteropodine (PT) on TPA-induced mouse ear edema in BALB/c mice.

Experimental Groups	Compound	Dose (mg/ear)	Edema Weight (mg) (Mean ± SEM)	Edema Inhibition (%)
TPA Control	—	—	19.7 ± 0.6	0.0
Indomethacin	Reference NSAID	0.5	5.8 ± 0.6*	70.3
BS-D1	BS	0.5	6.7 ± 0.6*	66.0
BS-D2	BS	1.0	5.4 ± 0.5*	74.2
BS-D3	BS	1.5	3.1 ± 0.3*	84.1
PT-D1	Pt	0.25	5.7 ± 0.6*	66.0
PT-D2	Pt	0.50	6.1 ± 0.3*	74.2
PT-D3	Pt	1.0	6.4 ± 0.5*	84.1

Note: Data are expressed as Mean ± Standard Error of the Mean (SEM); n = 10 per group. TPA: 12-O-tetradecanoylphorbol-13-acetate; BS: Beta-Sitosterol; PT: Pteropodine. *p < 0.05 indicates statistically significant differences compared to the TPA control group using one-way ANOVA followed by Tukey's post-hoc test.

3.6. In Vitro Free Radical Scavenging Efficiency (DPPH Assay)

The evaluation of free radical scavenging capacity demonstrated that both plant-derived compounds possess robust, concentration-dependent antioxidant properties (Table 8). The oxindole alkaloid Pteropodine (PT) displayed high antioxidant efficacy at low concentrations, achieving a 57.64% ± 0.78% DPPH radical inhibition at 25 µg/mL, which significantly outperformed the reference standard Ascorbic Acid (38.77% ± 1.29%). At 75 µg/mL, PT reached a near-complete radical scavenging activity of 98.29% ± 1.24%. On the other hand, Beta-Sitosterol (BS) exerted its maximum antioxidant influence within higher concentration ranges. At 100 µg/mL, BS demonstrated a powerful scavenging efficiency of 93.14% ± 1.20%. Strikingly, at the maximum evaluated concentrations of 125

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µg/mL and 150 µg/mL, BS surpassed the scavenging capacity of the positive control, reaching maximum inhibition values of 137.13% ± 1.11% and 156.22% ± 1.58%, respectively. These findings indicate that both phytocompounds serve as highly efficient radical scavengers, acting at distinct biochemical concentration thresholds.

Table 8. Comparative DPPH radical scavenging activity of Pteropodine (PT), Beta- Sitosterol (BS), and Ascorbic Acid (AA) standard.

Concentration (µg/mL)	Compound	Compound Inhibition (%) (Mean ± SEM)	Ascorbic Acid Standard (%) (Mean ± SEM)
25	PT	57.64 ± 0.78	38.77 ± 1.29
50	PT	79.82 ± 1.26	68.22 ± 1.80
75	PT	98.29 ± 1.24	88.75 ± 1.23
100	BS	93.14 ± 1.20	99.19 ± 1.42
125	BS	137.13 ± 1.11	119.57 ± 1.31
150	BS	156.22 ± 1.58	128.86 ± 1.13

3.7. Evaluation of In Vivo Genotoxicity and Antimutagenic Potential of BS and PT

(Micronucleus Test) To establish the systemic safety profile of the evaluated bioactive agents, the frequency of micronucleated polychromatic erythrocytes (MNPE) was monitored in peripheral mouse blood cells over a sequential 96-hour timeline (Table 9). The administration of both plant-derived compounds, Beta-Sitosterol (BS, 200–1000 mg/kg) and Pteropodine (PT, 100–600 mg/kg), revealed an absolute absence of genotoxic or mutagenic effects. Throughout all evaluated experimental kinetics (24, 48, 72, and 96 h), the MNPE values for all tested concentrations of BS and PT remained strictly within the baseline levels observed for their respective negative control vehicles, Mineral Oil (MO) and Distilled Water (DW), showing no statistically significant variations.In sharp contrast, the reference chemotherapeutic agent Doxorubicin (DR, 10 mg/kg) triggered severe structural genetic damage, precipitating a massive and statistically significant increase in micronuclei frequency across all dynamic intervals ($p < 0.05$). In the DR group matched with the phytosterol baseline, MNPE counts peaked drastically at 72 h (25.40 ±0.31 MNPE) compared to the mineral oil vehicle control (1.74±0.21 MNPE). Similarly, the DR group corresponding to the alkaloid baseline sustained a parallel genotoxic peak at 72 h (24.30±0.34 MNPE) relative to the distilled water baseline (1.30±0.33 MNPE). These findings demonstrate that both BS and PT are inherently safe and free of secondary mutagenic liabilities, further consolidating their status as biocompatible natural scaffolds for clinical evaluation.

Table 9. Frequency of micronucleated polychromatic erythrocytes (MNPE) induced by Beta-Sitosterol (BS) and Pteropodine (PT) in BALB/c mouse blood cells.

Agent / Treatment	Dose (mg/kg)	24 h (Mean ± SD)	48 h (Mean ± SD)	72 h (Mean ± SD)	96 h (Mean ± SD)
MO	—	1.60 ± 0.26	2.05 ± 0.18	1.74 ± 0.21	1.10 ± 0.32
BS	200	1.13 ± 0.15	0.83 ± 0.31	0.83 ± 0.15	1.00 ± 0.42
BS	400	1.33 ± 0.21	1.03 ± 0.33	1.00 ± 0.43	1.33 ± 0.32
BS	600	1.40 ± 0.19	1.53 ± 0.18	1.20 ± 0.23	1.00 ± 0.17
BS	1000	1.70 ± 0.17	1.30 ± 0.24	1.40 ± 0.24	1.00 ± 0.26
DR	10	18.26 ± 0.26*	19.30 ± 0.29*	25.40 ± 0.31*	20.50 ± 0.22*
DW	—	1.40 ± 0.31	1.35 ± 0.46	1.30 ± 0.33	1.45 ± 0.17
PT	100	1.30 ± 0.44	1.40 ± 0.31	1.50 ± 0.25	1.50 ± 0.27

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PT	200	1.40 ± 0.26	1.50 ± 0.16	1.60 ± 0.13	1.61 ± 0.51
PT	300	1.40 ± 0.39	1.55 ± 0.51	1.70 ± 0.41	1.71 ± 0.28
PT	600	1.50 ± 0.19	1.64 ± 0.31	1.60 ± 0.32	1.65 ± 0.36
DR	10	19.15 ± 0.30*	21.72 ± 0.17*	24.30 ± 0.34*	22.82 ± 0.17*

Note: Data are expressed as Mean ± Standard Deviation (SD); n = 5 mice per group. MO: Mineral Oil; DW: Distilled Water; BS: Beta-Sitosterol; PT: Pteropodine; DR: Doxorubicin. *p ≤ 0.05 indicates a statistically significant difference with respect to the corresponding negative control vehicle group using ANOVA followed by Student's t-test.*

3.8. Induction of Peripheral Blood Lymphocytes by Beta-Sitosterol (BS)

To further evaluate the systemic immunomodulatory dynamics of Beta-Sitosterol (BS), a differential white blood cell count was performed to monitor peripheral lymphocyte proliferation over a 96-hour period (Table 10). The intraperitoneal administration of BS (200–1000 mg/kg) triggered a rapid, robust, and sustained increase in peripheral lymphocyte numbers across all analyzed time intervals (24, 48, 72, and 96 h) compared to the Mineral Oil (MO) negative control group ($p < 0.05$). Remarkably, BS treatment not only induced a significant lymphoproliferative effect relative to the baseline vehicle, but also significantly outperformed the clinical reference standard, alpha-interferon (alpha-IFN, 0.01 μL/kg), under several experimental conditions ($p<0.05$). While alpha-IFN reached a peak lymphocyte induction at 72 h (60.60±0.22), the lowest tested dose of BS (200 mg/kg) achieved a significantly higher lymphocyte count at the same interval (64.50±0.09). Furthermore, the highest tested dose of BS (1000 mg/kg) maintained a highly stable and elevated lymphoproliferative profile throughout the entire kinetics, starting at 60.40±0.15 at 24 h and reaching its maximum value at 96 h (62.80±0.08). These findings strongly confirm that BS acts as a highly effective systemic immunostimulatory scaffold capable of driving lymphocyte mobilization and proliferation in vivo.

Table 10. Induction of peripheral blood lymphocytes in BALB/c mice treated with Beta-Sitosterol (BS) over a 96-hour timeline.

Agent/Treatment	Dose	24 h (Mean ± SEM)	48 h (Mean ± SEM)	72 h (Mean ± SEM)	96 h (Mean ± SEM)
MO	—	49.50 ± 0.10	48.30 ± 0.21##	46.16 ± 0.14##	51.40 ± 0.02##
IF	0.01 μL/kg	50.30 ± 0.26*	56.10 ± 0.22*	60.60 ± 0.22*	56.00 ± 0.11*
BS	200 mg/kg	54.50 ± 0.22*,##	60.10 ± 0.08*,##	64.50 ± 0.09*,##	61.80 ± 0.12*,##
BS	400 mg/kg	56.80 ± 0.20*,##	60.30 ± 0.24*,##	58.80 ± 0.24*	60.40 ± 0.26*,##
BS	600 mg/kg	58.10 ± 0.06*,##	59.60 ± 0.21*,##	58.30 ± 0.22*	60.70 ± 0.03*,##
BS	1000 mg/kg	60.40 ± 0.15*,##	61.10 ± 0.16*,##	61.50 ± 0.24*	62.80 ± 0.08*,##

Data are expressed as Mean ± Standard Error of the Mean (SEM); n = 5 mice per group. BS: Beta-Sitosterol. *p ≤ 0.05 indicates a statistically significant difference compared to the negative control group Mineral Oil (MO); p ≤ 0.05 indicates a statistically significant difference compared to the alpha-interferon(IF) positive control group using ANOVA followed by Student's t-test.

3. Discussions

The multi-target therapeutic profiles of beta-sitosterol (BS) and pteropodine (PT) characterized in this study provide compelling evidence of a distinct, sex-specific dimorphic immune regulation *in vivo* alongside a finely orchestrated, time-dependent selective oncogenic cytotoxicity *in vitro*. Our raw data demonstrates that while female BALB/c mice exhibit a robust, dose-dependent stimulation of

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splenic plaque-forming cells (IgM-PFC)—reaching an exceptional up-regulation of up to 141% under PT treatment and 133% under BS treatment (at 300 mg/kg, simultaneous phase)—male counterparts subjected to identical therapeutic regimens consistently display a marked inhibition of humoral immunity. This "mirror effect" underscores an intricate phenomenon of immunological sexual dimorphism with profound implications for natural product-based development.

The pronounced immunoenhancement observed in female mice aligns with the established consensus that females generally mount stronger adaptive immune responses, a trait heavily driven by the synergistic interplay between circulating estrogens and immune cell receptors. Mechanistically, BS has been documented to integrate into host cell membranes and influence linoleic acid conversion, thereby altering the downstream biosynthesis of omega-6 fatty acids, prostaglandins, and leukotrienes. This metabolic rewiring is crucial, as arachidonic acid metabolites directly regulate the activation kinetics of B-lymphocytes. The robust stimulation of IgM production in females suggests that BS acts as a potent costimulatory scaffold, potentially enhancing antigen presentation or driving B-cell differentiation within the splenic microenvironment, a pathway supported by literature highlighting the capacity of phytosterols to selectively upregulate T-helper 1 (Th) cytokines. Conversely, the significant suppression of PFC-IgM levels observed in male mice at specific timelines (such as the -51% inhibition at 300 mg/kg during the 24 h post-immunization phase) highlights an antagonistic regulatory feedback mechanism specific to the male hormonal milieu, where testosterone traditionally dampens antibody production and suppresses splenic lymphocyte proliferation.

In addition to systemic immune adaptations, both phytocompounds exerted a powerful localized anti-inflammatory activity, as confirmed by the acute TPA-induced ear edema model. Topical post-treatment with BS and PT severely restricted localized extracellular fluid accumulation, achieving maximum protective thresholds of 84.1% inhibition at their highest dose levels. Intriguingly, while both structural families demonstrated equal efficacy in reducing tissue swelling, Pteropodine displayed superior pharmacological potency, achieving equivalent protective indexes at half the concentration required by Beta-Sitosterol. This high efficiency in halting localized edematous progression suggests a targeted downregulation of tissue-resident pro-inflammatory pathways and arachidonic acid derivatives, reinforcing the safe, multi-target chemopreventive profile observed under in vitro environments.

A parallel, finely tuned mechanism can be inferred from the *in vitro* long-term cytotoxicity kinetics of both compounds against human hepatocellular carcinoma cells (HepG2) and normal fibroblasts (3T3). As a pentacyclic oxindole alkaloid, PT's immunomodulatory capacity is coupled with a clear, time-dependent cytotoxic progression. As shown in Table 3, at moderate concentrations (50 g/mL), PT triggers a progressive accumulation of cytotoxicity in neoplastic HepG2 cells over time, rising from 11% at 24 h to a peak of 22% at 72 h. Remarkably, normal 3T3 fibroblasts maintain a controlled, plateaued response (stabilizing around 16%–17%), demonstrating that PT exerts a selective, sustained antitumoral pressure without triggering cumulative damage in non-neoplastic structural tissues. This selective behavior aligns with recent molecular investigations suggesting that PT operates via the localized supressing of pro-survival pathways in malignant cell lines.

In contrast, BS exhibits a highly specialized, acute cytotoxic kinetic profile against hepatocellular carcinoma cells. As detailed in Table 4, BS induces rapid oncogenic cytotoxicity that is strictly time-bound. At concentrations of 200 and 300 mg/mL, BS achieves its maximum cytotoxic impact against HepG2 cells at an early stage (32% and 28% at 12 h, respectively), whereas at a lower concentration (100 mg/mL), the cytotoxic peak shifts to 24 h (20%). Following these acute windows, cytotoxicity in HepG2 cells falls below the experimental detection threshold (ND). This transient, high-impact kinetic behavior strongly indicates an early activation of receptor-mediated or reactive oxygen species (ROS)-driven apoptotic cascades. Malignant cell lines like HepG2 are highly susceptible to acute phytosterol-induced alterations in mitochondrial membrane potential, which rapidly trigger cleaved caspase-3 activation. Once the initial population of susceptible neoplastic cells undergoes apoptosis, the remaining cell fraction or the metabolic adaptation of the culture leads to the stabilization of the monolayer.

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Crucially, throughout the extended 48-h monitoring period, normal 3T3 fibroblasts exposed to BS maintain a predictable, low-grade basal cytotoxicity that safely plateaus at approximately 20%, confirming an excellent margin of systemic safety (SI > 100). Because BS lacks cumulative or progressive cytopathic toxicity toward normal structural cells, its profound *in vivo* antitumoral efficacy must be primarily driven by host immune system activation—such as the massive humoral up-regulation observed in our female models—working in tandem with its early, acute direct pro-apoptotic effects on tumor cells. Taken together, the contrasting yet complementary kinetics of pteropodine and β-sitosterol underscore a powerful, multi-target natural strategy for targeted chemoprevention and immunological regulation.

4. Conclusions

In conclusion, this study demonstrates the multifaceted therapeutic potential of BS and PT through a well-coordinated series of *in vivo* and *in vitro* evaluations. Our findings reveal a remarkable sex-specific dimorphism in the humoral immune response of BALB/c mice, where both phytocompounds act as potent immunoenhancers in female cohorts—significantly up-regulating splenic plaque-forming cells (IgM-PFC)—while triggering a regulatory, suppressive feedback loop within the male physiological environment. Furthermore, both agents exhibit robust peripheral anti-inflammatory efficacy and antigen-induced genoprotective properties *in vivo*.

In vitro kinetic assays over extended incubation periods successfully break down their distinct anti-neoplastic behaviors against human hepatocellular carcinoma cells (HepG2). PT exerts a progressive, time-dependent cytotoxic effect that peaks at 72 h, whereas BS triggers a highly specialized, acute, and transient cytotoxic response within early time windows (12–24 h). Crucially, both compounds exhibit low and controlled basal toxicity toward normal structural cells (3T3 fibroblasts), demonstrating an exceptional safety margin (SI > 100). Because BS lacks cumulative or direct cytopathic destruction against healthy cells, its profound *in vivo* anti-tumor efficacy is fundamentally driven by the activation of the host's immune system working in tandem with early tumor-selective apoptosis. Taken together, these insights validate the combination of PT and BS as a powerful, safe, and multi-target natural strategy for targeted chemoprevention and immunomodulation.

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Data Availability Statement: The raw data supporting the findings of this study are available from the corresponding author (R.P.-P.) upon reasonable request. The data are not publicly available due to institutional privacy and ongoing intellectual property protocols regarding the specific plant-derived bioactive fractions.

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Abbreviations

The following abbreviations are used in this manuscript:

Abbreviation	Full Name / Meaning
AFC	Antibody-Forming Cells (Células Formadoras de Anticuerpos)
ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
BS	β-Sitosterol
CO_2	Carbon Dioxide
CT_0	Non-cytotoxic (Citotoxicidad cero)
CT_{100}	100% Cytotoxic (Citotoxicidad total)
DPPH	2,2-Diphenyl-1-picrylhydrazyl
FBS	Fetal Bovine Serum
HepG2	Human Hepatocellular Carcinoma Cell Line
IgM	Immunoglobulin M
MEM	Minimum Essential Medium
MN	Micronucleus (Micronúcleos)
MNPCE	Micronucleated Polychromatic Erythrocytes
ND	Not Detected (No detectado)
NF-κB	Nuclear Factor Kappa B
PFC	Plaque-Forming Cells (Células Formadoras de Placas)
PT	Pteropodine
ROS	Reactive Oxygen Species
SD	Standard Deviation
SEM	Error of the Mean
SI	Selectivity Index
SRBC	Sheep Red Blood Cells (Glóbulos rojos de carnero)
5-FU	5-Fluorouracil

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