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RESEARCH ARTICLE

Development, Characterisation, and Pharmacological Assessment of *Eclipta alba* Phytosomes as Potential Anti-cancer formulation

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Abstract

Background: *Eclipta alba* leaf extract has demonstrated promising anticancer potential; however, its clinical application is limited by poor bioavailability and rapid metabolism. **Aim:** This study aimed to enhance the therapeutic efficacy and bioavailability of *Eclipta alba* leaf extract by encapsulating it within phytosomes and evaluating its antiproliferative effects on 4T1 breast cancer cells, as well as its safety *in vivo*. **Methods:** Phytosomes were synthesised using the nanoprecipitation technique and characterised by dialysis membrane studies and Dynamic Light Scattering (DLS). Key parameters such as particle size, zeta potential, Polydispersity Index (PDI), encapsulation efficiency, and total phenolic content were evaluated. Drug release profiles were assessed, and *in vitro* cytotoxicity and antiproliferative activity were determined using the MTT assay. Acute toxicity was assessed in Swiss albino mice according to OECD guidelines. **Results:** The prepared phytosomes showed a particle size of 296 ± 0.29 nm, zeta potential of −40.1 ± 1.19 mV, and a PDI of 0.106 ± 0.002, indicating good stability and uniformity. The encapsulation efficiency was 90.32 ± 0.11%, and the total phenolic content was 50.81 ± 0.02 mg GAE/g. Drug release exhibited a biphasic pattern with sustained release following an initial burst. Phytosomes displayed higher antiproliferative activity (IC_{50} = 7.73 ± 2.87 µg/mL, selectivity index > 3) against 4T1 cells compared to both the free extract (IC_{50} = 212.9 ± 1.30 µg/mL) and doxorubicin (IC_{50} = 68.35 ± 3.508 µg/mL), with statistically significant results ($p<0.001$). Acute toxicity studies confirmed safety up to 2000 mg/kg. **Conclusion:** Phytosomes loaded with *Eclipta alba* leaf extract significantly enhance therapeutic efficacy and show a favourable safety profile, suggesting their strong potential as a novel drug delivery system for breast cancer treatment.

Major Findings: Phytosomes loaded with *Eclipta alba* leaf extract demonstrated enhanced antiproliferative activity and reduced cytotoxicity against 4T1 breast cancer cells compared to the free extract and doxorubicin. They were well tolerated *in vivo* at doses below 2000 mg/kg, indicating their potential as a safe and effective drug delivery system for breast cancer therapy.

Keywords: Breast Cancer, Drug Delivery, 4T1 Cells, MTT Assay, Nanoprecipitation, Phytosomes, Polyphenols

1. Introduction

As the most common cancer killer of women, breast cancer poses a serious threat to public health around the world[1,2]. Worldwide, developing nations bore a disproportionate share of the burden in 2020, with a projected 2.3 million cases and 685,000 fatalities[1,2]. Due to limited access to early identification and treatment, these nations experience a higher mortality-to-incidence ratio[3]. Factors include shifting reproductive patterns, lifestyle changes, and longer life expectancy are associated with the dramatic rise in breast cancer cases in developing countries[4]. Current adjuvant treatments, including chemotherapy, immunotherapy, and radiation therapy, while effective, often present adverse side effects, can lead to drug resistance, and pose a significant financial burden, particularly in resource-constrained settings[5,6]. This underscores the urgent need for alternative, efficacious, and affordable therapeutic strategies. Plant-derived compounds offer

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a promising avenue in this pursuit, often exhibiting fewer side effects at appropriate dosages. The successful utilisation of plant-based agents like Docetaxel, derived from *Taxus baccata*, in existing cancer treatments further supports this approach.

Bhringaraj, or *Eclipta alba*, has shown encouraging anticancer effects in a number of preclinical investigations[7,8]. *Eclipta alba* contains a number of bioactive substances, including apigenin, wedelolactone, ursolic acid, oleanolic acid, luteolin, and eclalbasaponins[8]. The plant's anticancer effects may be enhanced by these chemicals. Scientists have discovered that *Eclipta alba* extracts can stop the growth of many cancer cell lines, such as colon cancer[9] and breast cancer[7]. Two of the anticancer strategies that have been seen involve preventing angiogenesis, the process by which tumours acquire new blood arteries, and promoting apoptosis, a process by which cancer cells undergo programmed cell death[7]. Researchers saw no harmful side effects from using an alcoholic extract of *Eclipta alba*, which had strong antioxidant and anticancer benefits. The complete plant extract also showed anticancer effects on colon cancer cells *in vitro*, according to another research[9].

Nanomedicine, the application of nanotechnology to medicine, offers innovative approaches to disease diagnosis, treatment, and prevention[10]. In cancer therapy, nanomedicine aims to enhance the efficacy and delivery of anticancer agents, mitigating limitations of conventional treatments[11,12]. Nanoparticles, engineered structures with dimensions between 1 and 100 nanometers, can be designed to act as drug delivery vehicles[13,14]. One such example is the use of phytosomes, a type of hybrid nanostructure, to encapsulate and deliver bioactive compounds, enhancing their bioavailability and therapeutic impact[15,16]. Phytosomes represent a promising strategy within cancer nanomedicine for improving the delivery and effectiveness of anticancer agents[17,18]. They can be engineered to passively target tumours through the enhanced permeability and retention effect, where nanoparticles accumulate preferentially in tumour tissues due to leaky vasculature[19]. Furthermore, nanomedicine approaches can be tailored to actively target cancer cells by conjugating nanoparticles with targeting moieties like antibodies or peptides[10,20].

Phytosomes, a novel drug delivery system, offer a promising approach to improve the bioavailability and efficacy of plant extracts[21]. They are created by binding standardised plant extracts to phospholipids, such as phosphatidylcholine, resulting in lipophilic complexes that can easily cross lipid-rich biological membranes. This enhanced lipophilicity improves the absorption and bioavailability of the active plant constituents. Phytosomes offer several advantages, including enhanced absorption and bioavailability, improved drug delivery to target tissues, protection of the drug from degradation, and reduced toxicity.

The anticancer potential of *Eclipta alba*, combined with the enhanced delivery capabilities of phytosomes, presents a novel approach to cancer therapy.

2. Materials and Methods

High-purity dichloromethane (99%) and ethanol were obtained from SD Fine Chemicals, Indore, while soy phosphatidylcholine was obtained from Qualigens Chemicals. Sigma-Aldrich, Mumbai, supplied the MTT reagent and Trizol reagent. The 4T1 murine mammary carcinoma and Vero cell lines were purchased from the CCMB, Pune. Cell culture media and supplements, including Dulbecco's Modified Eagle Medium, 10% fetal bovine serum, 1% L-glutamine, and 1% antibiotic solution, were all acquired from Rankem, India.

2.1 Preparation of Extract

Eclipta alba leaves, procured from Shobhasavi Ayurvedics and Agros, Bhopal, India, were authenticated by the Department of Botany, Shri Krishna University, and a voucher specimen (AN/2008-2009/04) was deposited for reference. Following seven days of air-drying, the leaves were pulverised. A microwave-assisted extraction technique was then employed for efficient extraction of bioactive compounds from the powdered plant material[22]. Briefly, 10 g of powdered leaves were subjected to microwave irradiation in 100 mL of ethanol for 10 min at 60°C, with a power level of 400 W.

2.2 Phytosome Preparation

Eclipta alba phytosomes were prepared using a thin-layer hydration technique[23]. Soy phosphatidylcholine was dissolved in dichloromethane, while the *Eclipta*

alba extract was dissolved in 90% ethanol (Table 1). These two solutions were combined in a round-bottom flask and sonicated for 10 min to ensure thorough mixing and facilitate complex formation. The resulting mixture was then subjected to solvent evaporation using a Büchi Mini Spray Dryer B-290. Nitrogen gas flow, controlled by the spray dryer at specific inlet and outlet temperatures (100°C) and pump/aspirator rates, facilitated the removal of the organic solvents. This process resulted in the formation of a thin, uniform film of the *Eclipta alba* phytosome complex. The thin-layer hydration method is preferred for its simplicity and efficiency in producing homogenous phytosome complexes[23].

3. Characterisation of *Eclipta alba* Phytosomes

3.1 Percentage Entrapment Efficiency

Eclipta alba leaf extract luteolin flavonoids' entrapment effectiveness (% EE) within the phytosome complex was ascertained by means of ultracentrifugation[24]. A 90-min centrifugation run at 4°C using 15,000 rpm was performed on the phytosome preparation. The supernatant containing unencapsulated luteolin was collected after centrifugation and subjected to UV spectrophotometry at 340 nm to determine the luteolin flavonoid content. This method allowed for the quantification of free luteolin and, subsequently, the calculation of % EE[25].

3.2 Zeta Potential, Polydispersity Index, and Particle Size Distribution

The dynamic light scattering analysis was performed using a Malvern Zetasizer Nano system to determine the particle size distribution, zeta potential, and polydispersity index of the phytosome complex[26,27].

Table 1. Composition of phytosome formulation of *Eclipta alba* extract

Ingredients	Amount used
Soy phosphatidylcholine	1000 mg
Eclipta alba extract	500 mg
Dichloromethane	25 ml
Ethanol (90%)	100 ml
Distilled water	100 ml

The DLS measurements were conducted using the following parameters: conditions: temperature, wavelength, scattering angle, refractive index, viscosity, and temperature (25°C)[28].

3.3 Fourier Transform Infrared Spectroscopy

Fourier-transform infrared spectroscopy was employed to analyse the *Eclipta alba* leaf extract-phytosomes complex, providing insights into its chemical stability and structure[29,30]. A JASCO 4700 ATR-FT/IR instrument was used, scanning over the wavenumber range of 400-4400 cm^{-1}. To minimise interference from moisture, freshly prepared potassium bromide pellets were used. The sample (0.5 mL) was mixed with the KBr and pressed into a pellet, which was then analysed.

3.4 *In Vitro* Drug Release of Luteolin from Prepared Phytosomes

Eclipta alba phytosomes were tested for their *in vitro* drug release characteristics using a modified dynamic dialysis approach. The release medium was a 0.02 M phosphate-buffered saline solution with a pH of 7.4, and the dialysis bag had a molecular weight cutoff of 4000 Da. In the dialysis bag, 10 mg of phytosomes were mixed with 2 mL of PBS. After that, the bag was placed in an Erlenmeyer flask with 100 mL of PBS, kept at 37°C, and swirled at 100 rpm. The complete release medium was removed and replaced with new PBS at prearranged intervals of 0, 8, 12, 24, and 72 h. A 0.45 **µm** Millipore filter was used to filter the obtained sample. The amount of released luteolin flavonoid in the filtrate was determined by spectrophotometer at 340 nm, with fresh PBS serving as a reference. Several kinetic models were used to match the data, including zero-order, first-order, Higuchi, and Korsmeyer-Peppas, and the cumulative proportion of polyphenol release was plotted against time[31].

3.5 Cell Viability Test

The MTT assay was used to investigate the cytotoxic activity of *Eclipta alba* phytosomes against both the 4T1 murine mammary cancer and the Vero (E6) cell lines[32,33]. In a nutshell, cells that were multiplying exponentially were trypsinised, counted by trypan blue exclusion, and then seeded in 96-well microtiter plates with 100 **µL** of minimum necessary media + 10% fetal bovine serum at a density of 1×10^6 cells/mL. Cells were

treated with *Eclipta alba* phytosomes with increasing concentrations (10-100 µg/mL) after incubating them for 24 h at 37°C in a humidified environment with 5% CO_2. The wells were supplemented three times with progressively diluted phytosomes, beginning at a concentration of 100 **µL**. The plates were incubated at 37°C for an extra 2 h after being incubated for 72 h, after which 50 **µL** of MTT dye was added to every well. To dissolve the formazan crystals produced by live cells, 100 **µL** of dimethyl sulfoxide was added later. Utilising a Thermo Fisher Multiskan Go Spectrophotometer, the absorbance was ascertained at 570 nm. As a positive control, doxorubicin was used. The term CC_{50}, which stands for the concentration that causes 50% cell death, was used to express the cytotoxicity compared to the untreated cells (the negative control). The IC_{50} of *Eclipta alba* phytosomes against 4T1 cells was calculated with the help of GraphPad Prism 6. Every experiment was carried out three times.

3.6 *In Vivo* Acute Toxicity Study

Female Swiss albino mice weighing 18-22 g and kept in a controlled environment with a temperature of 25°C and lighting provided by the PBRI animal house were utilised in this study. The mice were allowed free access to food and drink. Each of the nine groups consisted of six mice per group, who were identified by their unique tail marks. Following OECD recommendations with minor adjustments, the acute toxicity research was carried out[34,35]. The mice were given a single oral dosage of the test drug in PBS at concentrations of 50, 300, or 2000 mg/kg body weight, administered at a volume of 0.2 mL, after a 3-h fasting period during which they were given water as they needed it. The first five hours of each observation were devoted to tracking general behaviour, changes in body weight, and death; subsequent assessments were performed daily for the duration of the study. The following parameters were included in these observations: death rate, symptoms of acute toxicity, behavioural changes (such as aggression, paralysis, vocalisation, restlessness, agitation, convulsions, ataxia, diarrhoea, piloerection, catatonia, altered locomotion, grooming, fasciculation, sleep patterns, coma, prostration, hypoxia, hyperactivity)[36]. Days 1, 7, and 14 were the days when body weights were recorded. After 14 days of observation, the animals were put to sleep, and a necropsy was conducted. We looked for obvious problems in the brain, ovaries, liver, kidneys, spleen, lungs, and intestines[37,38].

4. Results and Discussion

The Soxhlet extraction method produced a dark brown extract of *Eclipta alba* with a notable yield of 12.65% w/w. This relatively high extraction efficiency is attributed to the continuous solvent circulation inherent to the Soxhlet system, which ensures sustained contact between the fresh solvent and plant material, thereby facilitating the exhaustive extraction of bioactive phytoconstituents. The concentrated extract obtained was subsequently utilised for the formulation of *Eclipta alba* phytosomes, aimed at enhancing the bioavailability and therapeutic efficacy of the active components. The prepared phytosomal formulations were further subjected to comprehensive physicochemical characterisation, including evaluation of particle size, zeta potential, entrapment efficiency, drug content, and *in vitro* release behaviour.

4.1 Percentage Encapsulation Efficiency

The percentage encapsulation efficiency (% EE) of luteolin flavonoid within the phytosome complex was a critical parameter indicative of the success of the encapsulation process and the stability of the phytosome formulation[39,40]. This study demonstrated a high encapsulation efficiency (88.25±2.3%), suggesting that the phytosomes effectively entrapped luteolin, protecting it from degradation and enhancing its bioavailability.

4.2 Zeta Potential, Polydispersity Index, and Particle Size Distribution

The hydrodynamic diameter of *Eclipta alba* phytosomes, obtained from five DLS measurements, ranged from 137.6 ± 1.47 nm to 299 ± 0.16 nm (Figure 1). This size range falls within the microsphere category (<500 nm). The polydispersity index, ranging from 0.116 ± 0.003 to 0.208 ± 0.015, indicates a homogeneous particle size distribution, consistent with the criterion for optimal homogeneity (PDI<0.5)[41].

The zeta potential is a crucial indicator of colloidal system stability, including phytosomes. A zeta potential value of -41.1 ± 1.21 mV for *Eclipta alba* phytosomes suggests good physical stability due to the strong

		Size (d.nm):	% Intensity:	St Dev (d.nm):
Z-Average (d.nm): 299.0	Peak 1:	327.8	100.0	102.4
PdI: 0.116	Peak 2:	0.000	0.0	0.000
Intercept: 0.901	Peak 3:	0.000	0.0	0.000
Result quality : Good				

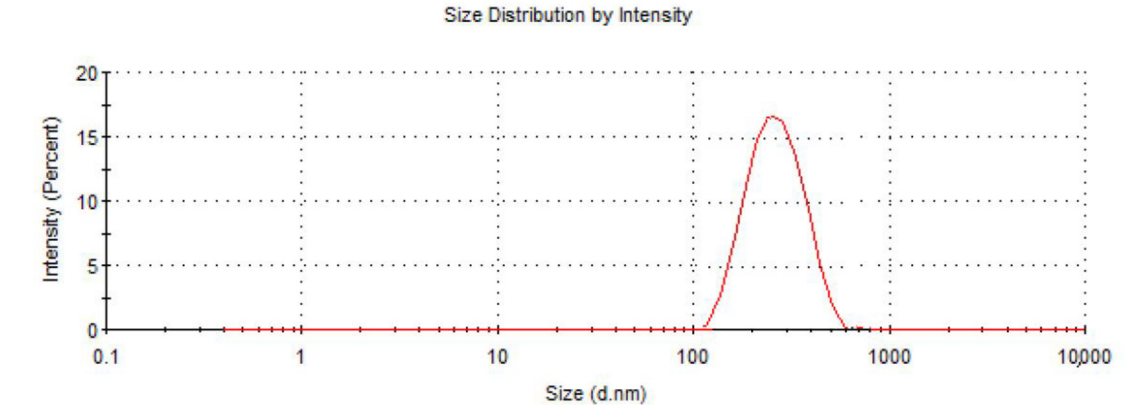


Figure 1. The mean particle size and PDI of optimised *Eclipta alba* phytosomes.

		Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV): -41.1	Peak 1:	-41.1	100.0	7.35
Zeta Deviation (mV): 7.38	Peak 2:	-33.6	45.2	4.12
Conductivity (mS/cm): 0.036	Peak 3:	0.00	0.0	0.00
Result quality : Good				

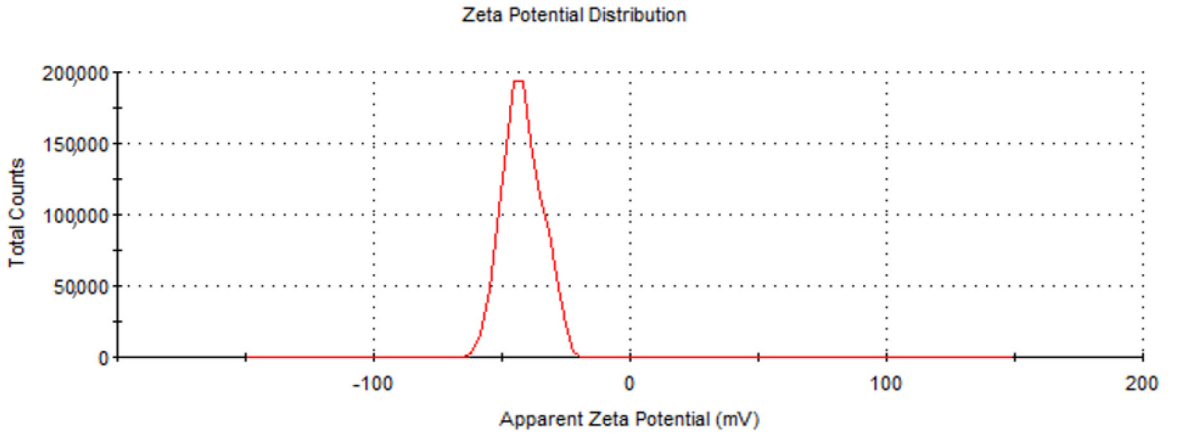


Figure 2. Zeta potential of optimised *Eclipta alba* phytosomes.

electrostatic repulsion between particles, preventing aggregation (Figure 2)[42-44]. Values above ±30 mV generally indicate good stability[45-47], while values closer to zero increase the risk of aggregation[48,49]. This repulsion arises from the negative surface charge of the phytosomes.

4.3 Fourier Transform Infrared Spectroscopy

To explore possible interactions between luteolin and phospholipids in the *Eclipta alba* phytosome complex, Fourier-transform infrared spectroscopy was used. The 400-4000 cm^{-1} region of the infrared spectra

showed important functional groups of luteolin, phosphatidylcholine, and the phytosome complex. The aliphatic alcoholic (-OH) group, which is characteristic of flavonoids, is accompanied by a wide peak at 3281 cm^{-1}. Indicative of unsaturated molecules often present in fatty acids, the shielding of peaks at 1622 cm^{-1} (C=C) implies that phytosome production was successful. Additional evidence for phytosome production can be found in the long-chain bands of the phospholipid molecule at 2960, 2924, and 2861 cm^{-1}. The stretching vibration of the C-N bonds in the phytosomes' choline moiety is responsible for the band at 1260 cm^{-1}. The successful synthesis of the complex is indicated by the presence of the spectral band at 1025 cm^{-1}, which is related with the symmetric stretching of the C-O-C vibration found in phospholipids and flavonoids. The presence of hydrophobic and electrostatic contacts between phospholipids and polyphenols, as indicated by the absorption at 1616 cm^{-1}, verifies the development of phytosomes[50,51].

4.4 *In Vitro* Drug Release Study

The improved phytosome formulation exhibited controlled and sustained release of medication properties in *in vitro* drug release profiles. As a result of the medication being trapped on the surface, the formulation showed a rapid release of 44.32% of the drug within the first 8 h[52-54]. Figure 3 shows that the following release was slower, gradually increasing to 73.98% over the following 24 h. Diffusion of the medicine from inside the phytosome complex is responsible for this prolonged release[55-57]. The release data was most closely matched by the Korsmeyer-Peppas model (R^2 = 0.9306) among the kinetic models that were tested. Consistent with previous findings and typical of controlled-release systems, this points to the presence of erosion and diffusion as governing processes for drug release[58,59].

4.5 Cytotoxicity Studies

Cytotoxicity studies revealed that both *Eclipta alba* leaf extract and *Eclipta alba* phytosomes exhibited CC_{50} values greater than 90 **µg**/mL, indicating lower toxicity compared to doxorubicin. The CC_{50} values for the Vero cell line ranged from 68.35 ± 3.51 to 212.9 ± 1.30 **µg**/mL. The inhibitory concentrations (IC_{50}) were determined as 3.04 ± 0.27 **µg**/mL for doxorubicin, 7.73 ± 2.87 **µg**/mL for *Eclipta alba* phytosome complex, and 39.84 ± 0.10 **µg**/mL for the free extract. The selectivity index, calculated as the ratio of CC_{50} to IC_{50}, was found to be 2.07 ± 1.30 for doxorubicin, 12.79 ± 0.50 for *Eclipta alba* phytosomes, and 5.34 ± 0.13 for the free extract. A higher SI indicates a greater safety margin, suggesting that *Eclipta alba* phytosomes exhibit a better safety profile compared to both doxorubicin and the free extract[34].

The results indicate that *Eclipta alba* phytosomes demonstrate selective cytotoxicity against breast cancer cells (4T1) while exhibiting lower toxicity toward normal cells compared to free polyphenols and doxorubicin[60-62]. Doxorubicin, with a selectivity index ≤ 3, showed toxicity to 4T1 cells. At low concentrations

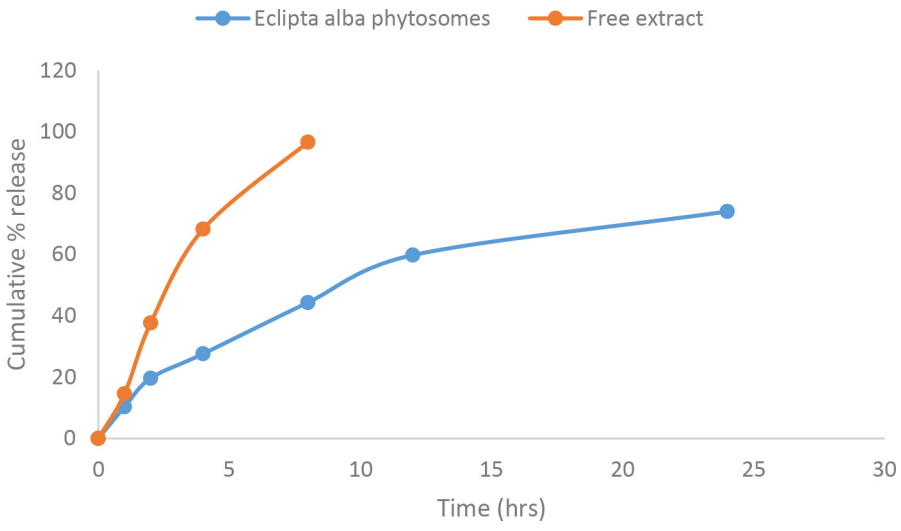


Figure 3. *In vitro* drug release profile of *Eclipta alba* phytosomes.

(0.14 ***µg***/mL), *Eclipta alba* phytosomes had a limited effect on cell growth, but at higher doses (0.41-100 ***µg***/mL), the inhibitory effect on 4T1 cell proliferation increased with increasing dose. Although *Eclipta alba* phytosomes did not have the same level of activity as the positive control, there was a notable disparity in the cytotoxicity of the extract and *Eclipta alba* ($p<0.05$)[63,64]. Due to their dose-dependent effect and selective cytotoxicity, *Eclipta alba* phytosomes show promise as a targeted treatment for breast cancer.

4.6 *In Vivo* Toxicity Studies

During a 14-day observation period, Swiss albino mice that were given oral doses of *Eclipta alba* phytosomes and free extract up to 2000 mg/kg did not show any symptoms of acute toxicity or mortality[65]. Nobody noticed any changes to their weight or any abnormalities in the organs that were autopsied. This safety profile in living organisms is consistent with research showing that *Eclipta alba* phytosomes do not harm normal cells *in vitro*[66-70]. These results provide credence to the idea that oral dosages of *Eclipta alba* phytosomes up to 2000 mg/kg are safe (Table 2). Positive findings from this study suggest that *Eclipta alba* phytosomes may have therapeutic potential; however, this is the first report on their acute toxicity in Swiss albino mice.

Acute toxicity studies are critical in preclinical research to assess the safety margin of new therapeutic agents, and they serve as a preliminary guide for dose selection in further pharmacological and toxicological studies. Following OECD guidelines (with slight modifications), mice were administered single oral doses of 50, 300, and 2000 mg/kg body weight of either the free extract or phytosomal formulation of *Eclipta alba*. These doses were selected in accordance with internationally accepted toxicity screening protocols, particularly OECD Test Guideline 423, which is designed to classify substances based on their toxicological profile following acute exposure.

Throughout the 14-day observation period, no mortality or significant clinical signs of toxicity were observed in any group, including those administered the highest dose of 2000 mg/kg. The behavioural parameters monitored included a wide spectrum of neurological and physiological signs—such as aggressiveness, tremors, convulsions, vocalisation, ataxia, diarrhoea, and altered sleep or grooming patterns—and none were exhibited by the treated animals. This absence of adverse effects strongly indicates the non-toxic nature of both the free extract and phytosomal form of *Eclipta alba* under the experimental conditions.

Table 2. Acute oral toxicity assessment of *Eclipta alba* phytosomes and free extract in female Swiss Albino mice

Group	Treatment	Dose (mg/kg, p.o.)	Number of mice	Mortality	Behavioural/ toxicity signs	Body weight changes	Gross organ abnormalities (Day 14)
G1	Control (PBS)	0	6	0/6	None observed	No significant change	None
G2	Free extract of *Eclipta alba*	50	6	0/6	None observed	No significant change	None
G3	Free extract of *Eclipta alba*	300	6	0/6	None observed	No significant change	None
G4	Free extract of *Eclipta alba*	2000	6	0/6	None observed	No significant change	None
G5	*Eclipta alba* Phytosomes	50	6	0/6	None observed	No significant change	None
G6	*Eclipta alba* Phytosomes	300	6	0/6	None observed	No significant change	None
G7	*Eclipta alba* Phytosomes	2000	6	0/6	None observed	No significant change	None
G8	Vehicle Control (PBS, fasting)	0	6	0/6	None observed	No significant change	None
G9	Historical control (unfasted, PBS)	0	6	0/6	None observed	No significant change	None

Additionally, no significant changes in body weight were recorded across all treatment groups, suggesting that the test substances did not interfere with normal metabolic functions or general health status of the mice. This is an important observation, as body weight changes often reflect underlying systemic toxicity. Necropsy performed at the end of the study revealed no visible abnormalities in major organs including the liver, kidneys, spleen, lungs, brain, intestines, or ovaries. These findings further reinforce the non-toxic nature of the phytosomal and free extract formulations at the administered doses. The lack of organ-specific toxicity is particularly relevant given that phytoconstituents are often metabolised in the liver and kidneys—organs typically most vulnerable to toxic insult.

5. Conclusion

The development of *Eclipta alba* phytosomes addresses the limitations of conventional herbal extracts by enhancing solubility, bioavailability, and stability, thereby improving therapeutic efficacy. Comprehensive *in vitro* and *in vivo* studies have confirmed the safety and efficacy of *Eclipta alba* phytosomes, demonstrating their potential as a promising therapeutic agent. The absence of acute toxicity and adverse effects at high doses further strengthens their potential for clinical translation. Future research should focus on long-term toxicity studies, detailed mechanistic investigations, and clinical trials to fully realise the therapeutic benefits of *Eclipta alba* phytosomes.

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7. Author Contribution

Conceptualisation and project execution: Vivek Daniel; Initial draft preparation, review, and editing: Prachi Maheshwari. All authors have read and agreed to the published version of the manuscript.

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