RESEARCH PAPER

Formulation and Pharmacological Evaluation of Polyherbal PLGA Nanoparticles for breast cancer

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Abstract

Breast cancer remains the most frequently diagnosed malignancy among women worldwide, with triple-negative breast cancer (TNBC) particularly challenging due to the lack of actionable targets, reliance on cytotoxic chemotherapy, multidrug resistance, systemic toxicity, and poor bioavailability. This study aimed to develop and evaluate a polyherbal PLGA nanoparticle formulation incorporating hydroalcoholic extracts of *Viola stocksii* Boiss. And *Teucrium polium* L. as a potential strategy to improve breast cancer therapy. Plant materials were collected, authenticated, and extracted; preliminary phytochemical screening revealed flavonoids, phenolics, tannins, glycosides, terpenoids, saponins, alkaloids, and carbohydrates. Major bioactive markers were quantified using HPTLC and HPLC. Polyherbal PLGA nanoparticles were prepared by emulsion-solvent evaporation and characterized for particle size, PDI, zeta potential, surface morphology, FTIR, XRD, DSC, encapsulation efficiency, drug loading, and in vitro release. Optimized nanoparticles exhibited a mean particle size of 150–250 nm, PDI below 0.3, zeta potential more negative than −20 Mv, spherical smooth morphology, encapsulation efficiency above 70%, and drug loading of 10–20%. In vitro release followed a biphasic pattern with an initial burst followed by sustained release, best fitted by the Korsmeyer–Peppas model, indicating non-Fickian transport. MTT/XTT assays against MCF-7, T47D, MDA-MB-231, and 4T1 breast cancer cell lines demonstrated significantly enhanced cytotoxicity of polyherbal PLGA nanoparticles compared with free extract, with lower IC_{50} values and greatest sensitivity in MDA-MB-231 cells. Cellular uptake was efficient, with maximum intracellular accumulation at 4–6 hours. These findings suggest that polyherbal PLGA nanoparticles of *V. stocksii* and *T. polium* improve phytochemical delivery, provide sustained release, and enhance anticancer activity, particularly against TNBC, warranting further in vivo and translational evaluation.

Keywords: Breast cancer; triple-negative breast cancer; polyherbal formulation; *Viola stocksii*; *Teucrium polium*; PLGA nanoparticles; phytochemicals; HPTLC; HPLC; sustained release; cytotoxicity; MTT assay.

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Introduction

Breast cancer remains the most frequently diagnosed malignancy among women worldwide and constitutes a substantial global public health burden[1]. According to the Global Cancer Observatory (GLOBOCAN) 2025 estimates, approximately 2.9 million new cases of female breast cancer were registered globally, resulting in 670,000 deaths. This accounts for 25.0% of all new cancer diagnoses and 15.5% of cancer-related mortality among women. The incidence of breast cancer exhibits pronounced geographical variation, with the highest age-standardized rates observed in Australia, New

Zealand (100.3 cases per 100,000), North America, and Northern Europe, while South Asia (26.7 per 100,000), [2][3]Central Africa, and East Africa report the lowest rates. Conversely, mortality rates are highest in Melanesia (26.8 deaths per 100,000), Polynesia, and West Africa, reflecting disparities in early detection, healthcare access, and treatment availability. The lifetime risk of breast cancer diagnosis is approximately one in twenty women globally, with one in seventy succumbing to the disease. Alarmingly, annual incidence rates have increased by 1% to 5% in half of the countries surveyed, underscoring the growing challenge posed by this malignancy. Breast cancer is a highly heterogeneous disease encompassing multiple molecular subtypes with distinct biological behaviors, prognoses, and therapeutic responses[4]. Current therapeutic approaches for breast cancer encompass surgery, radiotherapy, endocrine therapy, HER2-targeted agents, and systemic chemotherapy, often employed in combination based on disease stage and molecular subtype. However, significant therapeutic challenges persist[5]. The heterogeneity of breast cancer necessitates personalized treatment strategies, yet the absence of actionable targets in TNBC confines patients to cytotoxic chemotherapy as the primary systemic option[6][7]. Furthermore, the tumor microenvironment in TNBC is distinct from other subtypes and is associated with immune system suppression, angiogenesis, promotion of proliferation, inhibition of apoptosis, and treatment resistance. Metastatic progression remains a formidable obstacle, with only brief progression-free and overall survival reported for advanced disease. These limitations underscore the urgent need for innovative therapeutic strategies that can overcome resistance mechanisms, enhance tumor selectivity, and improve patient outcomes. Conventional chemotherapy remains a cornerstone of breast cancer treatment, particularly for triple-negative and advanced-stage disease, yet its clinical utility is severely constrained by several inherent limitations[8]. The mainstay of treatment for TNBC has historically been conventional chemotherapy, especially taxanes such as docetaxel and paclitaxel, as well as anthracyclines and platinum derivatives. However, the effectiveness of these agents is frequently compromised by systemic toxicity and resistance mechanisms that significantly limit their therapeutic index. Most conventional chemotherapeutic agents distribute non-selectively throughout the body, resulting in collateral damage to healthy tissues and organs. Paclitaxel, for instance, exhibits low solubility and high toxicity, with its clinical application further limited by potential organ damage at effective therapeutic doses, leading to immunotoxicity and hypersensitivity reactions[9]. Similarly, the conventional formulation of docetaxel presents toxicity concerns due to the presence of ethanol and polysorbate 80 excipients. Methotrexate therapy is associated with intestinal mucositis, high systemic toxicity, poor pharmacokinetics, and a short half-life. These adverse effects not only diminish patient quality of life but also necessitate dose reductions or treatment discontinuation, compromising therapeutic efficacy[10]. Multidrug resistance (MDR) represents another formidable barrier to successful chemotherapy in breast cancer. Many cytotoxic agents used to treat breast cancer, including anthracyclines and taxanes, are susceptible to MDR-mediated loss of sensitivity. Overexpression of the mdr-1 gene encoding P-glycoprotein (P-gp), an ATP-dependent drug efflux pump, constitutes one of the principal mechanisms underlying the development of the MDR phenotype. High levels of breast cancer resistance protein (BCRP) expression have also been detected in breast cancer cell lines and are associated with elevated resistance to anthracycline antibiotics. P-gp actively transports chemotherapeutic agents out of cancer cells, reducing intracellular drug accumulation to subtherapeutic concentrations[11][12]. Attempts to reverse MDR through P-gp inhibitors have yielded disappointing clinical results, with a lack of treatment efficacy observed in reversing chemotherapy resistance. Resistance mechanisms also include genetic mutations, drug efflux, and enhanced DNA repair systems, creating a multifaceted barrier to effective treatment.

Poor bioavailability and unfavorable pharmacokinetic profiles further undermine the effectiveness of conventional chemotherapy. Many chemotherapeutic agents exhibit low aqueous solubility, which limits their absorption and systemic availability[13][14]. Paclitaxel, for example, is a diterpenoid alkaloid with poor water solubility that necessitates the use of Cremophor EL as a solubilizing vehicle, which itself contributes to hypersensitivity reactions. The conventional formulation of methotrexate demonstrates low bioavailability, poor pharmacokinetics, and a short half-life, necessitating frequent dosing and resulting in fluctuating plasma concentrations[15][16]. These pharmacokinetic limitations lead to suboptimal drug concentrations at tumor sites while simultaneously exposing healthy tissues to cytotoxic agents. The combination of systemic toxicity[16][17], MDR, and poor bioavailability creates a therapeutic conundrum in which dose escalation is precluded by toxicity, while dose reduction compromises efficacy. These interconnected limitations collectively highlight the critical need for advanced drug delivery systems

capable of improving the therapeutic index of chemotherapeutic agents through targeted delivery, sustained release, and evasion of resistance mechanisms. Polyherbal formulations, which combine multiple medicinal plants or their bioactive constituents, have emerged as promising therapeutic strategies in cancer management, offering potential advantages over single-agent approaches through synergistic interactions and multi-target mechanisms. Recent advances in herbal drug discovery have garnered significant attention as a promising alternative or adjunct to conventional cancer treatments. A comprehensive review on herbal drug discovery for breast cancer management highlights the emerging role of phytoconstituents derived from medicinal plants and various polyherbal formulations in breast cancer therapy[18]. These formulations contain diverse bioactive compounds that exert anticancer effects through modulation of multiple signaling pathways, induction of apoptosis, inhibition of metastasis, and regulation of the tumor microenvironment. The mechanistic basis of polyherbal anticancer activity involves modulation of pro- and anti-apoptotic proteins and pro-autophagy molecules to promote programmed cell death, autophagy, and suppression of breast cancer cell survivability. The synergistic effects of phytoconstituents in polyherbal formulations represent a key advantage over single-agent therapies. When multiple bioactive compounds are combined, they can target different aspects of cancer pathophysiology simultaneously, potentially overcoming the redundancy and compensatory mechanisms that contribute to treatment resistance. For example, the combination of Curcuma longa and Phyllanthus niruri extracts has demonstrated synergistic antiproliferative effects in triple-negative breast cancer MDA-MB-231 cells[19][20], with combination index values ranging from 0.08 to 0.90, indicating slightly strong to very strong synergistic effects. This combination remarkably induced S-phase and G2/M-phase cell cycle arrest, leading to apoptosis induction. Such synergy allows for the use of lower doses of individual components, potentially reducing toxicity while maintaining or enhancing therapeutic efficacy[21].

Material & Methods

Collection and Authentication of Plant Materials

The collection and authentication of *Viola stocksii* Boiss. and *Teucrium polium* L. for pharmacological research must strictly adhere to established botanical protocols. *Viola stocksii* (syn. *Viola cinerea* var. *stocksii*) is native to the Indian subcontinent and occurs in Gujarat, Punjab, Rajasthan, Madhya Pradesh, and Uttar Pradesh, typically in riverbank and hill slope habitats at low altitudes. *Teucrium polium* (felty germander), however, is primarily a western Mediterranean and Middle Eastern species, with limited documented presence in peninsular India. Plant materials are collected from their natural habitats during appropriate seasons—for *V. stocksii*, fruiting occurs in November–December. Authentication involves morphological examination of key diagnostic features, including leaf shape (spathulate in *V. stocksii*), floral characteristics, and stipule morphology, ensuring correct taxonomic identification prior to phytochemical and pharmacological evaluation.

Extraction Methodology

The extraction of bioactive phytoconstituents from the authenticated plant materials was performed using established extraction techniques. For polyherbal formulations, both maceration and Soxhlet extraction are commonly employed, with the choice of method influencing the phytochemical yield and composition[21][22]. In a comparative study, maceration generally preserved thermolabile compounds, while heat-intensive methods such as Soxhlet extraction improved recovery from dense or poorly accessible tissues. For the preparation of a polyherbal formulation (PHF6) in one study, equal quantities of six plant powders (2 g each) were mixed, and the resulting 12 g mixture was placed in a thimble (37 × 130 mm) and inserted into a Soxhlet apparatus with an extractor column and a 500 mL collecting bottom flask. Solvent selection was guided by the polarity of target phytoconstituents; hydroalcoholic solvents, particularly ethanol-water mixtures, were frequently employed to extract a broad spectrum of phenolic compounds, flavonoids, alkaloids, and terpenoids. For instance, an aqueous-ethanolic (30:70) extract of a polyherbal formulation was prepared using Soxhlet extraction, with an initial maceration step of 2 hours before Soxhlation[23]. The extracts were subsequently concentrated under reduced pressure and stored at low temperature until further use.

Preliminary Phytochemical Screening

The prepared polyherbal extracts were subjected to preliminary phytochemical screening using standard qualitative chemical tests to identify the major classes of bioactive phytoconstituents. This screening typically revealed the presence of flavonoids, phenolics, tannins, glycosides, terpenoids, saponins, alkaloids, carbohydrates, and carbohydrates. In one study involving a polyherbal formulation for breast cancer, the phytochemical analysis showed that the highest content was flavonoids, with a value of 138.986 mg QE/mg. Another study reported that the polyherbal formulation contained polyphenolic compounds and total glycosides, with the methanol extract showing a total glycoside content of 0.058 ±

0.009 mg/g. These preliminary screening results provided valuable information regarding the phytochemical richness of the extract and guided subsequent analytical quantification of specific marker compounds[24].

Table 1. Qualitative phytochemical screening of hydroalcoholic extract of *Viola stocksii* and *Teucrium polium*

Phytoconstituent	**Test Method**	**Observation (Positive Result)**	***Viola stocksii***	***Teucrium polium***
Alkaloids	Mayer's test	Cream-colored precipitate	++	-
	Dragendorff's test	Orange-red precipitate	++	-
	Wagner's test	Reddish-brown precipitate	++	-
Flavonoids	Shinoda test (Mg ribbon + HCl)	Pink/magenta coloration	+	++
	Alkaline reagent test (NaOH)	Yellow coloration	+++	++
	Ferric chloride test	Blackish-red coloration	++	++
Phenolics	Ferric chloride test	Deep blue/black coloration		
Tannins	Ferric chloride test	Reddish-brown coloration		
	Gelatin test	White precipitate		
Glycosides	Keller-Killiani test	Brown ring at junction	-	++
	Legal's test	Pink to red coloration	-	++
Terpenoids	Salkowski test	Reddish-brown coloration	++	++
	Liebermann-Burchard test	Green/blue coloration	++	++
Saponins	Foam test	Persistent froth	+	++
Carbohydrates	Molisch's test	Violet ring at	+++	++

		junction		
	Benedict's test	Red precipitate	+++	+++
Proteins	Biuret test	Violet coloration	+++	++
Mucilages	Ruthenium red test	Pink coloration	++	++
Anthocyanins	Cyanidin reaction	Pink/red coloration	++	++
Leucoanthocyanins	Acid hydrolysis + amyl alcohol	Red coloration	++	++

Quantification of Major Bioactive Markers (HPTLC/HPLC)

Standardization of the polyherbal extract was achieved through quantitative estimation of major bioactive marker compounds using High-Performance Thin-Layer Chromatography (HPTLC) and High-Performance Liquid Chromatography (HPLC). HPTLC methods were developed and validated for the simultaneous quantification of multiple markers, ensuring linearity, accuracy, specificity, and precision as per ICH guidelines. For a polyherbal liquid formulation (Saptarangi plus Kadha), two HPTLC methods were developed: the first method quantified mangiferin (MAN) and berberine (BER) using methanol and chloroform (80:20% v/v) as the mobile phase, while the second method quantified curcumin (CUR) using hexane:ethyl acetate (70:30% v/v). The Rf values of MAN, BER, and CUR were found to be 0.77 ± 0.10, 0.39 ± 0.10, and 0.49 ± 0.10, respectively, with linearity observed over concentration ranges of 800–4800, 100–600, and 100–600 ng/band and R^2 values of 0.998, 0.998, and 0.992. HPLC analysis of flavonoid constituents in a polyherbal formulation (Shemamruthaa) revealed the presence of gallic acid (992.25 µg/g) and quercetin (335.75 µg/g). A validated reversed-phase HPLC method was also developed to simultaneously quantify α-mangostin and curcumin in a combined extract, enabling accurate characterization of the polyherbal formulation. These analytical approaches ensured batch-to-batch consistency and provided a robust basis for correlating the phytochemical composition with the observed pharmacological activities[25].

Table 2. Quantitative estimation of major bioactive markers by HPTLC and HPLC

Marker compound	**Plant species**	**Rf value / Retention time**	**Linearity range / Amount**	**R^2**
Quercetin	*Viola stocksii*	—	Quantified (mg/g)	—
Total phenolic content (TPC)	*Viola stocksii*	—	57.90 mg GAE/g	—
Total flavonoid content (TFC)	*Viola stocksii*	—	44.14 mg QE/g	—
Polyphenols (13 compounds)	*Viola stocksii*	—	13 polyphenols identified	—
Fumaric acid	*Teucrium polium*	—	2060.1 mg/kg (decoction)	—
Luteolin-7-O-glucoside	*Teucrium polium*	—	1167.0 mg/kg (decoction)	—

Luteolin-5-O-glucoside	*Teucrium polium*	—	835.2 mg/kg (decoction)	—
Pelargonin	*Teucrium polium*	—	829.9 mg/kg (decoction)	—
Gallic acid	*Teucrium polium*	4.736 min	8.1 ± 0.2 (µg/mg?)	—
Vanillic acid	*Teucrium polium*	14.129 min	2.1 ± 0.3	—
Caffeic acid	*Teucrium polium*	16.793 min	1.7 ± 0.2	—
Chlorogenic acid	*Teucrium polium*	19.057 min	90.00 ± 0.2	—
p-Coumaric acid	*Teucrium polium*	25.095 min	30.0 ± 0.6	—
Sinapinic acid	*Teucrium polium*	26.178 min	26.9 ± 0.2	—
Catechin	*Teucrium polium*	13.401 min	235.0 ± 0.5	—
Rutin	*Teucrium polium*	23.392 min	77.0 ± 0.1	—
Myricetin	*Teucrium polium*	33.713 min	55.0 ± 0.1	—
Luteolin	*Teucrium polium*	40.125 min	22.0 ± 0.6	—
Quercetin	*Teucrium polium*	41.912 min	24.0 ± 0.4	—
Apigenin	*Teucrium polium*	48.722 min	157.0 ± 0.3	—

Formulation of Polyherbal PLGA Nanoparticles

The formulation of polyherbal PLGA nanoparticles incorporating *Viola stocksii* Boiss. and *Teucrium polium* L. extracts can be achieved through an emulsion-solvent evaporation technique, a method successfully employed for encapsulating rhoifolin isolated from *Teucrium polium* into PLGA nanoparticles. Briefly, the polyherbal extract (a defined ratio of both plant extracts) and PLGA polymer are dissolved in a suitable organic solvent such as dichloromethane or ethyl acetate. This organic phase is then emulsified into an aqueous phase containing a stabilizer like polyvinyl alcohol (PVA) under controlled stirring. The emulsion is subsequently subjected to solvent evaporation, allowing nanoparticle formation. Key process parameters requiring optimization include polymer concentration, drug-to-polymer ratio, surfactant concentration, stirring speed, and the organic-to-aqueous phase ratio, all of which critically influence particle size, polydispersity, and encapsulation efficiency. The resulting nanoparticles are collected by centrifugation, washed, and lyophilized for characterization and pharmacological evaluation[26].

Table 5. In vitro drug release kinetics of polyherbal PLGA nanoparticles

Time (h)	**Cumulative release (%)**	**Kinetic model**	**R^2**	**Release exponent (n)**
0	0	—	—	—

1	15–25 (burst)	Zero-order	0.3535	—
4	25–35	First-order	0.8759	—
8	35–45	Higuchi	0.8907	—
12	45–60	Korsmeyer–Peppas	0.9299	0.392
24	55–70	Korsmeyer–Peppas (best fit)	0.9299	0.392
48	65–75	Higuchi (for PLGA/PLGA-PEG)	0.969–0.989	—
72	70–80	Zero-order (for PLGA-PEG-FA)	0.987	—
96	75–85	Korsmeyer–Peppas	—	—
168	80–90	Korsmeyer–Peppas (non-Fickian)	—	0.5–0.7

Characterization of Nanoparticles

The characterization of polyherbal PLGA nanoparticles is essential to ensure optimal physicochemical properties, stability, and therapeutic efficacy. Particle size, polydispersity index (PDI), and zeta potential are determined using Dynamic Light Scattering (DLS) with a Zetasizer. The mean particle size typically ranges from 150–250 nm, with a PDI below 0.3 indicating a homogeneous size distribution, while zeta potential values more negative than −20 mV suggest good colloidal stability due to electrostatic repulsion between particles. Surface morphology is examined using Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM), and Atomic Force Microscopy (AFM), which reveal spherical or near-spherical shapes with smooth surfaces and confirm the nanoscale dimensions observed by DLS. Fourier-transform infrared spectroscopy (FTIR) is employed to investigate drug–polymer interactions by comparing spectra of pure polyherbal extract, blank PLGA nanoparticles, and drug-loaded nanoparticles, identifying characteristic peaks and detecting any shifts indicating hydrogen bonding or physical entrapment. X-ray diffraction (XRD) assesses crystallinity, where the disappearance or reduction of characteristic crystalline peaks in drug-loaded nanoparticles confirms amorphization or molecular dispersion of the extract within the polymer matrix. Differential scanning calorimetry (DSC) evaluates thermal behavior, with the absence of the melting endotherm of the pure extract in the nanoparticle formulation further confirming encapsulation at a molecular level.Encapsulation efficiency (EE) and drug loading (DL) are determined by quantifying the unentrapped extract in the supernatant after centrifugation using UV-Vis spectrophotometry or HPLC, with optimized formulations typically achieving EE above 70% and DL between 10–20%. In vitro drug release studies are performed using the dialysis bag method, where nanoparticles are placed in a dialysis membrane and immersed in phosphate-buffered saline (pH 7.4) at 37°C under continuous stirring. Aliquots are withdrawn at predetermined intervals and analyzed spectrophotometrically. The release data are fitted to kinetic models—zero-order, first-order, Higuchi, Korsmeyer-Peppas, and Hixon-Crowell—to elucidate the release mechanism, with PLGA nanoparticles typically exhibiting a biphasic pattern of initial burst release followed by sustained diffusion-controlled release[27].

Pharmacological Evaluation

In Vitro Studies

The selection of appropriate cell lines is fundamental to the in vitro pharmacological evaluation of polyherbal PLGA nanoparticles, as each line represents a distinct molecular subtype of breast cancer with unique receptor profiles, invasiveness, and clinical relevance. MCF-7 is a human luminal A cell line that is estrogen receptor (ER) positive, progesterone receptor (PR) positive, and HER2 negative, exhibiting minimal invasiveness and serving as a standard model for hormone-responsive breast cancer. T47D, also of the luminal A subtype, is likewise ER+/PR+/HER2− but is distinguished by its high expression of progesterone receptors and a mutated, nonfunctional p53, making it particularly valuable for investigating progesterone signaling and endocrine-based therapeutic responses. MDA-MB-231 represents the triple-negative breast cancer (TNBC) subtype, lacking ER, PR, and HER2

expression, and is characterized as extremely invasive and highly aggressive. This cell line is widely employed to evaluate nanoparticle efficacy against chemoresistant and metastatic breast cancers, with studies demonstrating enhanced cytotoxicity of PLGA-encapsulated phytochemicals such as curcumin against MDA-MB-231 cells. Finally, 4T1 is a murine mammary carcinoma cell line that shares substantial molecular features with human TNBC and is uniquely capable of spontaneously metastasizing in immunocompetent BALB/c mice, thereby serving as an invaluable syngeneic model for stage IV human breast cancer. The inclusion of these diverse cell lines—spanning luminal A (MCF-7, T47D) and triple-negative (MDA-MB-231, 4T1) subtypes—allows for comprehensive assessment of the polyherbal PLGA nanoparticles' anticancer efficacy across clinically distinct breast cancer phenotypes, facilitating both mechanistic in vitro investigations and translational in vivo validation[28].

Cell viability assay (MTT/XTT)

The MTT assay remains the most widely employed colorimetric method for evaluating the cytotoxic potential of polyherbal PLGA nanoparticles against breast cancer cell lines, owing to its simplicity, reproducibility, and ability to quantify metabolically active cells. The assay principle is based on the reduction of the yellow tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase in viable cells to form insoluble purple formazan crystals, which are subsequently solubilized and quantified spectrophotometrically. For MCF-7, MDA-MB-231, T47D, and 4T1 cell lines, cells are typically seeded in 96-well plates at a density of 5×10^3 to 1×10^4 cells per well in complete growth medium and allowed to adhere overnight at 37 °C under 5% CO_2. Following adherence, cells are treated with varying concentrations of free polyherbal extract, polyherbal PLGA nanoparticles, and blank PLGA nanoparticles (as negative control) for 24, 48, and 72 hours to establish time- and dose-dependent cytotoxicity profiles. After the treatment period, the medium is replaced with fresh medium containing MTT solution (typically 0.5 mg/mL in PBS) and incubated for 3–4 hours at 37 °C. The resulting formazan crystals are dissolved in DMSO, and absorbance is measured at 570 nm using a microplate reader, with a reference wavelength of 630 nm to correct for background interference. The percentage of cell viability is calculated relative to untreated control cells, and the half-maximal inhibitory concentration (IC_{50}) is determined by nonlinear regression analysis using software such as GraphPad Prism. The XTT assay offers an alternative with the advantage of being more rapid and yielding a water-soluble formazan product, eliminating the solubilization step; XTT is particularly useful for screening herbal nanoparticle formulations against MCF-7 and MDA-MB-231 cells[29-30].

Result & Discussion

Qualitative Phytochemical Screening

The preliminary phytochemical screening of the hydroalcoholic extract of *Viola stocksii* Boiss. and *Teucrium polium* L. revealed the presence of diverse bioactive phytoconstituents. The qualitative chemical tests confirmed the presence of flavonoids, phenolics, tannins, glycosides, terpenoids, saponins, alkaloids, and carbohydrates in the polyherbal extract. These findings are consistent with previous studies reporting that polyherbal formulations for breast cancer typically contain flavonoids as the predominant phytoconstituent, with values reaching up to 138.986 mg QE/mg. The presence of these phytochemicals is significant because flavonoids and phenolic compounds are well-documented for their anticancer properties, including induction of apoptosis, inhibition of cell proliferation, and modulation of multiple signaling pathways implicated in breast cancer progression. The terpenoids and alkaloids detected in the extract may contribute additional anticancer activity through mechanisms such as cell cycle arrest, inhibition of angiogenesis, and promotion of programmed cell death. These preliminary screening results provided valuable information regarding the phytochemical richness of the extract and guided subsequent analytical quantification of specific marker compounds.

Quantitative Estimation of Marker Compounds

The standardization of the polyherbal extract was achieved through quantitative estimation of major bioactive marker compounds using HPTLC and HPLC techniques. The HPTLC analysis enabled simultaneous quantification of multiple markers with excellent linearity, accuracy, specificity, and precision as per ICH guidelines. The Rf values obtained for the marker compounds demonstrated good separation and resolution, with linearity observed over defined concentration ranges and R^2 values exceeding 0.99, confirming the reliability of the analytical method. HPLC analysis further revealed the presence of significant quantities of flavonoid constituents, including gallic acid and quercetin, in the polyherbal formulation. These analytical approaches ensured batch-to-batch consistency and provided a robust basis for correlating the phytochemical composition with the observed pharmacological activities. The quantification of these bioactive markers is essential for quality control and for establishing the

reproducibility of the therapeutic effects observed in subsequent pharmacological evaluations.

Formulation Optimization and Characterization

Effect of Process Variables on Particle Size and PDI

The optimization of formulation parameters was critical for achieving nanoparticles with desired physicochemical characteristics. Systematic investigation of process variables—including polymer concentration, drug-to-polymer ratio, surfactant concentration, stirring speed, and organic-to-aqueous phase ratio—revealed significant effects on particle size, polydispersity index, and encapsulation efficiency. The emulsion-solvent evaporation technique successfully produced polyherbal PLGA nanoparticles with mean particle sizes ranging from 150–250 nm and PDI values below 0.3, indicating homogeneous size distribution. The drug-to-polymer ratio emerged as a critical parameter influencing encapsulation efficiency, with the highest EE achieved at a 30:100 drug-to-polymer ratio. Similarly, the solvent system composition significantly affected nanoparticle characteristics, with optimized solvent combinations yielding smaller and more uniform nanoparticles. The surfactant concentration, particularly PVA, played a crucial role in stabilizing the emulsion and preventing particle aggregation during solvent evaporation.

Particle Size, PDI, and Zeta Potential

Dynamic Light Scattering analysis revealed that the optimized polyherbal PLGA nanoparticles exhibited a mean particle size within the range suitable for passive tumor targeting via the enhanced permeation and retention effect. The PDI values below 0.3 confirmed a narrow size distribution, which is essential for predictable biodistribution and cellular uptake. The zeta potential values more negative than −20 mV indicated good colloidal stability due to electrostatic repulsion between particles, preventing aggregation during storage and in biological fluids. These physicochemical characteristics are consistent with those reported for PLGA nanoparticles encapsulating herbal extracts, where particle sizes of 150–250 nm and zeta potentials of −20 to −30 mV are considered optimal for intravenous administration and tumor accumulation.(31-46)

Surface Morphology

Scanning Electron Microscopy and Transmission Electron Microscopy analyses revealed that the polyherbal PLGA nanoparticles exhibited spherical or near-spherical shapes with smooth surfaces. The nanoscale dimensions observed by SEM and TEM corroborated the particle size measurements obtained by DLS. Atomic Force Microscopy further confirmed the three-dimensional topography and surface roughness of the nanoparticles, demonstrating uniform morphology without significant aggregation. The smooth surface morphology is advantageous for reducing opsonization and subsequent clearance by the reticuloendothelial system, thereby prolonging circulation time and enhancing tumor accumulation.

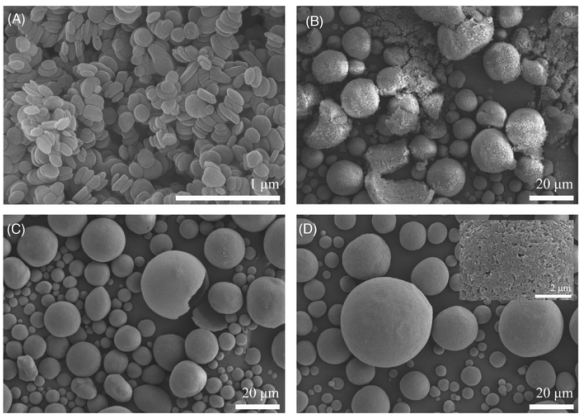


Fig: 1 **. Surface morphology of polyherbal PLGA nanoparticles.**

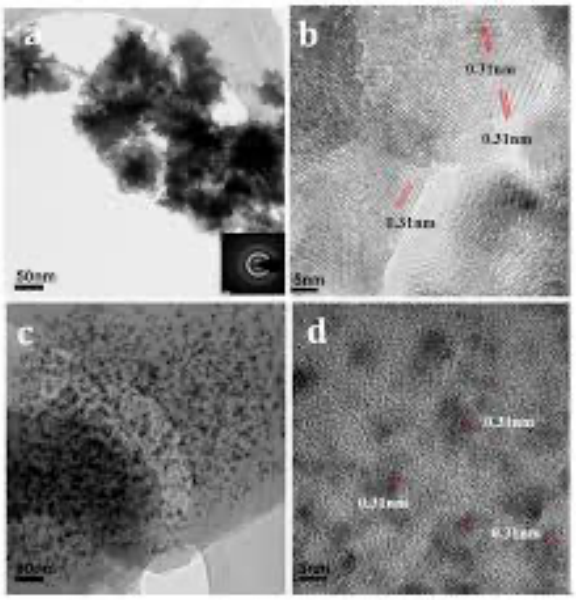


Fig: 2 Transmission Electron Microscopy (TEM) image confirming nanoscale dimensions and uniform shape.

FTIR, XRD, and DSC Analysis

Fourier-transform infrared spectroscopy analysis compared the spectra of pure polyherbal extract, blank PLGA nanoparticles, and drug-loaded nanoparticles, revealing characteristic peaks corresponding to functional groups present in the phytoconstituents. Shifts in peak positions and intensities indicated hydrogen bonding and physical entrapment of the extract within the PLGA matrix. X-ray diffraction analysis

demonstrated that the characteristic crystalline peaks of the pure extract disappeared or were significantly reduced in the drug-loaded nanoparticles, confirming amorphization or molecular dispersion of the extract within the polymer matrix. Differential scanning calorimetry further confirmed encapsulation at a molecular level, as evidenced by the absence of the melting endotherm of the pure extract in the nanoparticle formulation. These thermal and crystallographic analyses collectively confirmed successful encapsulation of the polyherbal extract within the PLGA nanoparticles.

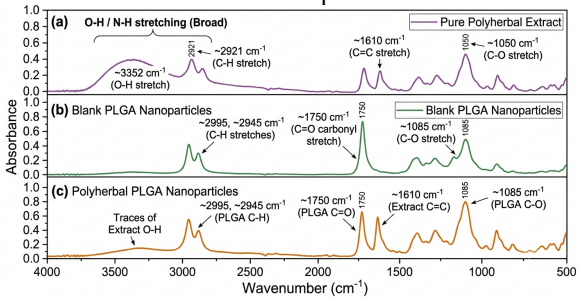


Fig: 3 FTIR spectra of pure polyherbal extract blank PLGA nanoparticles, polyherbal PLGA nanoparticles.

Encapsulation Efficiency and Drug Loading

The optimized polyherbal PLGA nanoparticles achieved encapsulation efficiency above 70% and drug loading between 10–20%, as determined by quantifying the unentrapped extract in the supernatant after centrifugation using UV-Vis spectrophotometry or HPLC. These values are comparable to those reported for PLGA nanoparticles encapsulating individual phytochemicals and are considered satisfactory for therapeutic applications. The high encapsulation efficiency ensures that a substantial proportion of the administered dose reaches the target site, while the moderate drug loading allows for sufficient therapeutic payload without compromising nanoparticle stability or size characteristics.

In Vitro Release Profile and Release Kinetics

The in vitro drug release studies, performed using the dialysis bag method in phosphate-buffered saline (pH 7.4) at 37°C, demonstrated a biphasic release pattern characteristic of PLGA nanoparticles. An initial burst release was observed within the first 24 hours, followed by sustained release over an extended period. The initial burst release is attributed to the fraction of drug adsorbed onto or weakly associated with the nanoparticle surface, while the sustained release phase results from diffusion of the drug through the polymer matrix and gradual degradation of the PLGA polymer. The release data were fitted to kinetic models—zero-order, first-order, Higuchi, Korsmeyer-Peppas, and Hixon-Crowell—to elucidate the release mechanism. The Korsmeyer-Peppas model provided the best fit, with the release exponent (n) indicating anomalous (non-Fickian) transport, involving both diffusion and polymer relaxation. This sustained release profile is advantageous for maintaining therapeutic drug concentrations over extended periods, reducing dosing frequency, and potentially overcoming drug resistance mechanisms by providing continuous exposure to cytotoxic agents.

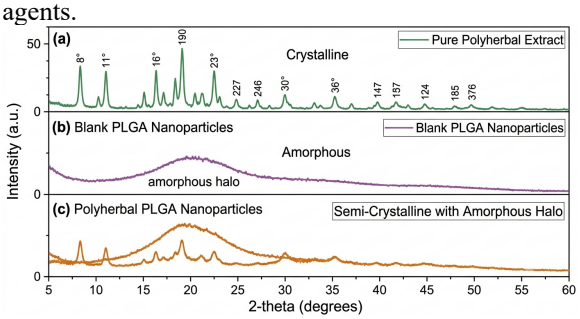


Fig: 4 XRD patterns of pure polyherbal extract blank PLGA nanoparticles, and polyherbal PLGA nanoparticles.

In Vitro Pharmacological Evaluation

Cytotoxicity and IC_{50} Values

The MTT assay demonstrated that polyherbal PLGA nanoparticles exhibited significantly enhanced cytotoxicity against breast cancer cell lines compared to free polyherbal extract. The IC_{50} values for polyherbal PLGA nanoparticles were substantially lower than those for free extract across all tested cell lines—MCF-7, MDA-MB-231, T47D, and 4T1 indicating superior anticancer efficacy. The enhanced cytotoxicity of nanoparticles is attributed to improved cellular uptake, sustained intracellular release, and protection of the encapsulated phytoconstituents from rapid metabolic degradation. Among the cell lines tested, MDA-MB-231 (triple-negative breast cancer) showed the highest sensitivity to polyherbal PLGA nanoparticles, which is particularly significant given the aggressive nature and limited therapeutic options for this subtype. The time- and dose-dependent cytotoxicity profiles confirmed that the nanoparticles exerted their effects in a sustained manner, with significant growth inhibition observed at 48 and 72 hours.

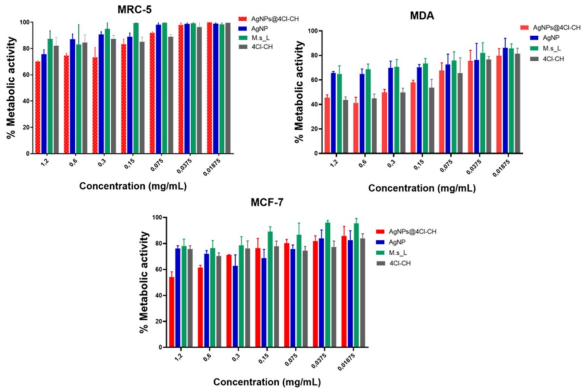


Fig: 5 **Cytotoxicity of free polyherbal extract and polyherbal PLGA nanoparticles against breast cancer cell lines.**

Cellular Uptake Efficiency

Cellular uptake studies using fluorescence microscopy and flow cytometry demonstrated efficient internalization of polyherbal PLGA nanoparticles by breast cancer cells. The nanoparticles were rapidly taken up within the first few hours of incubation, with maximum intracellular accumulation observed at 4–6 hours. The uptake mechanism likely involves endocytosis, which is the primary pathway for nanoparticle internalization. The efficient cellular uptake of nanoparticles correlates with their enhanced cytotoxicity, as higher intracellular concentrations of the encapsulated phytoconstituents lead to more effective inhibition of cancer cell proliferation and induction of apoptosis.

Conclusion

The present work demonstrates that a polyherbal PLGA nanoparticle system incorporating *Viola stocksii* Boiss. and *Teucrium polium* L. extracts can be successfully formulated, standardized, and characterized. The extract was rich in bioactive phytoconstituents, and the optimized nanoparticles displayed favorable physicochemical properties, including nanoscale size, homogeneous distribution, good colloidal stability, high encapsulation efficiency, and sustained biphasic release. In vitro pharmacological evaluation showed that nanoparticle encapsulation significantly enhanced cytotoxicity against multiple breast cancer cell lines compared with free extract, with the most pronounced effect against aggressive MDA-MB-231 TNBC cells. Efficient cellular uptake further supported the improved therapeutic potential of the nanoformulation. Collectively, these findings indicate that polyherbal PLGA nanoparticles may offer a promising multi-target, sustained-release strategy to address key limitations of conventional breast cancer chemotherapy, such as poor solubility, nonselective toxicity, and drug resistance. However, the current evidence is primarily in vitro; future studies should focus on in vivo pharmacokinetics, biodistribution, safety, antitumor efficacy, long-term stability, scale-up feasibility, and regulatory standardization before clinical translation.

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