

Targeting Breast Cancer: Encapsulation and *In-vitro* Studies of α -Amyrin from *Capparis zeylanica* L.

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ABSTRACT

Historically, *Capparis zeylanica* L. has garnered attention for its potential effectiveness in treating cancer. This has inspired the researchers to delve into the plant's properties in search of new and innovative compounds with anticancer properties. Therefore, the current study aimed to encapsulate and characterize α -amyrin phytochemical from leaf extract of *C. zeylanica* L. and study it for the *in-vitro* cancer cell line. The high-performance liquid chromatography (HPLC) method development of *C. zeylanica* L. extracts furnished α -amyrin, whose characterization was established by high-performance liquid chromatography (HPLC), fourier-transform infrared spectroscopy (FTIR), liquid chromatography-mass spectrometry (LC-MS). Molecular docking was performed using Auto Dock Vina software to validate the mechanism of action. The α -amyrin nanospheres were synthesized through the emulsion solvent diffusion method, employing various drug-polymer ratios ranging from 1:1 to 1:5, with pullulan as the chosen polymer. Compatibility was revealed no interaction using FTIR. The cytotoxic properties were assessed on MCF-7 cell lines using MTT assay. The production yield and entrapment efficiency of F1-F5 batches in the range of 43 to 68.36% and 70 to 82.5%, respectively. Batch F5 showed the highest production yield, and entrapment efficiency. The average particle size ranges from 241.3 to 603.2 nm. The F5 formulation had shown the highest drug release (90.40 \pm 0.88%). The IC₅₀ value for optimized formulation was 34.54 μ g/mL as observed in MCF-7 breast cancer, which was considerable as compared with 5-fluorouracil. *In-silico* studies revealed that α -amyrin showed good binding affinity to breast cancer PDBIDs and form a stable complex. In our study, α -amyrin has been successfully extracted and further validation through pharmaceutical formulation was done and warranted to solidify its potential as an effective anticancer agent.

Keywords: *Capparis zeylanica* L., Cytotoxicity, Breast cancer, α -Amyrin.

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INTRODUCTION

Cancer stands as one of the most devastating global diseases, according to a report by the World Health Organization (WHO). Within this grim statistic, India bears a significant burden, accounting for approximately 2.25 million cases with more than 100,000 new cases emerging annually. Projections from the Indian Council of Medical Research (ICMR) paint a distressing picture, forecasting over 1.7 million new cases and upwards of 800,000 deaths by the year 2020. The surge in cancer incidence can be attributed not only to the growth and aging of the population but also to various risk factors, including sedentary lifestyles, obesity resulting from unhealthy dietary habits, smoking, and shifting reproductive behaviors tied to modernization.¹⁻⁵ Encouragingly, the past couple of years have witnessed a decline in cancer-related mortality, owing to advances in comprehending tumor biology as

well as advancements in diagnostic tools and therapeutic approaches. The arsenal of cancer treatments encompasses surgical procedures, radiation therapy, and chemotherapy. However, these interventions often come at a cost, as they may inadvertently harm healthy cells and subject patients to considerable levels of toxicity.²

Conventional chemotherapy regimens used in cancer treatment frequently lead to undesirable side effects that curtail their therapeutic efficacy and undermine patient quality of life. In contrast, revisiting the ancient Indian medical system of Ayurveda highlights the presence of numerous potent natural compounds endowed with anticancer, anti-angiogenic, and anti-proliferative attributes.⁶ Natural products exhibit noteworthy characteristics, encompassing their remarkable chemical diversity, distinct chemical and biological properties, and reduced toxicity. These attributes position

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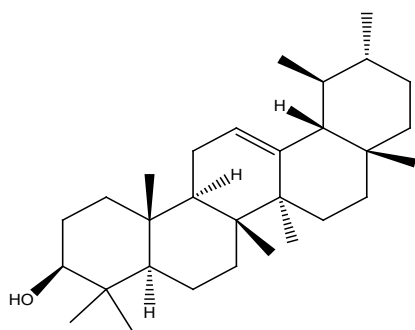


Figure 1: Chemical structure of α -amyrin

them as promising pathways for the exploration of innovative drug candidates. There is an increasing market demand for pharmaceuticals derived from natural bioactive compounds due to the side effects associated with synthetic medications. Phytochemicals have not only been valued for their nutritional benefits but also for their potential in preventing and treating chronic cancerous conditions.^{7,8}

Plant-derived phytochemicals present an appealing and comprehensive approach to drug action across various diseases with minimal side effects. As a result, our attention is drawn to the isolation and screening of potential phytochemicals for their suitability as drug candidates, particularly in addressing breast cancer. Employing a structure-based drug design approach using these phytochemicals promises to streamline the process and reduce uncertainties. Hence, the present research is focused on performing virtual docking simulations involving specific phytochemicals, which are being explored as potential lead compounds. The objective is to evaluate their binding strengths with a designated target protein identified by its PDBID. These interactions will be juxtaposed with the binding interactions exhibited by well-known anticancer medications. This will be followed by a subsequent *in-vitro* study using the MCF-7 cell line. The goal is to uncover potential candidates that hold promise for the development of next-generation pharmaceuticals targeting proteins implicated in breast cancer.⁹⁻¹²

Capparis zeylanica L., commonly referred to as Indian caper, holds a significant and esteemed position in the realm of medicinal plants. As a member of the Capparidaceae family, this extensively branching climbing or rambling shrub thrives throughout India and has been acknowledged for centuries as a valuable 'Rasayana' herb in traditional medicine. However, research on the leaves of *C. zeylanica* L. remains relatively scant. Surprisingly, there is a dearth of pharmacological investigations assessing the anticancer potential of *C. zeylanica* L. against this backdrop, the present study endeavors to contribute to our understanding of the anticancer attributes of *C. zeylanica* L. leaf extract through a comprehensive phytochemical analysis, bridging this knowledge gap.^{13,14} This research proved validation for the utility and potential of natural polymer-based encapsulation of α -amyrin as support for the treatment of breast cancer (Figure 1).

MATERIALS AND METHODS

Chemicals

α -Amyrin reference standard was procured from Chem-scene (China), HPLC grade water from Thermo Fisher, methanol LR grade was procured from Merck, ethyl acetate and hexane from Rankem and toluene Qualigens from Thermo Fisher Scientific.

Extraction of Leaves of *C. zeylanica* L.

The extraction process for the dried powder was conducted using two techniques: maceration and ultrasonication. Various batches were prepared using a combination of these methods, and separate batches were prepared using each technique individually. The ratios of 1:5 and 1:10 for the drug to solvent were employed, as detailed in Table 3. Different extracting solvents were utilized in these processes.¹⁵⁻¹⁶

High-performance Liquid Chromatography

Accurately weigh and transfer about 1.0 mg of α -amyrin reference standard to 1-mL centrifuge tube. Add 1-mL methanol as diluent and sonicated to dissolve to achieve 1000 μ g/mL concentration for the standard curve.

The 5 mg of dried column fraction was weighed and dissolved in 1-mL of diluent. Sonication was done to dissolve the fraction. The solution was filtered through 0.22 μ nylon filters and 20 μ L of the sample was injected to high-performance liquid chromatography (HPLC).

Procedure

HPLC analysis was conducted employing the Agilent 1100 system, utilizing an injection volume of 20 μ L. A photo-diode array detector set at a sensitivity of 0.001 and a wavelength of 205 nm was employed. The Hypersil gold column (250 x 4.6 mm, 5 μ) was maintained at a temperature of 27°C. The mobile phase consisted of a mixture of water and ACN in a ratio of 2:98. The analytes were eluted using a gradient elution method at a flow rate of 1-mL/min. Each diluted extract (20 μ L) was injected into the HPLC system thrice, and the average peak area was determined and used for quantification purposes. For comprehensive insights into the HPLC method development conditions, please refer to Table 1.¹⁷⁻¹⁸

Fourier Transform Infrared Spectroscopy Analysis

For the analysis of plant samples' spectral information, spectra were recorded using a Bruker FTIR spectrophotometer, covering a range of 400 to 4000 cm^{-1} . The drug and excipient compatibility studies were carried out by Fourier transform-infrared spectroscopy (FTIR).¹⁹⁻²¹

Molecular Docking

Molecular docking studies were executed to elucidate the binding affinity between the phytoconstituents α -amyrin from the *C. zeylanica* L. plant and targeted proteins associated with breast cancer. The initiation of molecular docking entailed creating 3D structures for the phytoconstituents, designated as ligands, which were subsequently prepared and energy minimized. The protein targets were obtained from the RCSB Protein Data Bank with identifiers epidermal growth factor receptor (2J6M), estrogen receptor (3ERT), human fatty acid

Table 1: Composition of nanosponges formulation batches of α -amyrin

Materials	F1	F2	F3	F4	F5
Drug: Polymer ratio	1:1	1:2	1:3	1:4	1:5
PVA (% w/v)	0.5	0.5	0.5	0.5	0.5
Dicholomethane (mL)	30	30	30	30	30
Triethyl citrate (mL)	0.5	0.5	0.5	0.5	0.5
Distilled water (mL)	100	100	100	100	100

synthase (3TJM), and progesterone receptor (4OAR). The corresponding 2D and 3D chemical structures, in structured data format (SDF), were retrieved from the PubChem-NCBI database. These SDF files were then transformed into Protein Data Bank (PDB) format using PyMOL, facilitating further analysis. The docking simulations were conducted using AutoDock Vina (version 1.1.2) on an IBM system equipped with an Intel Centrino Core2Duo CPU operating at 2.20 GHz and 4 GB DDR2 RAM. These AutoDock Vina simulations were executed on a Microsoft Windows 10 operating system. For the purpose of comparison and control, the chemical structures of both the natural substrate α -amyrin and known anticancer agents such as 5-fluorouracil and doxorubicin were also processed within the study.^{12, 22-24}

Method of Preparation of Nanosponges of α -Amyrin²⁵

Microsponges of α -amyrin were synthesized using the emulsion solvent diffusion method. The aqueous phase comprised polyvinyl alcohol, while the organic phase included the drug and pullulan polymer. The drug and pullulan polymer were dissolved in an appropriate organic solvent. This organic phase was then slowly added to the aqueous phase and stirred for two or more hours at 1000 rpm. Subsequently, the nanosponges were collected by filtration, washed, and dried either in ambient air at room temperature or in a vacuum oven at 40°C for 24 hours.

Preformulation Studies

Calibration curve of α -Amyrin

About 10 mg of α -amyrin was weighed and then added to a volumetric flask with a capacity of 10 mL. To prepare a stock solution with a concentration of 1000 μ g/mL, 1-mL of methanol was used to dissolve the medication, and 10 mL of pH 6.8 phosphate buffer (Stock solution I) was added to make the final volume. In order to prepare stock solution II, with phosphate buffer pH 6.8, 1-mL of this stock solution was diluted up to 10 mL. The respective amounts of stock solution II of 0.2, 0.4, 0.6, 0.8, 1, 1.2 and 1.4 mL at 208 nm, and the absorbance of these solutions were measured in comparison to a control sample.

Evaluation Studies of Prepared Nanosponges

Percentage yield

The percentage yield (PY) can be as determined by splitting the particle mass of nanosponges by the total mass of the drug and polymer.

$$PY (\%) = \frac{\text{Actual mass of nanosponge}}{\text{Theoretical mass of the drug and polymer}} \times 100$$

Encapsulation efficiency of drug

To calculate the entrapment efficiency accurately the quantity of nanosponges (10 mg) with 5 mL of methanol in a volumetric flask was shaken for 1-minute using a vortex mixer. The volume was made up to 10 mL. Then, the solution was filtered and diluted and the concentration of α -amyrin was determined spectrophotometrically at 208 nm.

$$\% EE = \frac{\text{Mass of drug used in preparation} - \text{Mass of drug in nanosponges}}{\text{Mass of drug used in preparation}} \times 100$$

Determination of particle size

The size of particles is maintained during polymerization to form free-flowing powders with fine aesthetic attributes. Particle size analysis of loaded and unloaded nanosponges performed by Malvern zeta sizer.

Scanning electron microscopy

An SEM(S-3400 N type II model) examined the surface structure and morphology.

In-vitro drug release studies

In-vitro drug release studies were conducted in triplicate using the USP Paddle method at 100 rpm and $37 \pm 0.2^\circ\text{C}$ in 900 mL of phosphate buffer (pH 6.8). Each experiment utilized 100 mg of the formulated nanosponges. Samples were collected at specific time intervals, with measurements taken every 1-hour over a 12-hour period. Spectrophotometric analysis was performed at 208 nm. The fresh dissolution medium was replenished each time a sample was withdrawn to maintain a constant volume.

Cell lines

The human breast cancer cell line MCF-7 were purchased from Biocyte Research lab, Sangli, India, whereas MCF-7 cells were maintained in DMEM-F12 medium supplemented with hydrocortisone (0.5 μ g/mL), insulin (10 μ g/mL), and hEGF (20 ng/mL).

MTT assay

The evaluation of *in-vitro* cytotoxicity for both pure α -amyrin and the standard 5-fluorouracil against MCF-7 human breast cancer cells was conducted through the MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide) assay. Initially, MCF-7 carcinogenic cell lines were cultured in Dulbecco's Modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) and maintained at $37 \pm 1^\circ\text{C}$ in a 5% CO_2 environment. Subsequently, 1-mL of the cultured cell suspension ($\sim 5 \times 10^4$ cells/mL) was seeded into a 24-well plate and allowed to incubate undisturbed overnight. The samples under investigation were individually added to pre-treated 96-well plates. Following this, 100 μ L of MTT solution (5%, w/v) was introduced into the respective wells,

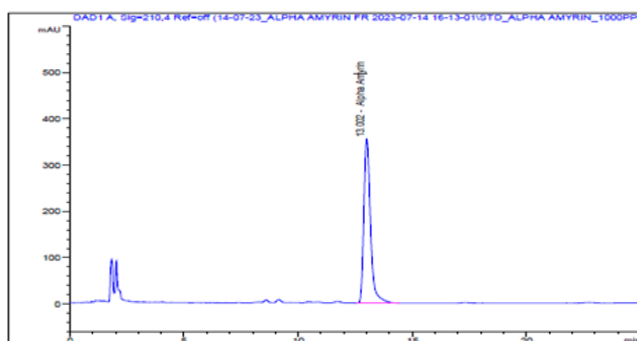
followed by another incubation at 37°C for 4 hours. After incubation, the absorbance of each sample was measured at 490 nm using an ELISA microplate reader (Thermo Fisher Scientific, USA). The percentage cell viability was calculated using the following expression:

$$\% \text{Cell Viability} = \frac{\text{Mean absorbance of test sample}}{\text{Mean absorbance of control}} \times 100^{26-28}$$

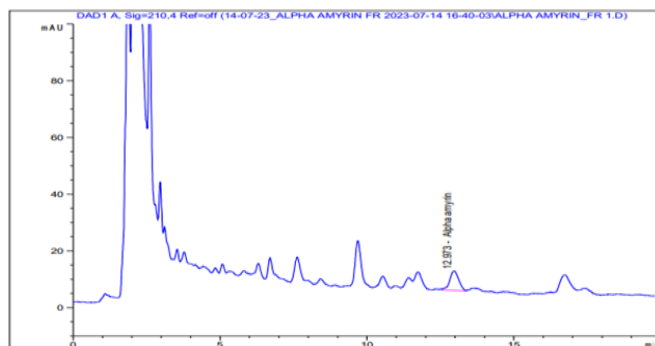
RESULTS AND DISCUSSION

Extraction of Leaves of *C. zeylanica L.*

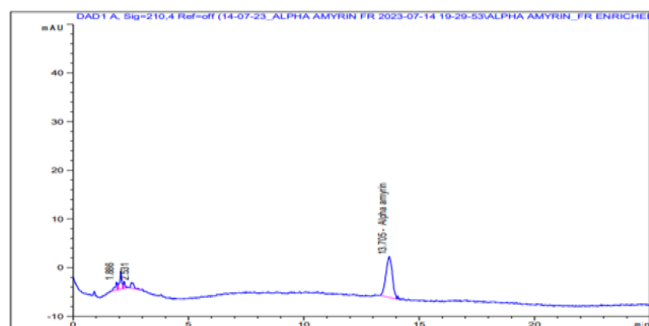
The extraction batches showed the highest yield (21.74%) in 100% methanol. Hence bulk batch was done with 100% methanol. As explained, the α -amyrin was present in the water fraction yielded 102 mg (0.102%) from 100% methanolic extract.



A. Standard α -Amyrin 1000 ppm



B. Fraction of α -Amyrin



C. Enriched α -Amyrin from extract

Figure 2: HPLC chromatogram (A) Standard α -amyrin 1000 ppm (B) Fraction of α -amyrin (C) Enriched α -amyrin form extract

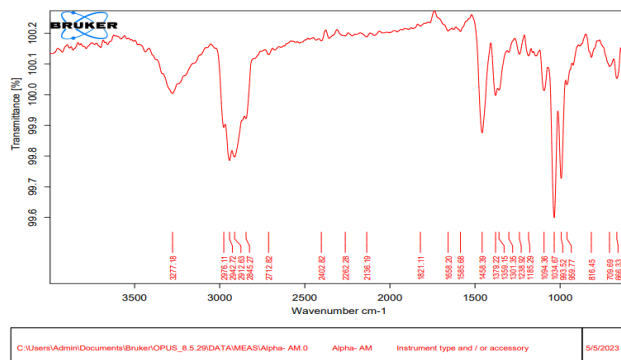


Figure 3: IR spectra of α -amyrin from leaves extract of *C. zeylanica L.*

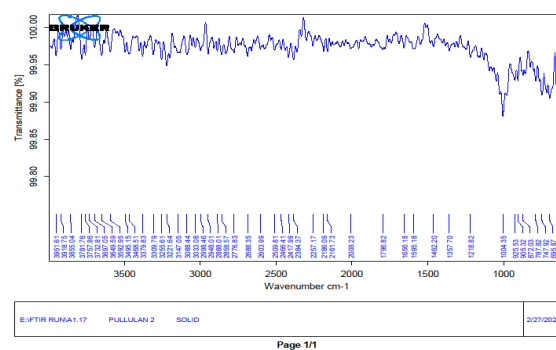


Figure 4: IR spectra of pullulan polymer

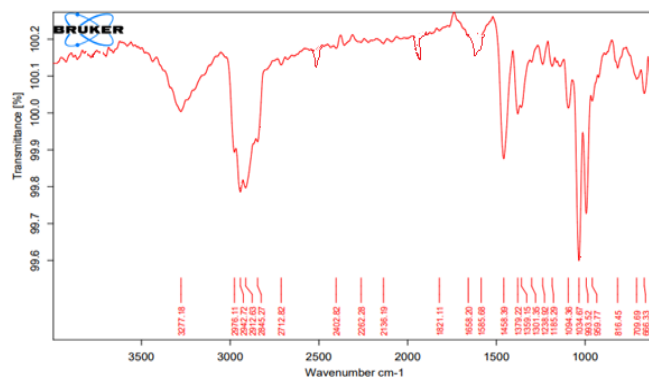


Figure 5: IR spectra of physical mixture of α -amyrin and pullulan polymer

High-Performance Liquid Chromatography

The elution was started with 5, 30, and 50% methanol in chloroform, respectively. Fractions were dried and checked on HPLC for enrichment. The dried fraction showed enrichment of α -amyrin. The developed HPLC method showed a peak at 13.7 minutes. The chromatograms for the same are attached with the report, as shown in Figure 2.

Fourier Transform Infrared Spectroscopy

FTIR spectrum of α -amyrin, which showed transmittance bands at 2976, 2942, 2912 and 2845 cm^{-1} indicated the presence of an aliphatic alkyl moiety while the other bands were assigned for 1734 (C=O), 1458 (C=C); 1359 (CH_3 -/ CH_3CO), 1238 ($-\text{C}-\text{O}$) and 1094, 1034, 993, 969, 816, 606 cm^{-1} (C-H) (Figure 3).

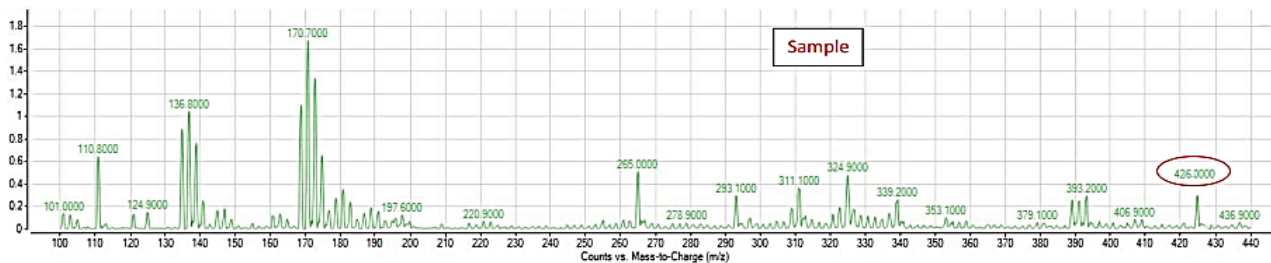


Figure 6: Mass spectra of isolated α -amyrin from leaves extract of *C. zeylanica L.*

Table 2: Binding affinity of α -amyrin and reference standard

Compound name	Binding affinity (PDBID)			
	2J6M	4OAR	3ERT	3TJM
α -Amyrin	-12.2	-14.5	-12	-13.2
Doxorubicin	-7.8	-9.9	-7.7	-8
5-Fluorouracil	-5.1	-5.7	-5.5	-5.3

The drug and polymer showed compatibility between them (Figures 4 and 5).

Mass spectroscopy

EI-MS: m/z 426 (10%, M+) calculated for $C_{30}H_{50}O$ as shown in Figure 6.

Molecular Docking

Molecular docking analyses were undertaken to elucidate the binding interactions involving the phytochemical α -amyrin, sourced from *C. zeylanica L.*, with protein structures identified by PDBIDs: 2J6M, 3ERT, 3TJM, and 4OAR, alongside 5-fluorouracil and doxorubicin for comparison. The obtained results revealed noteworthy docking scores for α -amyrin across all PDBIDs, ranging from -12 to -14.5 kcal/mol, as documented in Table 2. The stable three-dimensional conformations of the ligands in relation to breast cancer were visually represented in Figure 7.

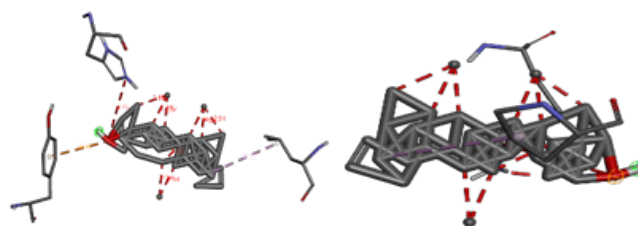


Figure 7: 3D confirmations of the α -amyrin with breast cancer PDBIDs (4OAR, 3TJM)

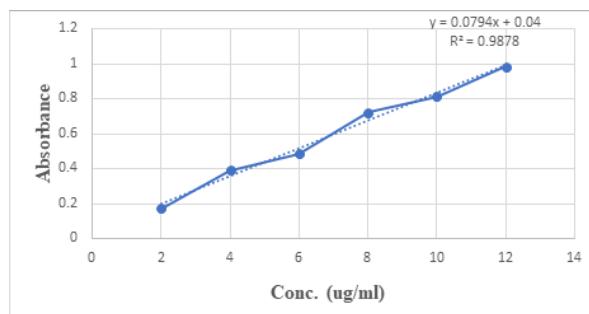


Figure 8: Calibration curve of α -amyrin

Table 3: Percentage yield of F1 to F5 batches

Batch No.	Percentage yield (%)
F1	43
F2	51.62
F3	62.89
F4	66.89
F5	68.36

Preformulation Studies

Calibration curve for alpha-amyrin showed linearity (0.9878) as shown in Figure 8.

Evaluation Studies of Nanosponges Formulation

Percentage yield

The production yield (PY) can be determined by calculating raw materials' initial weight and the nanosponges' final weight. The percentage yield of different batches was determined by weighing the nanosponges after drying. The percentage yields of different formulations were found to be in the range of 43 to 68.36% as shown in below Table 3.

Entrapment efficiency

The drug entrapment efficiency found in the range of 70 to 82.5%. As the concentration of polymers increases, the drug entrapment efficiency also increases (Table 4).

Particle size analysis

The average particle size was obtained in a range of 241.3 to 603.2 nm. The change in the concentration of polymer results

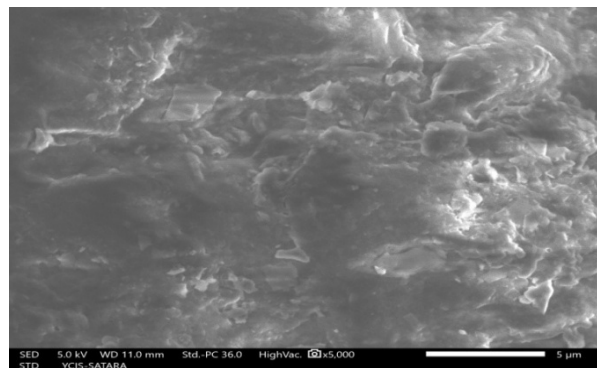


Figure 9: SEM image of nanosponges

Table 4: Entrapment efficiency of F1 to F5 batches

Batch No.	Entrapment efficiency%
F1	70
F2	74.2
F3	76.5
F4	80
F5	82.5

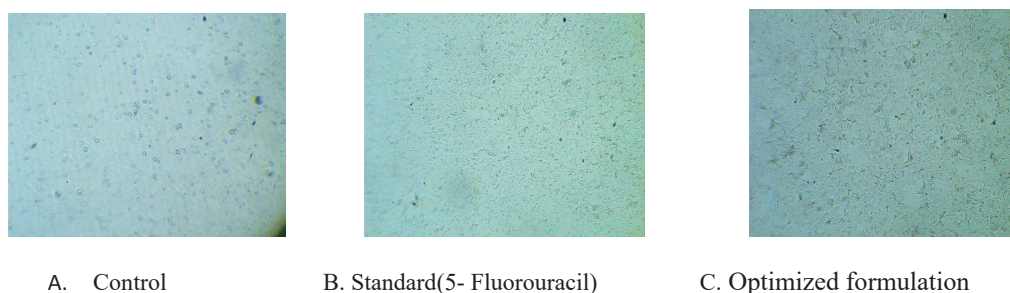
Table 5: Particle size of F1 to F5 batches

Batch No.	Particle size (nm)
F1	241.3
F2	349.5
F3	449.7
F4	512.56
F5	603.2

Table 6: *In-vitro* drug release of nanosponges formulations (F1 to F5)

Time in hrs/%CDR	F1	F2	F3	F4	F5
1	10.07 ± 0.90	11.99 ± 1.12	13.99 ± 1.11	11.07 ± 0.91	15.25 ± 0.78
2	15.32 ± 0.85	23.58 ± 0.80	21.58 ± 0.82	16.32 ± 0.83	19.48 ± 0.82
3	25.36 ± 0.92	32.72 ± 0.99	30.72 ± 0.99	22.36 ± 0.90	33.79 ± 1.02
4	39.98 ± 0.65	40.347 ± 1.2	39.347 ± 1.3	34.98 ± 0.66	51.93 ± 0.98
5	49.92 ± 1.12	50.38 ± 1.50	47.38 ± 1.51	42.92 ± 1.12	65.03 ± 1.14
6	55.23 ± 1.01	58.3 ± 0.98	55.30 ± 0.97	50.23 ± 1.01	69.3 ± 1.32
7	64.71 ± 1.11	65.72 ± 1.18	63.72 ± 1.18	62.71 ± 1.11	77.74 ± 1.26
8	70.98 ± 1.20	75.98 ± 0.96	72.98 ± 0.92	72.98 ± 1.20	82.15 ± 1.0
9	75.26 ± 0.74	81.52 ± 1.25	80.52 ± 1.23	75.26 ± 0.74	85.78 ± 0.9
10	87.24 ± 0.90	88.28 ± 1.24	85.28 ± 1.22	86.24 ± 0.80	90.40 ± 0.88

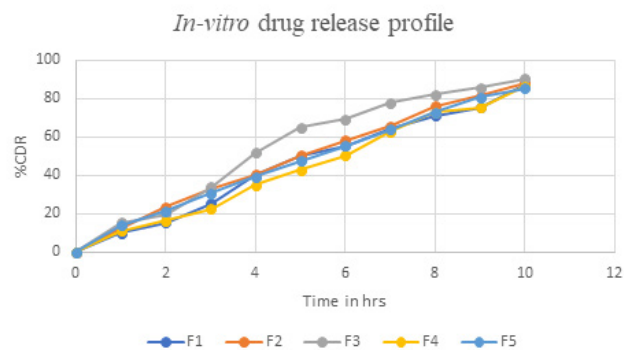
Average ± SD n = 3

**Figure 11:** Cell line study of α -Amyrin with MCF-7 breast cancer cell line A. Control B. Standard C. Optimized formulation

in a variation of particle size of nanosponges. The average particle size of formulation batch F3 showed minimum particle size, i.e., 449.7 nm, while formulation batch F5 showed maximum particle size, i.e., 603.2 nm. An increase in polymer concentration leads to an increase in nanosponges' particle size (Table 5).

Scanning Electron Microscopy

SEM micrographs of nanosponges showed porous structure. It could be advocated that inward diffusion of DCM and pullulan produce porosity for Nanosponges as shown in Figures 9 and 10.

**Figure 10:** *In-vitro* drug release study of nanosponge formulations (F1 to F5)

In-vitro Drug release of Nanosponges Formulations (F1 to F5)

The results of *in-vitro* release studies are shown in Figure 10. α -amyrin nanosponges showed %CDR from 87.24 ± 0.90 , 88.28 ± 1.24 , 90.40 ± 0.88 , 86.24 ± 0.80 and 85.28 ± 1.22 for F1 to F5 batches, respectively and succeeded to sustain the release of the drug for 10 hours as shown in Table 6. Hence, it was concluded that the drug was released slowly from nanosponge formulation when incorporated as an entrapped rather than an unentrapped form.

Cell Line Study

At the different doses (10–100 µg) of different compounds carried out for antitumor activity against MCF-7 cellline. The %inhibition for 10, 40, 100 µg/mL was reported as 40.48, 50.54 and 54.75%, respectively. The IC₅₀ value for optimized formulation was 34.54 µg/mL were, observed in MCF-7 breast cancer, which was better than 5-Fluorouracil, having 39.22 µg/mL. The samples showed considerable activity as compared to the standard compound as shown in Figure 11.

CONCLUSION

In our research, we isolated α -amyrin from the leaf extract of *C. zeylanica* L. and subjected it to characterization through techniques such as HPLC, IR, and LC-MS. The binding affinity with breast cancer PDBIDs was best compared to standard drugs.

Nanosponges formulation of α -amyrin exhibited good evaluation parameters. Furthermore, our *in-vitro* cell line experiments revealed that α -amyrin formulation exhibited anticancer activity comparable to the reference compound's. Based on these results, α -amyrin was identified as a promising candidate with potential as an anticancer agent. The outcomes of our investigation strongly suggest that α -amyrin formulation holds promise as an effective anticancer activity and also warrants further exploration for breast cancer.

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