Nanoemulsion Myricetin preparation increases the anticancer efficacy against Triple-negative Breast Cancer cells

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Abstract

Background and purpose: Myricetin (3,5,7-trihydroxy-2-(3,4,5-trihydroxyphenyl)chromen-4-one) is a polyhydroxyfavonol compound widely found in nature has been shown to possess anticancer effects in various cancers. Despite its efficacy, poor water solubility and low oral bioavailability hinders its therapeutic application. To overcome these limitations, Nanoemulsion (NE) emerged as a promising approach that combines the advantages of NE into a single delivery system. The present study aimed to investigate the advantage of myricetin loaded NE over and above native Myricetin. Experimental Approach: The nano-emulsion was formulated using Capryol 90 as oil, Tween 20 as a surfactant, and Transcutol HP as a co-surfactant. Further comparative analysis of Myricetin and nano-emulsified Myricetin (Myr-NE) were carried out in triple negative breast cancer (MDA-MB-231) cells for anticancer activity. Key results: The optimized Myr-NE had a zeta potential of -6.35±0.3, an average particle size of 89.32±2.8 nm, PDI of 0.105±0.02, and a spherical shape as confirmed in transmission electron microscopy. Diffusion-dominant drug release was observed with 95.49±2.84 % Drug release for 24hrs, 2-fold higher than Myr-suspension. When nano-emulsified, Myricetin exhibited efficient inhibition of cell proliferation, clonogenicity, and increased apoptosis with IC50 of 37 μM, a dose ~2.5 fold lower than native Myricetin. Mechanistic insights reveal that Myr-NE induced more ROS generation and considerably inhibited AKT and mTOR activation, leading to enhanced anticancer activity. Conclusions & Implications: In conclusion, these findings suggest that the therapeutic efficacy of Myricetin significantly improved through a novel Myr-NE formulation which may be a promising therapeutic approach for treating TNBC.
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Data Availability Statement:
The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

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Experimental Approach: The nano-emulsion was formulated using Capryol 90 as oil, Tween 20 as a surfactant, and Transcutol HP as a co-surfactant. Further comparative analysis of Myricetin and nano-emulsified Myricetin (Myr-NE) were carried out in triple negative breast cancer (MDA-MB-231) cells for anticancer activity.

Key results: The optimized Myr-NE had a zeta potential of -6.35±0.3, an average particle size of 89.32±2.8 nm, PDI of 0.105±0.02, and a spherical shape as confirmed in transmission electron microscopy. Diffusion-dominant drug release was observed with 95.49±2.84 % Drug release for 24hrs, 2-fold higher than Myr-suspension. When nano-emulsified, Myricetin exhibited efficient inhibition of cell proliferation, clonogenicity, and increased apoptosis with IC50 of 37 μM, a dose ~2.5 fold lower than native Myricetin. Mechanistic insights reveal that Myr-NE induced more ROS generation and considerably inhibited AKT and mTOR activation, leading to enhanced anticancer activity.

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Keywords: Myricetin, Myricetin Nanoemulsion, TNBC, Akt/mTOR pathway, Oxidative stress, Cell death

INTRODUCTION

The second leading cause of cancer-related mortality in women today is breast cancer, which accounts for 20% of all cancer types [1]. Breast cancer can be further categorized into many categories based on receptor expression or presence. Progesterone, Estrogen, and the human receptor for an epidermal growth factor (HER2) are hormone receptors that are typically present in cases of breast cancer; however, certain cases
of breast cancer lack these receptors. Triple-negative breast cancer (TNBC), makes up about 15-20% of all occurrences of breast cancer and is associated with poor clinical outcomes [1,2,3]. The most common problem with TNBC is the recurrence of disease and chemotherapy resistance. Common features of TNBC are metastasized easily, poor prognosis, pain, and a high mortality rate (approximately 20%) among all subtypes of breast cancers. Poor prognosis and less survival time show the heterogeneous nature of TNBC among other subtypes of breast cancer [1,4]. Chemotherapy treatment work effectively in the early stages, but the response becomes worse in advanced stages [1]. For efficient anticancer agents, it is necessary that they should have a maximal anticancer property with minimal side effects. Therefore, natural compounds are gaining interest as they have high therapeutic value with lesser side effects, and their biological efficacy provides a base to create more effective anticancer agents. Flavonoids are non-toxic plant based products generally present in vegetables, fruits, tea and wine. [5]. Myricetin is a naturally existing flavonol and a bioactive molecule presents in the bark of Myrica rubra and can be found in many other herbs like Vitis vinifera Linn, Dioscorea bulbifera L., Ardisia colorata Roxb, Carissa opaca and Cudrania tricuspidate [6]. Myricetin is also naturally found in many vegetables and several foods consumed daily in our diet like teas, berries, grapes, onions, beans and red wine [6-7]. Myricetin exerts strong antioxidative properties and many other biological properties like antidiabetic, antibacterial, and neuroprotective and potent anticancer activity [6-7]. Myricetin has shown anticancer potency in colon cancer [8], Glioblastoma [9], hepatocellular [10] and pancreatic carcinoma [11] through modulation of the phosphatidylinositol-3-kinase, mammalian target of rapamycin signaling (PI3K/AKT/mTOR) pathway. After so many biological properties and easily available natural flavonoids with non-toxic properties, Myricetin is limitedly used as a therapeutic agent due to its low solubility in water. Myricetin has low biological availability, approximately 10%, due to its poor aqueous solubility (16.60 μg/mL) and gastrointestinal tract (GIT) instability that limits its efficacy as a therapeutic agent [12]. Thus, other simple and effective methods are needed to enhance Myricetin solubility and bioavailability. Nanoemulsion is one of the most innovative and promising drug delivery technologies currently available. It has several advantages, including a long shelf life, simplicity of preparation, ready scalability, robust separation, flocculation, and coalescence stability, increased drug loading, protection of labile drugs, and increased drug absorption and permeation. [13-15]. NE, which are transparent systems of oil and water stabilized by an interfacial layer of surfactant and cosurfactant molecules with a droplet size of less than 100 nm, have very high thermodynamic stability. In these systems, water-rich and oil-rich domains are divided by surfactant-rich sheets, which can be categorized as oil-in-water (o/w), water-in-oil (w/o), or bicontinuous. Oil, water, surfactant, and cosurfactant are mixed in the right proportions to create these emulsions using the emulsification technique (phase titration). The process is nearly spontaneous and only needs moderate agitation to begin [15]. PI3K/AKT/mTOR pathway has been found in many cancers like colon cancer, glioblastoma, and many more but has recently been identified as one of the most important and major pathways involved in the TNBC development and chemoresistance and plays a huge role in the progression and metastatic development on TNBC. This pathway has been used as a molecular target for the design and development of potential therapeutic anticancer agents for the cure of TNBC [17–19]. Therefore, this study is designed to evaluate the effect of Myricetin and its NE formulation for its potential anticancer activity in TNBC through modulation of the PI3K/AKT/mTOR pathway.

MATERIALS AND METHODS

2.1 Materials

Myricetin was obtained from Cayman chemicals Co. (USA). Capryol 90 (propylene glycol monocaprylate), Tween-20, Tween 80, Transcutol HP, Transcutol P, Isopropyl myristate (IPM), Phurul oleique, Labrasol (PEG-8 caprylic/capric glycerides), Capmul MCM (mono-diglyceride of caprylic and capric acid) and Labrafac lipophile WL 1349 (triglycerides of caprylic and capric acids). Generously given by BASF. Cremophor ELP, Cremophor EL, and Cremophor RH40 (Ludwigshafen, Germany). The cell growth medium Dulbecco’s Minimum Essential Medium (DMEM) high glucose, sodium pyruvate, antibiotics (Penicillin G, streptomycin,) antifungal (Nystatin), Bicinchoninic acid (BCA), sulforhodamine B (SRB), were obtained
from Sigma Chemicals Co. (St Louis, U.S.A.), Fluorescent probes Calcein-AM, propidium iodide (PI), Mi-
toSOX, and CM-H2DCFDA were procured from Molecular Probes (Eugene, U.S.A.) Primary antibodies
mTOR (#2972S; CST), Phospho-mTOR (#2971S; CST), Bax (2772S; CST), Bcl2 (#MA5-11757; CST),
Phospho-Akt (#S473; CST), Pan-Akt (#C67E7; CST), Bcl-XL (#B9429; Sigma), β-actin (#612657; BD
transduction laboratories) whereas secondary antibodies (HRP conjugate) were purchased from Thermo
Fisher Scientific (USA).

2.2. Myr-NE preparation and optimization

2.2.1. Screening of components for NE preparation: Solubility and Miscibility studies: As
discussed in an earlier publication [19-20], successful screening of various excipients (Oil, surfactants, and
cosurfactants) was performed based on their drug solubility and miscibility ability, which is critical to the
formulation development and management of a NE stability. Oils such as (Capryol 90, Castor oil, Labrafac
Lipophile WL, Capmul MCM, Isopropyl myristate (IPM), Ethyl oleate, and Plurul oleique), surfactants
(Kolliphor EL, Cremophor ELP, Labrasol, Tween 80, and Tween 20) and co-surfactants (Polyethylene glycol
200 (PEG)), Transcutol HP, Transcutol P, and Propylene glycol) were used for screening. An excess amount
of Myricetin (drug) was added to an Eppendorf along with 1 mL of each excipient (Oil, surfactants, and
cosurfactants) and then kept on an isothermal shaker (SHEL LAB) for 72 hours; at 25 °C. After centri-
fugation (800g; 10mins.) supernatant was collected followed by processing through syringe filter (0.45 μm)
and the dilution of resulting supernatant was made in methanol. Further samples were run through a UV
spectrophotometer at λmax of 370 nm to determine how much of the drug had been solubilized. For misci-
bility studies, oil, surfactants, and co-surfactant were taken in equal amounts and vortex and kept for 24hr
for visual inspection to select suitable Smix and oil for the development of stable NE.

2.2.2. Construction of pseudo-ternary phase diagrams, and Placebo NE preparation

We developed the placebo NE through an aqueous microtitration procedure and then used a Remi cyclomixer
(Vortex) to evaluate the oils we had previously chosen with the surfactant: co-surfactant combination (Smix).
A clear blend of the chosen oil (Capryol 90) and Smix (Tween 20 and Transcutol HP) were prepared through
vertexing and diluted with double-distilled water. This step’s goal was to determine whether the oils were
compatible with the Smix and whether they were miscible with it. The amount of Smix was optimized
using different ratios (1:1, 2:1, 3:1, 4:1, 5:1, 1:2, 1:3, 1:4), which aided in a clear NE (Placebo) formation
in terms of turbidity and visual clarity following each dilution. Additionally, different oil and Smix ratios
were used to plot a pseudo-ternary phase diagram and achieve a larger NE region, including 16 different
combinations of oil and Smix ratios (1:9, 2:8(1:4), 3:7(1:2.3), 4:6(1:1.5), 5:5(1:1), 6:4 (1:0.7), 7:3(1:0.43),
8:2(1:0.25), 9:1(1:0.1), 1:2, 1:3, 1:3.5, 1:5, 1:6, 1:7, 1:8). Each point was carefully considered or examined
before being noted. The data obtained from the titration procedure were used to construct pseudo-ternary
phase diagrams. [19-20].

2.2.3. Preparation of Myr-NE

Myricetin-loaded nanoemulsion was prepared using the spontaneous emulsification method. The required
amount of Myricetin was first dissolved in a pre-selected oil; then, it was dissolved in a Smix. The obtained
blend was then micro-titrated with double-distilled water [20]. Transparent formulations, evaluated by visual
inspection, are taken for further thermodynamic stability studies.

2.2.4. Thermodynamic stability studies:

For the determination of thermodynamic stability of prepared Myr-NE formulations previously reported
method was slightly modified. This to analyse the NEs’ thermodynamic stability studies for phase separation,
clarity, and droplet size.[21].

• Heating cooling cycle: The test was used to investigate how the temperature affected the stability of
NEs. Over three separate cycles, NE formulations were kept at 4°C and 45°C for 48 hours. Formulations
that passed this test and remained stable were subjected to a freeze-thaw cycle test.
• **Freeze-thaw cycle:** In this process, NE formulations were kept between -21°C and 25°C for at least 48 hours over three separate cycles. Formulations that passed this test and were stable afterward underwent a centrifugation test.

• **Centrifugation test:** The prepared formulations were centrifuged (800g; 30mins.) and the formulations that did not exhibit any phase separation were chosen for further optimization.

• **2.3 Characterization of optimized Myr-NE**

A dynamic light scattering technique (DLS) was used to measure the Average Particle size (PS), zeta-potential (ZP), and Polydispersity index (PDI) (Malvern Zetasizer, Nano ZS Worcestershire, UK). The formulation was diluted at a ratio of 1:50, the system temperature was held constant at 25 °C, and the scattering angle was 90° during the analysis [12].

During the analysis, the composition was diluted down to a concentration ration of 1:50, and the temperature of the system was maintained at 25, and the dispersion angle was set at 90 degree.

• **2.4 Determination of drug content**

Drug content is an important parameter for the evaluation as it is used to determine the amount of drug entrapped in the nanoemulsion formulation. Myr-NE formulation’s drug content was measured by the UV-visible spectroscopy method. Each formulation’s 1 ml sample was centrifuged for 30 minutes at 3500 rpm. Following centrifugation, the supernatant layer was removed and transferred to another tube, where it was diluted with double-distilled water. Using UV- spectroscopy, the maximum absorbance was determined to be 370 nm [12].

• **2.5 Transmission electron microscopy (TEM)**

The surface morphology of the NE system containing Myricetin was evaluated using TEM (Philips Briarcliff Manor, Briarcliff Manor, USA) as described earlier [12]. To prepare the sample, copper grids were placed onto a piece of dust-free paraffin and a pre-diluted sample drop in the ration of 1:100 was pipetted into the grids. The copper grid was placed in the same paraffin sheet along with 2% phosphotungstic acid (a contrast agent). After the copper grids had been removed for a few minutes, they dried with Whatman filter paper and analyzed on TEM (operated at 200kV) [12].

• **2.6 Differential scanning calorimetry (DSC):**

DSC was performed for the analysis of melting point and solubilization, 2 mg sample of Myricetin, Myr-NE (lyophilized 5% w/v), and mannitol sample was sealed in an aluminum pan, and then the sample was allowed to heat at a temperature range of 100 - 450 oC with a constant rate of 10 oC/m. At a flow rate of 60 ml per minute, nitrogen gas was purged to maintain an inert atmosphere [20].

• **2.7 Fourier-transform infrared spectroscopy (FT-IR) examination of Myricetin, Myricetin-NE lyophilized (5% w/v), and mannitol was performed by using KBr pellet method, samples were weighed accurately, and 5 mg of each was blended with potassium bromide in 1:1 ratio followed by hydraulic press compaction for uniform pellet formation. Scanning of resulting pellet was performed between a 4000–500 cm1 wavenumber in the infrared spectrum of light. The FT–IR spectrum of the Myricetin powdered sample was recorded at a scan range of 400-4000 cm-1[20].**

• **2.8 In-vitro drug release study**

An in-vitro drug release study was performed as described earlier [12] with some modifications. The in vitro release of Myricetin and Myr-NE was performed using the dialysis membrane technique. A dialysis bag using an average molecular weight of 12000 Da was activated with pre-treatment. 3 mL of the optimized Myr-NE (5 mg Myricetin) and Myricetin suspension (5 mg) were filled in a dialysis bag, ends of the membrane were sealed and kept in a dissolution medium with a pH 7.4 PBS, which was stored in a water bath shaker (which was temperature controlled) at 37 +1.3degC and permitted to shake at 100 rpm. At each time point (0, 0.25, 0.5, 1, 2, 4, 8, 12, & 24 h), the needed amount of sample 5ml was removed and replaced with the same volume of fresh medium the samples’ absorbance was measured at a λ_max of 370 nm by UV spectrophotometry.
2.9 Sources of cell lines

Triple-negative breast cancer cells (MDA-MB-231) were obtained from National Centre for Cell Sciences (NCCS), Pune, India. MDA-MB-231 cells were routinely cultured in DMEM high glucose containing 10% fetal bovine serum (FBS; South American origin) and antibiotics, followed by incubation at 37°C in CO₂ incubator. All experiments were performed with exponentially growing cells.

2.9.1 Drug and formulation treatments

MDAMB231 cells were treated with Myricetin and Myr-NE for 24 and 48 hrs (continuous exposure) at different concentration ranges (10-150 μM) for IC₅₀ determination. The remaining experiments were carried out at 4 and 24 hrs using IC₅₀ values of 92μM and 37μM for Myricetin and Myr-NE, respectively.

2.9.2 SRB assay and IC50 determination

IC₅₀ values of Myricetin and Myr-NE were screened and estimated using sulforhodamine B (SRB) cell proliferation assay, as described earlier [22]. Briefly, 0.003×10⁶ cells were seeded in a 96-well plate. Myricetin and Myricetin loaded NE were then added to the cells at concentrations ranging from (10 to 150 μM). At the specified time of termination cells were processed for cell fixation and staining as per the previously described method [23]. Finally, absorbance was taken at 560 nm on a spectrophotometer (Biotek Instruments, USA). Graphs for both the treatment groups were plotted as concentration vs. percent inhibition response.

Immunoblotting, cell viability, and macro colony assay

Protein expression levels were measured for pan AKT, phospho-AKT (p-AKT), mTOR, phospho-mTOR (p-mTOR), Bax, Bcl-2, and Bcl-XL using immunoblotting as per the previously reported methodology [23]. Image J was used for the densitometric analysis, and the resulting bar graph presented the relative fold change in protein expression between the control and treatment groups. For cell viability assay, cells were seeded (density of 0.3×10⁶) in PD-60. Cells were treated with drug and formulation using their respective IC₅₀ values, and Calcein AM/PI staining was performed at 4 and 24 hrs post-treatment. Cells were stained with probe buffer containing MgCl₂(1mM), CaCl₂ (1mM), glucose (5mM), Calcein AM (3μM), and PI (2μM) in phosphate-buffered saline and incubated for 20 minutes at 37 °C in CO₂ incubator. Cells were washed, trypsinized, and resuspended in PBS for the acquisition on a flow cytometer (BD, FACS Aria Tm III Cell Sorter, USA). A macro colony assay was performed (post-plating method) for clonogenic survival analysis in MDA-MB-231 cells. Initially, cells were seeded with a density of 0.075×10⁶ in PD-35. After 48 hrs, cells were treated with Myricetin and Myr-NE with their respective IC₅₀ for 4 hrs., followed by washing with sterile PBS. Cells were trypsinized, counted, reseeded with very low density (100 cells/PD 60), and kept in a CO₂ incubator for 8 days to allow colony formation. After that, cultures were terminated, washed with PBS followed by crystal violet staining, and air dried, and colonies containing a minimum of 50 cells were considered survivors.

Cell cycle analysis, estimation of oxidative stress, and apoptosis assay

Cell cycle analysis cells were harvested at two different time intervals (4 and 8 hrs), by ethanol fixation (70% ethanol) and kept at -20. At the time of acquisition, cells were treated with RNase A (200 μg/ml; 30 min;37°C). Finally, PI (50μg/ml) was added, followed by data acquisition on the flow cytometer. An intracellular and mitochondrial RIOS were separately using CM-H2DCFDA (10μM) and Mito Sox (5μM), respectively, followed by incubation (37°C; 30 mins.). Finally, cells were washed and resuspended in PBS for acquisition on the flow cytometer. Apoptosis assay was performed using an Annexin V/PI staining kit (Invitrogen; Cat# VI3241) as per the manufacturer protocol, and data was acquired on the flow cytometer.

2.3 Statistical analysis

All the experiments were conducted at least three times in triplicates or quadruplicates. Data are presented as the mean, standard deviation ± SD (n=3). Statistical analysis was performed using Student’s t-test using software (GraphPad prism, version 8), and a value p [?] 0.05 was considered as statistically significant (*; #).
3. RESULTS

3.1 Myr-NE preparation and optimization

3.1.1. Screening of component for NE preparation: Solubility and Miscibility studies:

The solubility of Myricetin in various oil, surfactant, & co-surfactant was investigated (Fig. 1 A). The highest solubility of Myricetin among the oils phases was found in Capryol 90. Capryol 90 has permeation enhancer properties, good emulsification capacity, and have potential to easily solubilize water-insoluble drugs [24]. Therefore, the oil phase selected for the Preparation of Myr-NE was Capryol 90. Tween 20 was selected as a non-ionic surfactant due to its highest solubility among other surfactants as per solubility studies (less toxic, high HLB value), and more soluble in the aqueous phase [24, 15]. Transcutol HP is a hydrophilic co-surfactant that increased the spontaneity of nanoemulsion formation, and due to its highest solubilization capacity, it was selected as a co-surfactant [24]. Miscibility studies were also found to be clear and stable for the above-chosen excipients as per the visual inspection.

3.1.2. Construction of pseudo-ternary phase diagrams, Preparation of placebo NE

A pseudo-ternary phase diagram was used to further evaluate the combination of the chosen surfactants for the Smix ratio. Tween 20 and Transcutol HP were mixed (Smix) in seven different ratios as the surfactant and co-surfactant. The oil phase (Caproyl 90) was then mixed in 16 different ratios with each Smix ratio. The phase diagrams of various Smix to oil phase ratios are shown in (Fig. 1B). NE is indicated by dotted points. Following the Construction of the phase diagram for the NE Smix ratio 2:1 produced the highest NE region due to the highest HLB and amount of the surfactant. Other Smix ratios 1:1 and 3:1 shows good nanoemulsion area but the Smix ratio 1:4 produced the lowest NE area. Other ratios 1:2, 1:3, 1:4, 4:1 and 5:1 do not fulfill the criteria or have the minimum zone of NE, leading to droplet breakdown and disruption. The pseudo-ternary phase diagrams helped in finding the lowest and maximum percentages of oil and Smix to be used to produce a clear, translucent, and homogeneous NE, and the ratios of Smix exhibiting larger nanoemulsion area were then used to create a set of placebo formulations.

3.1.3 Preparation of Myr-NE

Based on the phase diagram, oil was sufficient for the solubilization of the drug, and Smix ratio was minimum further used to develop the drug-loaded formulation; ultrasonication was used to minimize the particle size; the method was similar to the prepared mention above placebo, and in this case, we have added the drug in the oil phase and titrated with water phase and evaluated for thermodynamic stability test (heating/cooling, freeze/thaw, centrifugation). Smix ratios (1:1, 2:1 and 3:1) showed good emulsifying properties among them 2:1 covered the maximum area under the pseudo-ternary phase diagram and was clearer and more transparent. More than 90 formulations were made from these Smix ratios, and visually observed the point at which formulations become turbid. Some transparent NE systems and concentrations of Smix ratios 4:1, 5:1, 1:2, 1:3, 1:4 show less shaded regions, which indicates the poor self-nano emulsifying ability of Smix at these ratios.

3.1.4 Thermodynamic stability studies

Thermodynamic stability of Myr-NE including heating-cooling, centrifugation, and the freeze-thaw cycle showed that NE formulations exhibit turbid and phase separation (Table 1). It is possible that this phase separation is attributed to Ostwald ripening, a process of free-surface energy in which tiny droplets combine through diffusion to form larger droplets or aggregates. Twelve Myricetin-loaded NE formulations were developed from these ratios (1:1, 2:1, and 3:1) and optimized to determine the thermodynamic stability of the Myr-NE systems (O/W), as shown in (Table 1). During the stress test, four NE formulations (FA-3, FA-4, FC-1, and FC-4) were found to have phase separation and cracking. The remaining eight NE formulations (FA-1, FA-2, FB-1, FB-2, FB-3, FB-4, FC-2, and FC-3) did not indicate phase separation, cracking, or creaming, and all were stable during the study, explicit and transparent. Thermodynamic stable NEs were further evaluated for average particle size, PDI & zeta potential.
3.2 Characterization of optimized Myr-NE

3.2.1 Average Particle size (PS), zeta-potential (ZP), and Polydispersity index.

All formulations were stable and showed good results, as shown in Table 2, but the best result was obtained from Formulation FB-2 due to maximum drug loading, low PDI, the minimum amount of Smix, and enough oil required for drug solubilization. The optimized Myr-NE formulation FB-2 showed an average particle size of 89.32±2.8 nm and PDI of 0.105±0.02 (Fig. 2 A). Due to the usage of non-ionic surfactants and co-surfactants, the zeta potential value was -6.35±0.3 Mv (Fig. 2 B). The optimized drug content of the Myr-NE was observed as 98.44±2.1%. The nano size, as previously mentioned, ensures a sufficient surface area and more effective drug delivery, absorption, and retention.

3.2.2 Transmission electron microscopy (TEM)

Transmission electron microscopy (TEM) images showed that the Myr-NE was circular in shape with an average particle size of less than 100 nm. (Fig. 2 C). Additionally, there was a clear correlation between the obtained size range and the zeta sizer’s observations. As a result, the optimized formulation is suitable for effective delivery due to its nanometric and globular sizes, respectively.

3.3 Differential scanning calorimetry (DSC)

DSC was further performed to examine the change in the melting point of Myricetin and Myr-NE. Fig. 3 A displays the peak of the Myricetin has a well-marked endothermic peak with a melting point of 358 degC & a fusion heat of 29.37 J/g. The Myr-NE formulation’s DSC thermogram identified a single peak at 175.587 degC with an area of 2922.906 mJ. The single peak may have been caused by the high mannitol content, as well as by the change of a Myricetin & Myr-NE from such a crystalline to an amorphous form, which did not show any notable peak.

3.4 Fourier Transforms Infrared Spectroscopy (FTIR)

The FTIR (KBr) spectra of the Myricetin. (Fig. 3 B) showed characteristic peaks (cm⁻¹) noted at, 3420.13 (O-H stretching, alcohol), 2930.07 (C-H stretching, alkane), 1660.41 (C=N stretching, amine), 1519.63 (N-O stretching, nitro compound), 1326.30 (S=O stretching, sulfone), 1168.16 cm⁻¹ (C-O stretching, ester) respectively (Fig. 3 B). Similarly, the spectra of the mannitol showed peaks (KBr) at 3401.33 (N-H stretching, amine), 3293.82 (O-H stretching, carboxylic acid), 2969.35, 2946.69 (C-H stretching, alkane), 1420.31 (O-H bending, alcohol), 1280.50 (C-O stretching, aromatic ester), 1080.90 (C-O stretching, primary alcohol), 703.89 (C=C bending, alkene), and 632.05 cm⁻¹ (C-Br stretching, halo compound). Whereas in the FTIR spectra of the lyophilized Myr-NE (KBr), the bands were observed at 3416.37 (N-H stretching, amine), 2963.09 (C-H stretching, alkane), 1419.19 (C-H bending, alkane), 181.79 (C=C bending, alkene), 699.55 (C=C bending, alkene), and 630.12 cm⁻¹ (C-Br stretching, halogen). Collectively, a lyophilized form of Myr-NE had somewhat different peak positions as than the original, indicating that it was amalgamated or complicated in nature. Given that it was utilized excessively to assure successful lyophilization of the Myr-NE, the mannitol (cryoprotectant) spectra’s peaks were remarkably similar to those of the Myr-NE.

3.5 In-vitro drug release study

The percent drug release profile for optimized Myr-NE, and Myricetin, were performed using a dialysis membrane in PBS (pH 7.4). (Fig. 4 A). After 24 hours, the % CDR of Myricetin release from NE and the free suspension was found to be 95.49±2.84 and 45.53±3.66%. The result showed that Myr-NE releases faster than free Myricetin due to its high solubility. Different numerical models were applied to study the release pattern of drugs from the NE system. The drug release kinetics of Myricetin showed best fitting Higuchi model with R² value 0.936.

3.6 Myricetin-loaded NE significantly reduces IC50 of Myricetin alone in TNBC cells.

After the chemical and structural evaluation of Myr-NE, we sought to determine its effect on triple-negative breast cancer (TNBC) cells and compared it with Myricetin alone. An in-vitro comparative assessment was
carried out in the MDA-MB-231 cell line. The half maximum inhibitory concentration (IC50) values were primarily screened at different concentrations ranging from 10 to 150 μM using a cell proliferation (SRB) assay at 24 and 48 hours (hrs) post-treatment (Fig. 5).

Myricetin alone and Myricetin loaded NE showed a concentration-dependent inhibition of cell proliferation in MDA-MB-231 cells (Fig. 5 A-D). The IC50 value of Myricetin was estimated as 92 μM at 24 hrs, while it increased to 107μM after 48 hrs. of drug treatment (Fig. 5 A&C). On the contrary, the IC50 values of Myr-NE were estimated as 37μM and 48.85μM at 24 and 48 hrs, respectively (Fig. 5 B&D). The IC50 value for Myr-NE was found to be decreased by more than 2-fold at both the tested time points compared to Myricetin alone (Fig. 5 A-D). These results demonstrate that Myr-NE have a profound inhibitory effect on TNBC cell proliferation and require considerably lower compound concentrations than Myricetin alone.

Myr-NE potentially inhibits AKT signaling and induces cell cycle arrest

Myricetin is well evident as a regulator of the PI3K/Akt signaling pathway, which binds to these kinases and prevents their phosphorylation which in turn inhibits the phosphorylation of downstream mTOR and eventually abrogates its activation in cancer cells. Therefore, we sought to compare the effects of Myr-NE with Myricetin alone on AKT signaling and cell cycle to effectively comprehend the mechanism by which Myr-NE significantly inhibited cell proliferation of MDA-MB-231 cells. In line with earlier findings, Myricetin showed a substantial decrease in AKT and mTOR phosphorylation with respect to control (Fig. 6 A). However, at the same time, treatment with Myr-NE confirmed an additional significant attenuation in both p-AKT and p-m-TOR with respect to Myricetin alone (Fig. 6 A). These results indicate that the pronounced inhibition of MDA-MB-231 cell proliferation (Fig.5) in Myr-NE is ascribed to significant downregulation of p-AKT and p-m-TOR as compared to Myricetin alone. Myricetin-induced inhibition of cell proliferation is reported to link with cell cycle arrest, which varies among sub-G1, S, and G2-M phases in different tissue-origin cancer cells [25-26]. We monitored the cell cycle progression at 4 and 8 hrs. post-treatment of Myricetin and Myr-NE in MDA-MB-231 cells (Fig.6 B). Myr-NE treated cells showed a substantial increase in the Sub-G1 population (~10%), compared to control, indicating the cell death induction as early as 4 hrs in MDA-MB-231 cells. In contrast, this effect was shifted to 8 hrs in Myricetin alone (9%) with respect to control (Fig. 6B). At 8 hrs the S phase arrest was observed in both the treatment groups, and increased proportion was estimated as Myricetin (21.6%) and Myr-NE (15.3%), compared to control. At this time, the Myr-NE treatment also showed a concomitant increase in the cellular proportion of the G2-M phase by 10% compared to the control, which was confined to only 4.4 % in Myricetin alone (Fig. 6 B). Although the combined effect on cell cycle arrest (S and G2-M) showed similar response in both the treatment groups (~46%); nevertheless, an increased cellular proportion in G2-M phase of Myr-NE treated cells indicate substantial DNA damage and corroborated the previous results of an efficient inhibition in the proliferation of MDA-MB-231 cells.

3.8 Myr-NE induces more cell death than Myricetin alone

The regulation of p-AKT and p-m-TOR by Myr-NE led us to investigate its effect on cell viability and compare it with Myricetin alone. Cell viability was performed using Calcein-AM/PI staining, which enables the simultaneous identification of live and dead cells based on their intracellular esterase activity and the integrity of the plasma membrane (Fig. 7 A-B). Myr-NE treatment substantially reduces the cell viability at both the tested time points of 4 and 24 hrs post-treatment with respect to control and Myricetin alone (Fig.7 A-B). However, Myricetin alone showed only a significant change in the loss of cell viability at 24 hrs post-treatment as compared to the control (Fig.7 B). At 24 hrs., the quantitative estimation was significantly correlated with the microscopic examination of Calcein-AM/PI in MDA-MB-231 cells (Fig.7 C). In context with the results pertaining to cell proliferation, it was intriguing to observe that treatment with Myr-NE causes a time-dependent shift in cell sensitization efficiency (from 4 to 24 hrs), whereas the effect of Myricetin alone was mostly confined to cell growth inhibition only. Further clonogenic assay was performed after the 4 hrs. treatment of both compounds (Fig.7 D). Although Myricetin treatment showed a significant reduction of 37% in clonogenicity as compared to control, nevertheless, Myr-NE resulted in an additional 25% (62% with respect to control) reduction over and above Myricetin alone. Previously it has been found that Myricetin induces cell death by inducing oxidative stress in cancer cells [27, 28]. Therefore, further analysis was carried
out for the estimation of both intracellular and mitochondrial oxidative stress using fluorescent probe CM-H2DCFDA and MitoSox, respectively, in MDA-MB-231 cells (Fig. 7 E). At both the tested time points of 4 and 24 hrs, Myricetin showed a substantial increase in cellular reactive oxygen species (ROS) generation including both intracellular and mitochondrial reactive ROS compared to the control (Fig. 7 E). However, Myr-NE further augmented these effects, with higher intracellular and mitochondrial ROS than Myricetin alone. The consequence of profound alteration in cellular redox status was further confirmed by cell death using Annexin V/PI assay at 24 hrs in both the treatment groups. Myr-NE induced a substantial increase in apoptosis estimated as ~ 56% as compared to 27% in Myricetin treatment alone (Fig. 7 F). In line with observed cell death, we found that Myr-NE induced significant upregulation of pro-apoptotic protein Bax and a considerable decrease in critical anti-apoptotic proteins Bcl-2 and Bcl-XL (Fig. 7 G), which in turn led to enhanced cell death in MDA-MB-231 cells. These results demonstrate that Myr-NE is advantageous over Myricetin alone in inducing oxidative stress, and the downregulation of anti-apoptotic proteins eventually results in a rapid decline in cell viability, clonogenicity, and accelerated cell death in TNBC cells.

4. Discussion

10-20% of invasive breast cancers are TNBCs, which are characterized by the under-expression of estrogen receptors (ER) and/or progesterone receptors (PR) and the over-expression of human epidermal growth factor 2 (HER2). [29]. TNBCs also resist HER2- and ER-targeted medications. Therefore, chemotherapy is the only adjuvant treatment after surgery. [28]. To combat this aggressive form of breast cancer, improved therapeutic strategies must be developed. The PI3K/AKT/mTOR pathway is one of the critical and active mechanisms that may be involved in the survival and chemo-resistant behaviour of TNBC. Since it is believed to play a major role in malignant transformation, this pathway represents a prospective molecular target for TNBC treatment [30]. Anticancer drugs must have maximum efficacy and minimal adverse effects to be effective. Phytochemicals are well known bioactive ingredients present in whole grains, fruits, vegetables, reported to reduce the possibility of cancer development [28, 31]. Moreover, earlier studies using both human and animal tissue origin cell lines have shown that natural phytoconstituents exhibits variety of anticancer properties which are novel, effective, potent, and safe [31, 32]. It is essential for there to be a maximum anticancer property alongside a minimum number of negative effects in order for the development of effective anticancer drugs. Flavonoids are chemicals produced by plants that have diverse biological effects and are normally non-toxic. Myricetin is a natural flavonol found in fruits, herbs, vegetables, wine, and tea. Myricetin is a phenolic phytochemical that inhibits invasion, causes apoptosis, and increases chemosensitivity in several human cancer cell lines. [33-35]. Despite the role of Myricetin as an anticancer agent, poor water solubility and low bioavailability limits its effective use. NE is the best option for enhancing solubility and bioavailability and achieving the best therapeutic effect of poorly soluble drugs. NE has many advantages like easy Preparation, low viscosity, rapid absorption, strong solubilizing effect, optical transparency, and thermodynamically stability [15]. Selection of components for the Preparation of NE is the most crucial part and also depends upon the solubility of Myricetin in different components. As per the Solubility studies (Fig. 1A), the highest solubility was observed for Myricetin in Capryol 90, Tween 20, and Transcutol HP compared to other oils, surfactant, and co-surfactant as well as miscibility studies for the combination of Capryol 90, Tween 20 and Transcutol HP shows no phase separation after a long time. Many NE formulations have been developed in previous studies using Capryol 90 as an oil phase and have shown to be an effective anticancer therapy in prostate cancer [14], Lung cancer [36]. Tween 20 is a non-ionic surfactant with lower toxicity, greater pH and ion stability, and a high HLB value. [15]. Drug release and absorption are determined by droplet size. Smaller droplets dissolve faster and provide more medication absorption surface area. Furthermore, (Fig. 1B) using pseudo-ternary phase diagrams, the combination (Smix ratio) of Tween 20 and Transcutol-HP was chosen to produce a clear NE using placebo formulations. As observed 2:1 ratio of Smix shows the highest dotted area as compared to other ratios; hence 2:1 was used as the optimal ratio for further NE formulations. Pseudo-ternary phase diagrams were also useful in determining the minimum and maximum oil and Smix proportions to produce a clear, transparent, and homogeneous NE [20]. Fig. 2A represents the particle size of Myricetin-NE was 89.76±1.32 nm, which is in the range of
NE. The PDI value for Myricetin-NE was 0.105 (Fig. 2A), PDI is another important element in assessing particle homogeneity. The smaller PDI ranges from 0.0 to 1.0, indicating a more stable, uniform NE. The zeta-potential value of Myricetin-NE was -6.33 ± 0.14 mV (Fig. 2B), indicating the stability of NE. Optimised Myricetin-NE formulations demonstrated excellent thermodynamic stability under stress circumstances, as determined by thermodynamic stability tests. According to the results, no precipitation, crystallization, or flocculation was seen (Table 1). TEM finding suggests that the formed Myricetin-NE were predominantly spherical, and the diameter of the particles was less than 100 nm which was in agreement with the findings of the DLS (Fig. 2B). The spontaneous creation of emulsions following dissolution increases Myricetin in vitro release rate and extent. Thus, this dissolution of Myricetin from Myricetin-NE may result in increased absorption and bioavailability. The result showed that Myricetin-NE released faster than free Myricetin due to its high aqueous solubility (Fig. 4A). In sequence, we did the comparative assessment of Myricetin and Myricetin alone in TNBC (MDA-MB-231) cells which are recognized as a particularly aggressive malignancy with poor prognosis and not susceptible to standard hormone therapy [37, 38]. Cytotoxicity studies suggest that IC50 of Myr-NE is lowered by 2-fold as compared to Myricetin alone which can be ascribed to greater absorption of the NE mainly owing to the lipid-mediated enhanced solubility (Fig. 5). The substantial loss to cell viability as early as 4 hrs following Myr-NE treatment was found in correlation with the early increase in percentage of Sub-G1 population (Fig. 6B, and Fig. 7A); whereas G2-M arrest indicated substantial DNA damage [39] confirm an efficient decline in cell proliferation of MDA-MB-231 cells (Fig. 5, and Fig. 6B). The overall effect of both the compound on cell cycles did not show considerable interchange; whereas a significant increase in cell death was observed in Myr-NE treated cells, compared to Myricetin alone (Fig. 7). Multiple lines of evidence demonstrate that the PI3K/Akt/mTOR pathway is critical to chemoresistance and survival of cancer cells, whereas Myricetin treatment is reported to induce cell death by inhibition AKT and mTOR activation in TNBC cells [1, 40]. Considering these facts our results showed that the effect of Myr-NE is more pronounced in the inhibition of AKT and m-TOR activation than Myricetin alone. Therefore, it is reasonable to speculate that Myr-NE-induced loss in cell viability and enhanced cell death is majorly attributed to the significant inhibition of AKT signaling in MDA-MB-231 cells [Fig. 7]. Notably, even after minimum exposure time (4 hours), the NE-loaded Myricetin also showed a considerable reduction in clonogenic survival, validating its long-term inhibition effect in TNBC cells (Fig 6D.). Earlier research reported that mitochondrial dysfunction and the BAX/Bcl-2 dependent pathway drive Myricetin-induced apoptosis in cancer cells [41,42]. Consistent with these findings, Myr-NE showed significant upregulation in Bax/Bcl-2 ratio and a substantial decrease in Bcl-XL as compared to Myricetin alone (Fig 6B-C.). Myricetin-induced oxidative stress was consistent with the previous reports [27,31]; nevertheless, the considerable elevation in mitochondrial ROS corroborated the dominant effect of Myr-NE treated cells over and above Myricetin in inducing mitochondrial dysfunction and enhanced cell death in MDA-MB-231 cells (Fig. 7). These results demonstrate that Myricetin-loaded NE may prove to be a promising therapeutic strategy since they have more potential for sensitization of TNBC cells than Myricetin alone. The results of the present study also provide a substantial basis for evaluating the in-vivo efficacy of Myr-NE and its effect on the PI3K/Akt/mTOR pathway.

5. Summary:

- Myr-NE exhibit high aqueous solubility and showed faster release than Myricetin alone.
- Myr-NE have a profound inhibitory effect on TNBC cell proliferation and require considerably lower compound concentrations than Myricetin alone.
- Myr-NE is advantageous over Myricetin alone in inducing oxidative stress, a rapid decline in cell viability, clonogenicity, and accelerated cell death in TNBC cells.
- Myr-NE mediated enhanced cell death in MDA-MB-231 is majorly attributed to the significant inhibition of AKT signalling than Myricetin alone.
- The findings of this work demonstrate that Myr-NE is an effective method for delivering Myricetin at levels for better clinical outcome.

Author contributions
PS: Conceptualization, investigation, methodology, data curation, Writing – original draft. SC: Formal analysis, writing – review & editing. MK: Formal analysis, writing – review & editing. YR: In-vitro investigation, review & editing. AB: In-vitro data analysis, review & editing. MA: Writing – review & editing. AM: Formal analysis – review & editing.

Competing Interests Statement: None

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References


Figure Legends:

Figure 1: Myricetin solubility study: Solubility study was performed in A.i) oils, A.ii) surfactants, and A.iii) co-surfactants. B) Representative pseudo ternary phase diagrams of an Oil (Capryol 90), mixture of surfactant (Tween 20) and cosurfactant (Transcutol HP) (Smix) at different ratios of water and phase, displaying the oil/water NE region (dotted area).

Figure 2. Myr-NE characterization: A. Particle size distribution profile of Myr-NE. Particle size is indicated as intensity-based Z-average hydrodynamic diameter (nm) determined from DLS measurements. B) Zeta potential value of optimized Myr-NE. C) TEM showing the average size and shape of the optimized Myr-NE.

Figure 3. FTIR analysis and melting point curve.


Figure 4. Comparative evaluation of in-vitro drug release

Graph indicating the in-vitro drug release profiles of the free Myricetin and Myr-NE as a function of time in PBS at pH=7.4.

Figure 5: Determination of IC-50 in MDA-MB-231 cells: IC-50 of Myricetin and Myr-NE were estimated using SRB cell proliferation assay in MDA-MB-231 cells. Cells were treated with the indicated concentration range of Myricetin and Myr-NE for 24 and 48 hrs (continuous exposure). IC50 was further calculated using non-linear regression (curve fit) for dose-response inhibition as inhibitor vs. response-variable slope on GraphPad Prism software (version 8). Data are presented as mean +- SD (n=4).

Figure 6: Myr-NE inhibits AKT signaling and cell cycle progression:

A. The immunoblot and densitometric evaluation indicates the relative fold change in the phospho-AKT (p-AKT) and phospho-mTOR (p-mTOR) after the normalization with respective pan-AKT and mTOR with respect to control. B. The representative cell cycle histogram shows the DNA content of the Sub-G1, G1, S, and G2-M phases at 4 and 8 hrs post-treatment in the indicated treatment groups. The data are expressed as the mean +- SD (n=3). Statistical analysis was performed using a Students t-test, and p-values presented as *p [?] 0.05 (control versus Myricetin) and #p [?] 0.05 (Myricetin versus Myr-NE).

Figure 7: Myr-NE treatment stimulates cell death in MDA-MB-231 cells
A-B. A representative dot plot indicating the distribution of Calcein AM-positive and PI-positive cells in two quadrants of indicated treatment groups at 4 and 24 hrs, post-treatment. C. Photomicrographs displayed here show the Calcein-AM (left panel), PI (middle panel) and merge staining (right panel) in MDA-MB-231 cells at 24 hrs post-treatment in the indicated treatment groups. D. The bar plot indicates the difference in clonogenicity (post-plating method); whereas a representative of clonogenic survival is presented for a better appreciation. E. The bar plot indicates the relative change in the level of intracellular ROS and mitochondrial ROS at the indicated time points. F. The representative scatters plot of the Annexin V/PI assay depicts the relative distribution of cell population in four different quadrants as Q1 (necrotic), Q2 (late apoptotic), Q3 (live), and Q4 (early apoptotic). The corresponding stacked column graph depicts the percentage of live and different apoptotic events retained in Q1-Q4 quadrants. G. The expression levels of Bax, Bcl-2, and Bcl-XL proteins in the total cell lysate of MDA-MB-231 cells. The densitometric evaluation was performed and presented as Bax/Bcl-2 ratio and relative change in the Bcl-XL with respect to control. Bar plots show the mean ± SD (n=3). Statistical analysis was performed using a student’s t-test, and p-values presented as *p [?] 0.05 (control versus Myricetin) and #p [?] 0.05 (Myricetin versus Myr-NE).

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