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Enhancing encapsulation of hydrophobic phyto-drugs naringenin and baicalein in polymeric nano-micelles

Pankaj Singla [a,*], Gloria Parokie [a,d], Saweta Garg [a], Sarbjeet Kaur [a,b], Inderpreet Kaur [b], Robert D. Crapnell [c], Craig E. Banks [c], Uwe Rinner [d], Corinne Wills [e], Marloes Peeters [a,**]

[a] School of Engineering, Merz Court, Claremont Road, Newcastle University, Newcastle Upon Tyne, NE1 7RU, United Kingdom
[b] Department of Chemistry, UGC-center for Advanced Studies-I, Guru Nanak Dev University, Amritsar, 143005, India
[c] Manchester Metropolitan University, Faculty of Science and Engineering, Chester Street, M1 5GD, Manchester, UK
[d] Institute of Biotechnology, Department of Life Sciences, IMC University of Applied Sciences Krems, Piaristengasse 1, 3500, Krems, Austria
[e] School of Natural and Environmental Sciences, Newcastle University, Bedson Building, Newcastle Upon Tyne, NE1 7RU, UK

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ABSTRACT

Pluronic micelles hold great potential to act as hydrophobic drug delivery carriers; however, there is a pressing need to optimize their use in commercial formulations. This is the first report that describes the loading of phyto-drugs naringenin (NAR) and baicalein (BAC) in different Pluronics F108, F127 and P84 using solvent evaporation method (S.Ev.M) and Direct dissolution method (D.D.M.). Pluronic P84 micelles were able to encapsulate significantly higher amount of both phyto-drugs as compared to other Pluronic micelles. S.Ev.M appreciably enhanced the encapsulation of NAR (19.2 ± 0.438 mg/mL) and BAC (2.593 ± 0.223 mg/mL) compared to D.D.M. (NAR, 10.95 ± 0.212 mg/mL, and BAC, 1.058 ± 0.049 mg/mL) in 5% w/v and 12% w/v Pluronic P84, respectively. SEM (Scanning Electron Microscopy) results showed a spherical morphology after the incorporation of NAR into Pluronic micelles and evidenced that S.Ev.M did not affect the morphology. Sustained release behavior of phyto-drugs was observed from the loaded Pluronic micelles, which was conformed via *in vitro* release studies. Finally, antioxidant activity was analyzed by $ABTS^{\bullet+}$ (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) scavenging assays, with both NAR and BAC loaded P84 micelles (IC_{50} 7.185 and 28.90 μg/mL) showcasing a marked increase in antioxidant properties compared to the pure phyto-drugs NAR and BAC (IC_{50} 13.25 and 53.68 μg/mL) or other Pluronic formulations. Interaction of phyto-drugs and Pluronic P84 has been screened using ^{1}H NMR Spectroscopy (proton nuclear magnetic spectroscopy) and revealed that the whole NAR molecule was encapsulated within the Pluronic micelles. These phyto-drugs hold great potential for use as nutraceuticals and other pharmaceutical applications but currently can't be used due to poor solubilization. Therefore, it can be suggested that preparation of drug loaded Pluronic formulations using S.Ev.M. would be more convenient, fast, and efficient method over D.D.M.

1. Introduction

Nutraceuticals (NUTs) are bioactive compounds commonly found in foods, which provide certain health benefits when taken orally [1]. Naringenin (NAR) and baicalein (BAC) are phyto-drugs belonging to the class of flavonoids and have been reported to possess several pharmacological properties including anticancer, antioxidant, anti-inflammatory, neuroprotection, antibacterial, antiviral, antiallergic and many more [2,3]. In addition to that, both NUTs are known to have protective effects on cardiovascular and microvascular diseases, hypertension, Parkinsonism and Alzheimer's diseases [4,5]. NAR (4,5,7-trihydroxyflavanone) is a colorless and flavorless flavanone mainly found in grapes and other citrus fruits like tomatoes (*Solanum lycopersicum*), bergamot (*Citrus bergamia*), oranges (Citrus sinensis), beans (*Phaseolus vulgaris*), water mint (*Mentha aquatica*), grapefruits (*Citrus paradise*) and tart cherries (*Prunus cerasus*) [6]. BAC (5,6,7-trihydroxyflavone) is a glycone baicalin (baicalein-7-O-glucoside), present in the roots of *Scutellaria baicalensis* (Chinese skullcap), *Scutellaria lateriflora,* as well as in

* Corresponding author.
** Corresponding author.
E-mail addresses: Pankaj.singla@ncl.ac.uk (P. Singla), marloes.peeters@newcastle.ac.uk (M. Peeters).

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Oroxylum indicum (Indian trumpet flower) [7]. However, the latent health benefits and advantages of NAR and BAC flavonoids in functional food and pharmaceutical goods is restricted because of their hydrophobicity and poor bioavailability [8,9]. Liang and colleagues have reported the nanocomposites consisting of carbon quantum dots for the delivery of NAR to improve the drug's solubility and its biological properties [10]. Additionally, glycyrrhizin nano-micelles, polymeric (Pluronic F68) micelles, cellulose based solid dispersions, and liposomes have also been explored to enhance the water solubility, bioavailability and stability of NAR [11]. Similarly, there are some literature reports in which cyclodextrin complexes, nano-formulations and glycyrrhizin nano-micelles have been investigated to improve the poor water solubility of BAC [12,13]. Nonetheless, alternatives are required to ameliorate the physical and chemical properties of NAR and BAC and other poorly-soluble flavonoids.

Pluronics, also known as poloxamers, are surface-active tri-block copolymers comprised of Polyethylene oxide (PEO)-Polypropylene oxide (PPO)-Polyethylene oxide (PEO). These materials are non-ionic, biocompatible, non-toxic, non-immunogenic and are commercially available [14]. They can self-assemble into nano-micelles ranging from 15 to 50 nm depending upon the type of Pluronic at/or above *cmc* (critical micelle concentration) and *cmt* (critical micelle temperature) [15]. Typically, PEO forming Corona region of Pluronic micelles can accommodate hydrophilic drugs and PPO-forming core region solubilize hydrophobic drug molecules [16]. Pluronics are extensively studied for the solubilization and loading of hydrophobic and hydrophilic drugs. Moreover, these are being used in targeted delivery for various drugs, gene delivery as well as for small interfering RNA [14,15]. Pluronics are being exploited for the different food formulations including micellar formulations, nano emulsions, hydrogels and many more. It was found that the hydrophobic core of Pluronics acts as a microenvironment to improve drug loading efficacy [16,17]. Curcumin encapsulated inside Pluronic micelles were found to have a very low hydrolytic breakdown rate as compared to curcumin in aqueous solution, making it a promising drug delivery agent [18]. Kadam and colleagues used Pluronics P103, P123, P84, and F127 for the testing its solubilization capacity for the hydrophobic drug Carbamazepine. Pluronic P103 was found to be the most effective Pluronic for solubilizing carbamazepine, attributed because P103 is more hydrophobic than the other copolymers, resulting in increased hydrophobic-hydrophobic interaction between the drug and P103 [19]. On the other hand, Raval et al. examined six different Pluronics (F88, P84, F127, P105, P103, and P123) for the solubilization of three widely used hydrophobic anticancer drugs, genistein, paclitaxel and quercetin. P103 and P123 showed the higher solubilization capacity as compared to other Pluronics employed in this study. The *in vitro* cytotoxicity results evidenced the improved anticancer activity of drug loaded P103 and P123 micelles as compared to free drugs on breast cancer cells (MCF-7) [20].

There is no report till date in which solubility of hydrophobic drugs in Pluronic systems has been compared using solvent evaporation method (S.Ev.M) and direct dissolution method (D.D.M.). Thus, phyto-drugs NAR and BAC were evaluated for their loading into different Pluronics F108, F127 and P84 to improve their water solubility and other properties including antioxidant potential. These phyto-drug loaded formulations are intended to administer through intravenous (IV) route as this is most convenient and well explored route for nano-formulations till date for examples Vyxeos™ in chemotherapy (by Jazz Pharmaceuticals) and Onivyde® (Ipsen) [21]. Pluronics formulations are the series of Pluronic chosen in this study was based on the hydrophobicity of the Pluronics; Pluronic F108, F127 and Pluronic P84 have 20%, 30% and 60% PPO (hydrophobic units) content respectively. The objective of selecting this set of Pluronics, is to decipher the impact of the hydrophobicity of Pluronic micelles on the encapsulation of the hydrophobic drugs (NAR and BAC). The locus of solubilization was determined using proton nuclear magnetic spectroscopy (^{1}H NMR). Formulations were screened employing Dynamic Light Scattering (DLS) and Scanning Electron Microscopy (SEM) to gain insight into the size and structure of these formulations respectively. Moreover, NAR and BAC loaded Pluronic formulations were screened for *in vitro* drug release and antioxidant activity using $ABTS^{\bullet+}$ scavenging assays. The obtained results showed the significantly higher encapsulation of both phyto-drugs in Pluronic P84 micelles as compared to other investigated Pluronics. Moreover, S.Ev.M method enhanced the loading of NAR and BAC in these Pluronic micelles as compared to D.D.M. Overall, the current study delineated that Pluronic P84 micelles can be employed for the loading of hydrophobic phyto-drugs as well as in pharmaceutical drug delivery applications.

2. Materials and methods

2.1. Materials

Pluronics P84, F127 and F108 were kindly provided as gift samples by BASF (Germany). Naringenin (NAR) and baicalein (BAC) were purchased from Alfa-Aesar Thermo Fisher Scientific, Heysham, UK. All other chemicals used were of analytical grade and used without further purification.

2.2. Methods

2.2.1. Loading and estimation of NAR and BAC

2.2.1.1. Solvent evaporation method (S.Ev.M.). Excess amount of Phyto-drugs (40 mg for NAR and BAC) and different Pluronics at variable concentrations (1–5% w/v in case of NAR; 6 and 12% w/v in case of BAC) were mixed and dissolved in vial containing 2 mL of ethanol and afterwards, the mixture was evaporated at 60 °C until a thin film was obtained. Deionized water (2 mL) was poured in the vial containing thin film and sonicated using RS pro ultrasonicator (for 10 min) at frequency of 40 kHz with ultrasonic power rating of 70W. After that, solutions were thoroughly stirred at 37 °C for 2 h to completely dissolve the thin film. The solution was filtered through 0.2 μm Polytetrafluoroethylene (PTFE) syringe filters (Fisher Scientific, Heysham, UK) to remove the unloaded phyto-drugs from the solutions. The samples of empty micelles produced via S.Ev.M for DLS and SEM measurements were prepared using the above-mentioned method without adding the Phyto-drugs.

2.2.1.2. Direct dissolution method (D.D.M.). The different Pluronic micellar solution (1–5% w/v in case of NAR; 6 and 12% w/v in case of BAC) were prepared in 2 mL of deionized water and excess amount of phyto-drug (40 mg for NAR and BAC) was added to it. Solutions were kept on constant stirring at 400 RPM at 37 ± 0.2 °C. After 12 h, the obtained solution was filtered (0.2 μm Polytetrafluoroethylene (PTFE) syringe filters) to remove the remaining undissolved phyto-drugs. This method of solubilizing drugs directly to the micellar solution by simple dissolution is called the Direct Dissolution Method (D.D.M.).

2.2.1.3. Estimation of phyto-drugs in micellar formulations. The λ_{max} of NAR and BAC was obtained at 292 nm and 275 nm using a Jenway 7200 UV–Visible scanning spectrophotometer. The stock solution of NAR and BAC was prepared in the ethanol and further diluted to the desired concentration with deionized water. Calibration curves of NAR and BAC were plotted at different concentrations (Fig. S1, supporting information) the molar absorption coefficient of NAR and BAC was determined to be 13.42 and 20.84 L mol^{-1} cm^{-1} respectively. These molar absorption coefficients were used to estimate loading of the phyto-drugs into these Pluronic micelles. The solution was further diluted if needed to measure the absorbance.

2.2.1.4. Drug loading capacity and thermodynamics of phyto-drugs encapsulation. The percent drug loading capacity (DLC) was calculated

by taking the ratio of amount of Phyto-drug encapsulated and weight Pluronic used.

$$\text{DLC}\ (\%) = \text{Encapsulated Phyto} - \text{drugs} \ / \ \text{Total weight of Pluronic x } 100 \quad \text{[Eq. 1]}$$

The partition coefficient (*P*) is the ratio of the drug encapsulated in the micellar phase to the drug solubilized in aqueous phase. It is an important property regarding the encapsulation of drugs in micelles.

$$P = S_{tot} - S_w / S_w \quad \text{[Eq. 2]}$$

Here S_w is the solubility of the drug in water and S_{tot} is the total solubility of the drug.

From the partition coefficient, the Gibbs free energy of drug encapsulation has been calculated using the formula below:

$$\Delta G^{\circ} = -RT \ln P \quad \text{[Eq.3]}$$

Where, R is the gas constant, T is the temperature in Kelvin and *P* is the Partition Coefficient.

2.2.2. Dynamic light scattering (DLS) and measurements

Dynamic light scattering (DLS) experiments were performed using a Malvern Zetasizer Nano ZS to measure the hydrodynamic diameter (D_h) of Pluronic and phyto-drugs loaded Pluronic micelles at 25 ± 0.1 °C. The pH values of all formulations were also measured together with polydispersity index (PDI). Each sample was run three times during the size measurement and all experiments were performed in duplicate. The instrument used a scattering angle of 173° and the laser wavelength of 632.8 nm. The size was measured at different times to evaluate the stability of the systems at 25 ± 0.1 °C. Zetapotential (ζ) of unloaded and loaded Pluronic micelles was determined with same instrument employed for DLS measurements using disposable folded capillary zeta cell (product code DTS1070, from Malvern Panalytical Limited UK). The analysis was done in triplicate. Samples for both size and zetapotential (ζ) measurement were not further diluted before the measurement.

2.2.3. In-vitro drug release

The drug-loaded micellar system (2 mL) was poured into a dialysis bag (Spectra/Por dialysis membrane with MWCO-12-14 kDa, normal flat width of 45 m and diameter 29 mm), which was sealed and subsequently placed inside a 100 mL phosphate buffer saline (PBS, pH 7.4) as a release medium. The total concentration of NAR and BAC was 16 mg and 0.902 mg per dialysis bag and was kept constant for all Pluronic formulations for the comparison. To active final concentrations in the dialysis bag (where needed) was diluted with deionized water. The buffer solutions were prepared by dissolving one PBS tablet (acquired from Fisher Bioreagents™). The typical composition of buffer was 137 mM Sodium Chloride,

10 mM Phosphate Buffer [potassium dihydrogen phosphate (8.1 mM) and disodium hydrogen phosphate (1.9 mM)] and 2.7 mM Potassium Chloride. The dialysis system was kept at a temperature of 37 ± 0.2 °C with constant stirring at 120 RPM, and release medium was changed after every 8 h to provide sink conditions. Aliquots (1 mL) were taken from the release medium at predetermined time interval (For NAR, samples were withdrawn every 1 h for 11 h, followed by 2 h until 23 h and then 4 h till 34 h, on the other hand for BAC, samples were taken for every 1 h till 16 h) and replenished with same volume of fresh release medium. The concentration of drug release was estimated using a UV/Visible spectrophotometer, using same respective molar absorption coefficient as represented in **section 2.2.1.3**. Cumulative percentage release was calculated using following equation:

$$\text{Cumulative \% release} = D_c \ / \ D.E._{tot} \text{ x } 100 \quad \text{[Eq. 4]}$$

where D_c is the total amount of drug present in the release medium (receiver) and $D.E._{tot}$ is the total drug encapsulated in the micelles.

2.2.4. $ABTS^{\bullet+}$ assay

2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) activity of NAR and BAC and their micellar formulations were determined. The ABTS radical cations ($ABTS^{\bullet+}$) were generated by dissolving ABTS (76.80 mg) and ammonium persulphate (APS, 11.81 mg) in 20 mL of deionized water and this well stirred mixture was stored overnight in dark conditions [22]. The solution was diluted with PBS buffer to obtain an absorbance of 0.700 ± 0.02 at 734 nm prior taking measurements. Briefly, 250 μL of the drug-loaded micelle formulations or pure phyto-drug solution in ethanol was added to the assay solution (2.25 mL). Different concentrations from 0.025, 0.5, 1, 2.5, 5, 7.5, 10, 15, 20 and 25 μg/mL were screened for NAR loaded micelles, and for BAC loaded micelles 0.5, 1, 2.5, 5, 7.5, 10, 15, 20, 25, 30, 40, 60 and 80 μg/mL were chosen. Moreover, the antioxidant activity of blank micelles was also determined at 5 and 12% concentration (% w/v). After storing this mixture in the dark for 7 min, the absorbance was measured. The percent $ABTS^{\bullet+}$ radical scavenging activity was calculated by Eq. (4) using PBS as a blank.

$$ABTS^{\bullet+} \text{radical scavenging}\ (\%) = [(A_0 - A_1) / A_0] \times 100 \quad \text{[Eq. 5]}$$

2.2.5. Scanning electron microscope (SEM)

Samples were drop-casted over the glass slides (1 × 1 cm) and left overnight for the evaporation of water. SEM images were taken using a Supra 40VP field emission scanning electron microscope from Carl Zeiss Ltd., Cambridge, UK with an average chamber and gun vacuum of 1.3×10^{-5} and 1×10^{-9} Torr respectively. Samples were mounted onto aluminum SEM pin stubs (12 mm diameter, Agar Scientific, Essex, UK).

2.2.6. Binding and interactional behavior of phyto-drugs with pluronics

The ^{1}H NMR (Proton Nuclear Magnetic Resonance) studies were performed on a Bruker Avance III HD 700 MHz nuclear magnetic resonance spectrometer with a Prodigy TCI cryoprobe operating at 700.13 MHz. Chemical shifts are reported in parts per million (ppm). For the interactional studies, phytodrugs were first dissolved in the DMSO-d_6 and further diluted in D_2O with final ratio of 5% (w/v) DMSO-D_2O.

3. Results and discussion

3.1. Loading and estimation of NAR and BAC

The molecular structure of NAR, BAC and Pluronics, in addition to schemes of the different drug loading methods, are depicted in Fig. 1a. To explore the effect of encapsulation process on loading capacity and efficiency of NAR and BAC phyto-drugs in Pluronic (F108, F127 and P84) micelles, two different methods *viz.* Solvent evaporation method (S.Ev.M) and Direct dissolution method (D.D.M.) were employed (Fig. 1b). The loading of both the phyto-drugs has been screened with different concentration of Pluronic micelles. It was observed that encapsulation of NAR was increased upon increasing the concentration of Pluronic micelles from 1 to 5% w/v (Fig. 2a). Unexpectedly, encapsulation of BAC was less pronounced, therefore, concentration from Pluronic micelles were increased and screened for 6–12% w/v (Fig. 2b). The solubilization of both NAR and BAC was found to be higher in Pluronic P84 as compared to other screened Pluronic micellar systems due to its hydrophobic nature [16]. The Hydrophilic–lipophilic balance (HLB) value for Pluronic P84, F127 and F108 is 14, 22 and 27 respectively, higher the HLB values greater the hydrophilicity, and these balance values are an indicative that the encapsulation efficiency of P84 for hydrophobic phyto-drugs (NAR and BAC) would be better than that of F127, F108 [19,20,23,24]. In 5% w/v and 12% w/v Pluronic P84, S.Ev.M significantly improved ($P < 0.05$) the encapsulation of NAR (19.2 ± 0.438 mg/mL) and BAC (2.593 ± 0.223 mg/mL) when compared to D.D.M. (NAR, 10.95 ± 0.212 mg/mL, and BAC, 1.058 ± 0.049 mg/mL) and other screened Pluronic micellar systems. The loading of BAC in Pluronic micelles was found to be significantly lower in comparison to NAR,

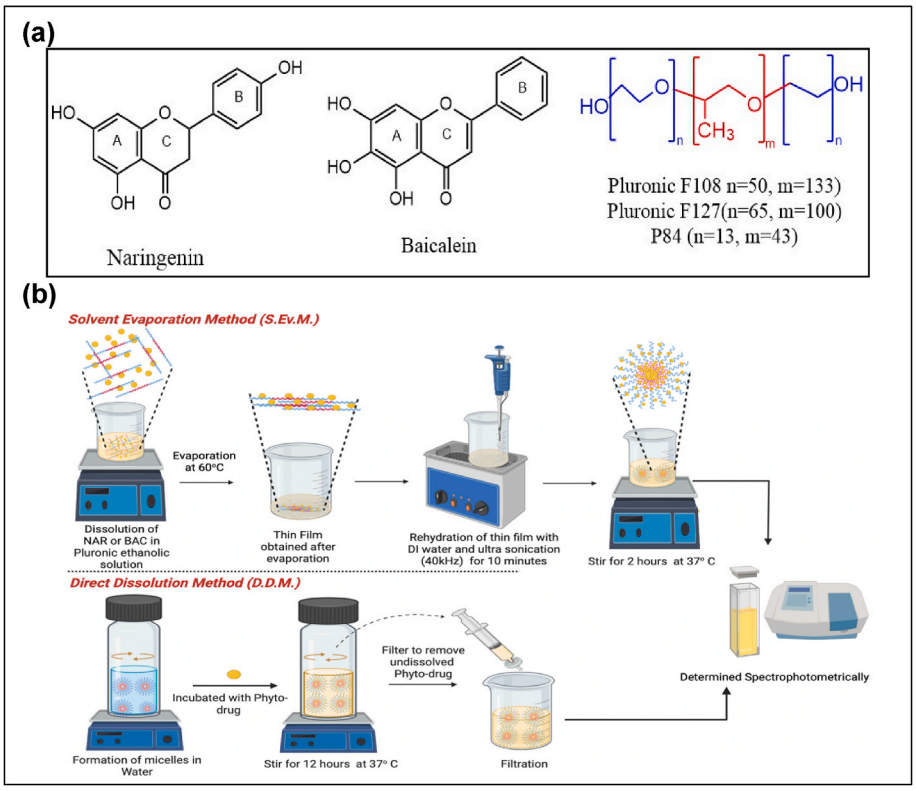

Fig. 1. **(a)** The molecular structure of NAR, BAC, and Pluronics P84, F108, and F127, **(b)** Method of drug solubilizing using solvent evaporation method (S.Ev.M.) and Direct dissolution method (D.D.M.).

this may be due to the structural differences between them. NAR is 4′,5,7-Trihydroxyflavanon, and BAC is 5,6,7-trihydroxyflavon; the major difference between flavanones and flavones is a saturated C ring (see Fig. 1a) while in flavones there is a double bond between the 2,3 positions of the C ring. Another difference between NAR and BAC is that there is no hydroxyl group present on the B ring of BAC (all three hydroxyl moieties are positioned on the A ring). In the case of NAR, two hydroxyl groups are present on the A ring and one hydroxyl group is present on the B ring [25]. Additionally, the results showed significantly higher solubilization of NAR and BAC with S.Ev.M. as compared to D.D.M. The possible reason behind higher solubilization is the energy input used during the S.Ev.M. and enthalpic and entropic contribution. After the evaporation of organic solvent (ethanol), the molecules of phyto-drug and Pluronic closely interacted with each other, on the other hand, this was missing in D.D.M. During the rehydration process, the use of sonication at a frequency of 40 kHz facilitated the formation of micelles in a Pluronic solution that consisted of a complex between a phyto-drug and the PPO and PEO regions of the Pluronic molecule. Additionally, the corona region of the Pluronic molecule played a role in stabilizing and dispersing the micelles in the solvent system. The energy input from sonication also helped to break down the thin film aggregates into smaller micelles. Other reason is enthalpic and entropic contribution, during the solvent evaporation of Pluronic and phyto-drugs, there were enthalpic and entropic interactions between drug and chains of Pluronics which facilitates the solubilization of phyto-drugs. This mechanism has been proposed by Xu and colleagues, where solvent evaporation induced and facilitated the formation of polymer patch nanoparticles by exquisite balance between enthalpic interaction and conformational entropy of grafted chains of polymers [26].

Solubility of NAR was ~2100 and ~1200 folds higher in Pluronic P84 micelles (5% w/v) with S.Ev.M and D.D.M. method respectively, as compared to NAR solubility in pure water. In the case of BAC, aqueous solubility was increased ~3200 and ~1300 in Pluronic P84 (12% w/v) with S.E.M and D.D.M. method respectively.

The drug loading capacity (DLC) of Pluronic micelles at higher concentration of both formulations were calculated (Fig. S2, supporting information). DLC was observed to be ~40% for NAR loaded P84 (at 5% w/v) micellar formulation, however, only ~2% DLC in 12% P84 micelles was observed for BAC. DLC for other Pluronic systems was lower than when the drug was loaded into Pluronic P84 micelles (Fig. S2, supporting information). Thermodynamics of phyto-drugs (NAR and BAC) encapsulation was determined in terms of the partition coefficient (P) and Gibbs free energy (ΔG°). The P values for NAR (1–5%) and BAC (6–12%) in the different Pluronics at various concentrations were calculated using solubilization data (Fig. 2 c, d). The higher values of P confirmed that Pluronic P84 micelles at concentration of 5% w/v and 12% w/v for NAR and BAC respectively provided a better hydrophobic environment to solubilize these phyto-drugs [27]. In addition to that, ΔG° was more negative in the case of Pluronic P84 at concentrations of 5% w/v and 12% w/v for NAR and BAC respectively.

3.2. Characterization of empty and drug loaded pluronic micelles

3.2.1. Dynamic light scattering (DLS) and zeta potential (ζ) measurements

Hydrodynamic diameter (D_h) of the optimal micellar formulation produced using S.Ev.M was measured by employing dynamic light scattering (DLS) measurements. The D_h values of empty micelles of Pluronic P84, F127 and F108 (5% w/v) at 25 ± 0.1 °C were found to 28.58, 32.39 and 51.30 nm respectively (Fig. S3 a, b, c, supporting information). After NAR loading, the D_h values for Pluronic P84, F127 and F108 (5% w/v) micelles were found to be 50.66, 59.11 and 108.23 nm respectively. The increase in the D_h was observed at 12% w/v concentration of Pluronic P84, F127 and F108 i.e. 33.27, 44.06 and 62.78 nm respectively. The D_h values were measured to be 42.83, 44.06 and 62.78 nm (Fig. S3 d, e, f) for Pluronic P84, F127 and F108 (12% w/v) respectively after the encapsulation of BAC drug. The polydispersity index (PDI) values and for all formulations were given in caption of Fig. S3 and found to be between 0.2 and 0.3, this means that

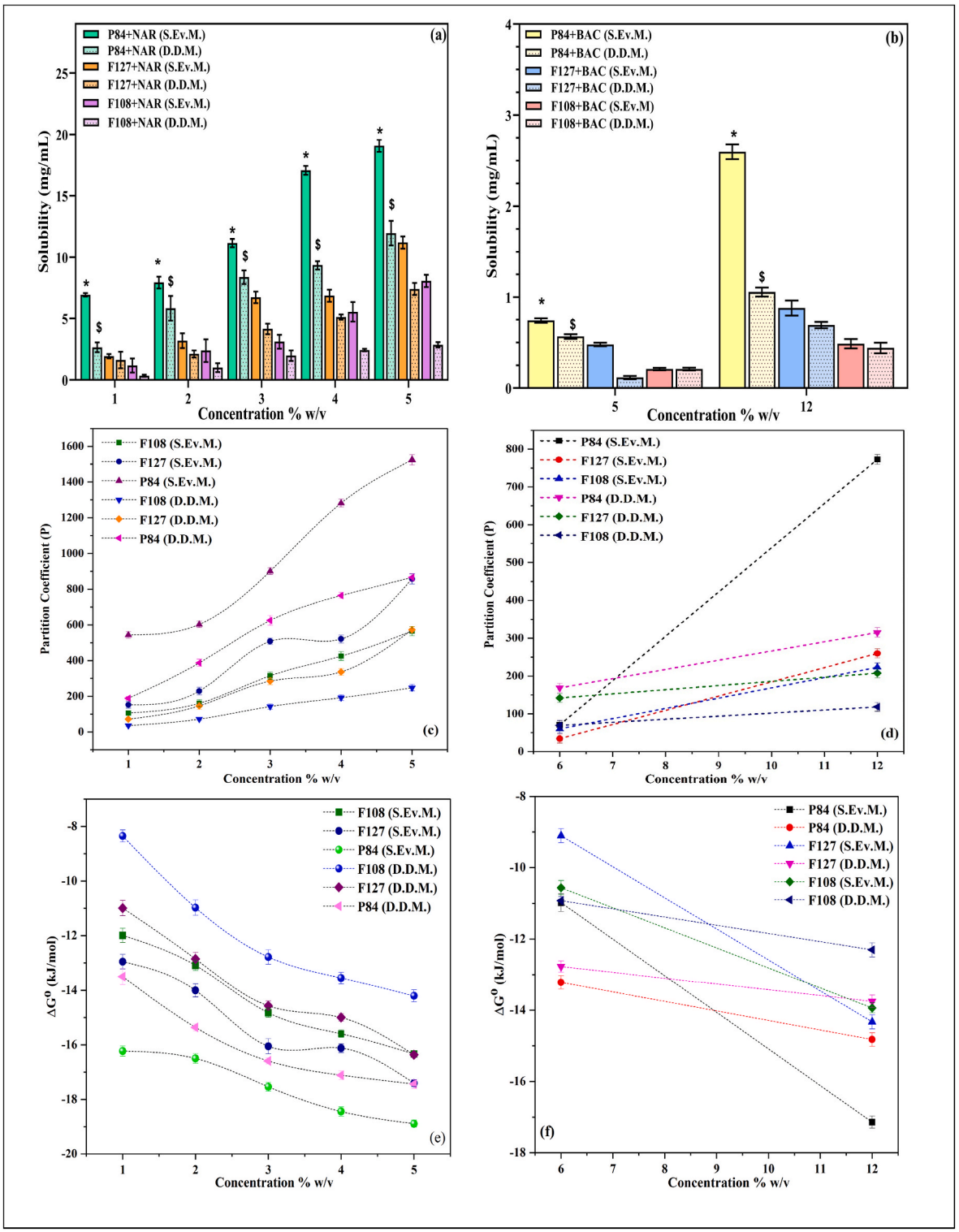

Fig. 2. Solubility of different phyto-drugs in different Pluronics (P84, F127 and F108) **(a)** NAR and (b) BAC, data was analyzed by two-way ANOVA followed by multiple comparison test.*P < 0.05 vs. S.Ev.M and D.D.M., $^{\$}$P < 0.05 vs. D.D.M. (n = 3); Partition coefficient (*P*) of **(c)** NAR and **(d)** BAC; Gibbs free energy ΔG^o (kJ/mol) of **(e)** NAR and **(f)** BAC. Data are represented as mean ± standard deviations (SD).

formulations are moderately homogeneous. There was a higher increase in D_h upon the loading of NAR as compared to BAC, which indicates the higher interactions between the NAR and Pluronic micelle surface and encapsulating the higher amount of NAR within the Pluronic micelle core and corona [28,29]. Zeta potential (ζ) measurements were also performed to probe the surface charge and stability of Pluronics and phyto-drug loaded Pluronic micelles, values for ζ potential as well as pH of the formulations have been tabulated in Table S1. Unloaded Pluronic micelles of 5% w/v P84, F127 and F108 exhibited ζ values (in mV) of -8.82 ± 0.43, -10.57 ± 0.58 and -11.94 ± 0.50, respectively, whereas for 12% w/v P84, F127 and F108 unloaded micelles ζ values for found to be -11.82 ± 0.43, -12.57 ± 0.58, and -12.94 ± 0.50 respectively. The loading of NAR to 5% w/v Pluronic P84, F127 and F108 increased the ζ values to -9.93 ± 0.65, -11.44 ± 1.52, -12.09 ± 0.79, respectively, on

the other hand, loading of BAC in 12% w/v Pluronic P84, F127 and F108 increases the zeta potential to −12.03 ± 0.65, −12.73 ± 0.52, −13.04 ± 0.22. Pluronics, despite being non-ionic, exhibit negative zeta potentials, indicating the presence of repulsive interactions among the micelles. These interactions prevent aggregation among the Pluronic micelles, resulting in their stability [30]. Table S1 demonstrates that phyto-drug loaded Pluronics an increase in ζ values, confirming the stability of the micelles. Moreover, to evaluate the stability of Pluronic formulations the Phyto-drug loaded Pluronic P84 micellar formulations (5% w/v P84 for NAR and 12% w/v P84 for BAC) were stored for 1, 4 and 8 weeks at 25 °C and size and visual turbidity was determined. There were no visual turbidity or size changes of the formulations (data not shown), for the storage of 8 weeks, but after that it show little turbidity. It witnessed that Pluronic P84 micelles formulations were stable for 8 weeks during storage. This information can be important in the development of drug delivery systems, as the size and stability of the delivery vehicle can impact the efficacy and safety of the drug.

3.3. In vitro appraisal

3.3.1. In vitro drug release

in vitro drug release is highly valuable for uncovering essential information about formulation structure and behavior, potential interactions between the drug and carrier composition, and how they impact the rate and mechanism of drug release [31,32]. The Pluronic formulation was examined using the dialysis release method in phosphate-buffered saline (PBS) with a pH of approximately 7.4, which is isotonic to blood. After 6 h, NAR loaded Pluronic P84, F127, and F108 micelles (at 5% w/v) showed a fast release of 32.55 ± 2.19%, 66.76 ± 1.93%, and 50.55 ± 1.97% respectively, followed the release rate slowed down (Fig. 3a). NAR was fully released from P84 micelles after 34 h, whereas, from F127 and F108 micelles, NAR took 26 h to be released completely. In the case of BAC, Pluronics P84, F127, and F108 (12% w/v) exhibited a faster drug release compared to NAR. BAC complete release was observed to be in 5 and 8 h in case of F127 and F108 micelles (Fig. 3b) respectively as compared to P84 micelles (16 h). Pluronic P84 is composed of 60% PPO component, and most of the drug is located within the PPO core. Therefore, the phyto-drugs must first pass through the PEO corona region before leaving the micelles and ultimately passing through the pores of the dialysis membrane to be released in the medium. This is the reason slower release of phyto-drugs in the P84 micelles was observed as compared to other F127 and F108 micelles. Sahu and colleagues noticed the slow and sustained release of Curcumin in Pluronic F127 and F68 micelles [33]. Zhao et al. also observed the sustained release behavior of Curcumin (phyto-drug) loaded from the Pluronic P123 and F68 mixed micelles [34]. In addition to that the phyto-drugs release of both NAR and BAC have been showed in the insets of respective Fig. 3a and **(b).** Four different release kinetics were conducted which are zero-order, first-order, Korsmeyer-Peppas, and Higuchi to analyze the release study, the theoretical background and equations of these kinetic release models is provided in the section S1 (supporting information). The highest R^2 value indicated the best release kinetics followed by these formulations and kinetics data is tabulated in Table S2. NAR loaded with P84, F108 (5% w/v) and BAC loaded with P84, F127 (12% w/v) followed the Higuchi release kinetics implying that that the drug release was diffusion controlled at this concentration [32]. Besides, NAR loaded F127 showed the first order release kinetics, confirming that the release was independent of concentration of NAR entrapped [35]. BAC loaded F108 micelles followed the Zero order release kinetics which revealed that the drug is released at a constant rate [36].

3.3.2. In-vitro antioxidant activity

The antioxidant activity of pure phyto-drugs NAR and BAC and their different Pluronic micellar formulations was deciphered through experiments with $ABTS^{\bullet+}$ radical scavenging assays (Fig. 3c and d). The antioxidant potential of pure drugs and drug loaded Pluronic micellar formulations was found to be increased in a concentration dependent manner. Pluronic formulations of NAR and BAC exhibited higher $ABTS^{\bullet+}$ scavenging activity as compared to the pure drug. However, higher $ABTS^{\bullet+}$ scavenging was observed in NAR and BAC loaded P84 micelles with 98.58 ± 1.36% and 95.6 ± 0.11% inhibition respectively as compared to NAR (81.92 ± 0.985%) and BAC (70.41 ± 2.71%) drugs (Fig. 3c and d). However, NAR-P84 micellar formulation was found to be a better antioxidant than BAC loaded Pluronic P84 micelles because it showed higher antioxidant activity at a very low concentration of 25 μg/mL. On the other hand, BAC loaded Pluronic P84 micelles showed the highest inhibition of $ABTS^{\bullet+}$ at 80 μg/mL. IC_{50} were also calculated for all systems mentioned above and depicted in Fig. 3e and f for NAR and BAC respectively. Results showed that the IC_{50} values of Pluronic formulations of NAR (7.185 μg/mL) and BAC (28.90 μg/mL) were lower than the pure phyto-drugs NAR and BAC with IC_{50} of 13.25 and 53.68 μg/mL respectively. Therefore, these results implied that encapsulation of these drugs in Pluronic formulations improved the overall antioxidant potential. Moreover, the $ABTS^{\bullet+}$ scavenging activity of unloaded Pluronic P84, F127 and F108 micelles (as a blank) at 5% w/v was found to be 3.235 ± 0.36%, 2.93 ± 0.12% and 2.165 ± 0.26% respectively and at 12% w/v values were observed to be 3.88 ± 0.33%. 3.285 ± 0.47% and 2.865 ± 0.26% respectively (Fig. S5). These results confers that the enhancement in the antioxidant was due to the overall improvement and solubilization of the Phyto-drugs (NAR and BAC).

The increase in $ABTS^{\bullet+}$ scavenging with NAR and BAC loaded Pluronic P84 micelles can be attributed to the improved water solubility of these drugs in Pluronic micelles, which imparts the better contact of these drugs with free radicals of $ABTS^{\bullet+}$. These results corroborated with our previous studies in which Pluronic mixed micellar formulations of clozapine and oxcarbazepine improved the antioxidant activity of pure drugs [37]. Similar results were observed by Sun et al., who also observed improved $ABTS^{\bullet+}$ scavenging activity of flavonoid Myricetin with polymeric micellar formulation as compared to the free drug [38]. As evidenced in literature, antioxidant activities of these phyto-drugs play an important role in several drug pathways including cancer, liver diseases and brain diseases [39]. Therefore, it can be suggested that these formulations could provide a better formulation system for such type of drugs being used for the treatment of various diseases in which antioxidant activity plays a major protective role.

3.4. Morphology of the micelles

To check whether S.Ev.M. technique for NAR loading had any impact on the morphology of Pluronic micelles, scanning electron microscopy (SEM) measurements were performed. Drug loading is higher in the case of Pluronic P84 and Pluronic F127; thus, these two micellar systems were subjected to perform SEM measurements. Pluronic F127 and P84 micelles showed the spherical morphology at 5% w/v concentration with average size of 58.33 nm and 40.33 nm respectively (Fig. 4a and b). Further, after NAR loading, the average sizes of Pluronic F127 and P84 micelles were found to be increased with average size of 75.66 nm and 61.66 nm (Fig. 4c and d) respectively. Moreover, SEM measurement evidenced that S.Ev.M. did not affect the morphology of Pluronic micelles, it is reported in the literature that Pluronic micelles exhibited spherical morphology [40].

3.5. Interactional behavior of NAR and BAC with pluronic micelles

^{1}H NMR spectroscopy was employed to understand the binding interactions and solubilization locus of NAR and BAC within Pluronic 84 micelles, which had the highest solubilization capacity. Stock solutions of NAR and BAC were prepared in DMSO-d_6 and further diluted in D_2O with final concentration of 1 mM NAR/BAC in solvent ratio of 5% v/v DMSO-D_2O. These solutions were recorded for their NMR spectra using TMSP-*d*4 (Trimethylsilylpropanoic acid) as internal standard (0.0 ppm)

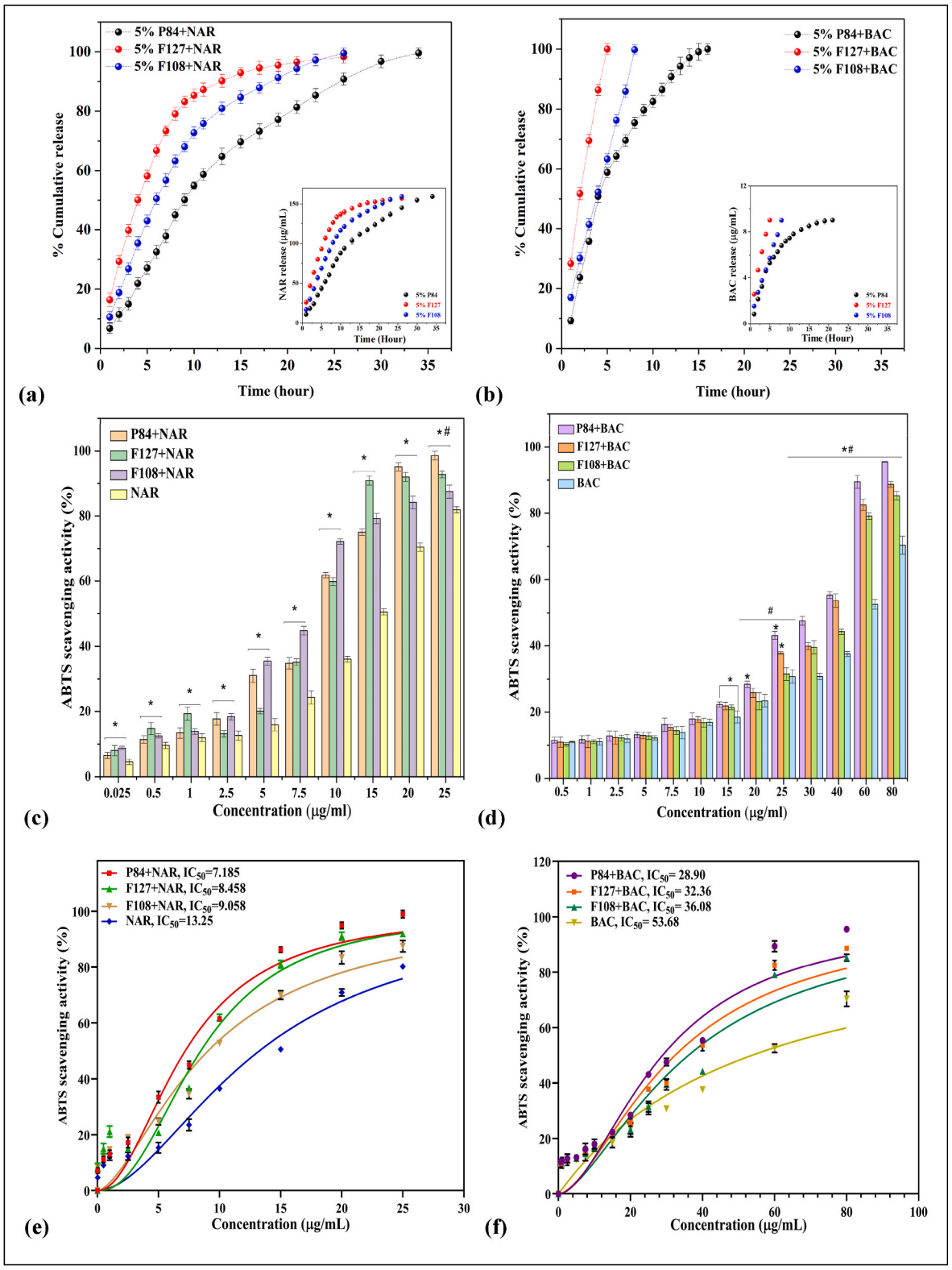

Fig. 3. *In vitro* release of **(a)** NAR (inset showed NAR release in µg/mL) and **(b)** BAC (inset showed BAC release in µg/mL) from different Pluronic micelles at concentartion 5% w/v and 12% w/v, respectively, at 37^{O}C ± 0.2, SD (n = 3); ABTS$^{\bullet+}$ scavanging activity of different Pluronic formulations of **(c)** NAR, *P < 0.05 vs. NAR and $^{\#}$P < 0.05 vs. P84+NAR and **(d)** BAC, *P < 0.05 versus BAC and $^{\#}$P < 0.05 versus P84+BAC; IC_{50} of **(e)** NAR **(f)** BAC different Pluronic formulations in ABTS$^{\bullet+}$ assay.

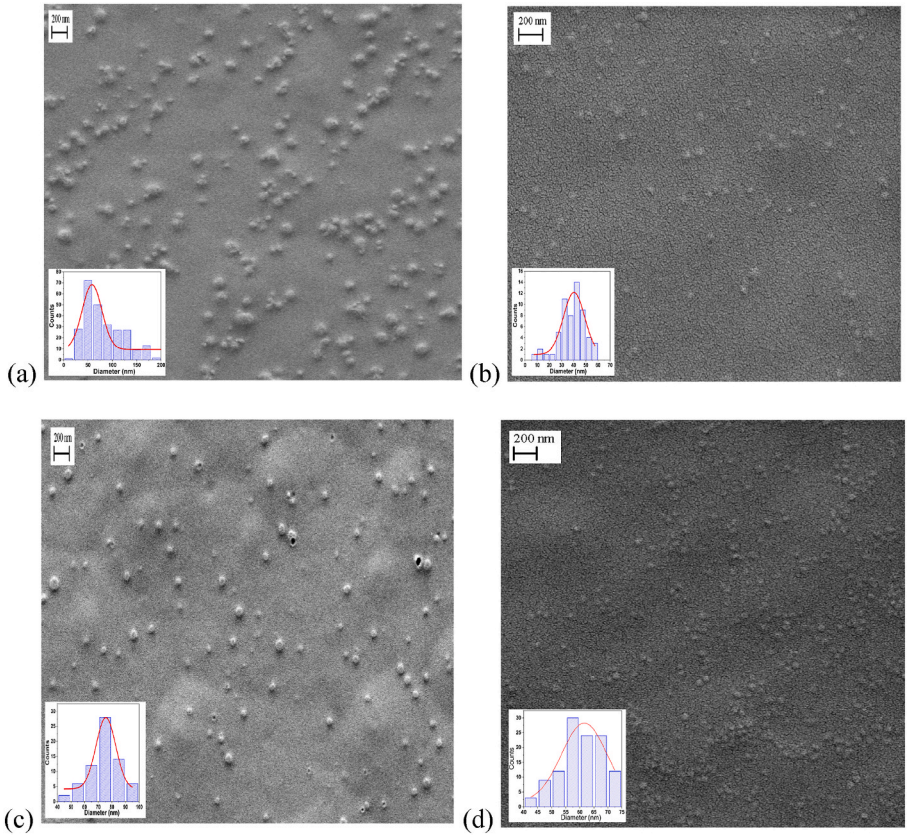

Fig. 4. SEM images of 5% w/v Pluronic micelles prepared by solvent evaporation method (S.Ev.M.) **(a)** Empty Pluronic F127 micelles **(b)** Empty Pluronic P84 micelles **(c)** NAR loaded Pluronic F127 micelles **(d)** NAR loaded Pluronic P84 micelles.

and titrated against Pluronic P84. Water suppression was used to remove the water signal. The hydrophobic drug BAC was observed to precipitate out in the chosen solvent system and therefore measurements were not possible with BAC. The typical NMR spectra of 1 mM NAR showed doublets at 7.45 and 6.97 ppm due to aromatic H's H-12, H-16 and H-13, H-15, respectively along with a singlet at 5.87 ppm due to H-2 and H-6 (Fig. S4). The peaks at 5.50 and 3.25 ppm correspond to H-9 and H-8, respectively. The peaks due to –OH hydrogens (H-18, H-19 and H-20) were resolved in pure DMSO-d_6 at 9.59, 10.80 and 12.16 ppm. However, no peaks corresponding to –OH groups were observed in the chosen solvent system which was likely due to rapid exchange of OH protons with deuterium which is not observable in a ^{1}H NMR spectrum (Fig. 5) [41,42].

As evidenced in literature, antioxidant activities of these phyto-drugs play an important role in several drug pathways including cancer, liver diseases and brain diseases [39]. Therefore, it can be suggested that these formulations could provide a better formulation system for such type of drugs being used for the treatment of various diseases in which

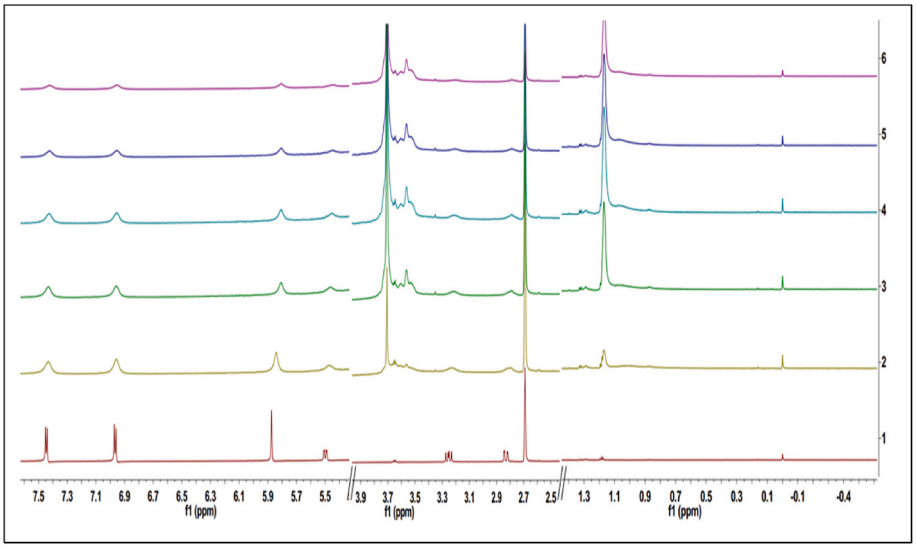

Fig. 5. ^{1}H NMR spectra of 1 mM NAR with succesive additions of Pluronic P84 (0–0.05 mM).

antioxidant activity plays a major protective role.

Upon sequential additions of P84 solution (up to 0.05 mM), the Ar–H's peaks showed upfield shifts to 7.41, 6.95 and 5.81 ppm along with broadening of signals. Also, the resonance signals at 5.50 and 3.25 ppm showed upfield shifts to 5.45 and 3.20 ppm, respectively. With each addition of P84, the peaks corresponding to protons of NAR are increasingly broadened while the peaks at 1.17 ppm belong to PPO-CH_3. The broad peaks at 3.50–3.65 ppm and the sharp peak at 3.71 ppm correspond to PPO-CH_2 and PEO-CH_2 groups of P84. Broadening of the peak evidenced that the protons of NAR molecule were in close proximity with PEO and PPO blocks of Pluronic P84 [43]. These NMR results emphasized that the probable solubilization locus (location of the NAR within micelles) is both PEO forming Corona and PPO forming core region [44].

3.5.1. Effect of temperature on the solubilization of NAR in pluronic micelles

NAR loaded Pluronic P84 micelles at 1% w/v were selected to scrutinize the effect of temperature using ^{1}H NMR in a 9:1H_2O:D_2O solution (Fig. 6). It is worth mentioning that we selected 1% w/v of Pluronic P84 instead of 5% w/v (which have highest solubilization capacity) for this study. Because at higher concentration of Pluronic P84, the signals from its protons were much stronger than NAR even at higher temperature screening, making it difficult to distinguish the NAR signals. At temperature of 283 K, the singlet at ~ 1.17 ppm was attributed to protons of PPO CH_3 groups and the additional peaks at ~3.36 and ~3.58 ppm belong to the protons of PPO CH groups and PPO CH_2 groups respectively. A sharp peak at ~3.72 ppm belongs to the protons of PEO CH_2 group. However, peaks of NAR could not be seen at this temperature. The micellar system was then subjected to an increase in temperature and the spectra were recorded. It was observed that the peak at 1.17 ppm was suppressed after increasing temperature from 283 to 293 K. Further increasing the temperature resulted in the formation of a new peak at 1.06 ppm, which intensified with sequential rise in temperature. This could be attributed to the change in microenvironment around the PPO-CH_3 region of Pluronic P84 due to interaction with hydrophobic NAR molecules. The signals at 1.17 and 1.06 ppm are due to hydrated and anhydrous state of the PPO-CH_3 groups. At lower temperatures, the chemical exchange rate between the two states was low, resulting in two well defined peaks while with increased temperature the chemical exchange rate accelerated, leading to unresolved signals [44]. In addition, peaks attributed to NAR were observed with the rise in temperature, thus leading to the conclusion that the signal of NAR solubilized in P84 can be seen at higher temperature due to higher chemical exchange rate.

4. Conclusions

This study demonstrated that the S.Ev.M. is a better method compared to D.D.M. for loading of hydrophobic flavonoids NAR and BAC without compromising size and morphology of Pluronic micelles. The results delineated that S.E_V.M. method would be a greater alternative to solubilize the hydrophobic drugs over D.D.M. and can be employed in pharmaceutical industries as it is more efficient, less processing time and easy deployable method. Pluronics were more favorable for the loading of NAR than BAC, as confirmed by encapsulation studies. DLS results showed that the loading of phyto-drugs resulted in swelling of the Pluronic micelles. *In vitro* sustained release behavior and higher antioxidant potential was evaluated for NAR and BAC loaded Pluronic formulation. It was demonstrated that Pluronic P84 exhibited greater sustained release (100% release in 36 h) and higher antioxidant potential for NAR and BAC. Furthermore, ^{1}H NMR results evidenced that NAR interact with both core and corona regions of Pluronic P84 micelles. From these results, it can therefore be suggested that this approach could be used for wider range of other water insoluble phyto and pharmaceutically important molecules.

Authors contributions

Dr Pankaj Singla designed and performed major experiments; wrote and proofread the manuscript. Gloria Parokie performed experiments such as UV–visible, DLS, antioxidant assays and did data acquisition. Saweta Garg, Sarbjeet Kaur and Professor Inderpreet Kaur analyzed and wrote the ^{1}H NMR and antioxidative part. Dr Robert Crapnel and Professor Craig E. Banks performed and discussed the SEM measurements. Dr Uwe Rinner proofread the manuscript and Corinne Wills performed ^{1}H NMR experiments. Dr Marloes Peeters designed and supervised the work, acquired the funding for laboratory space, equipment, chemicals and proofread the manuscript.

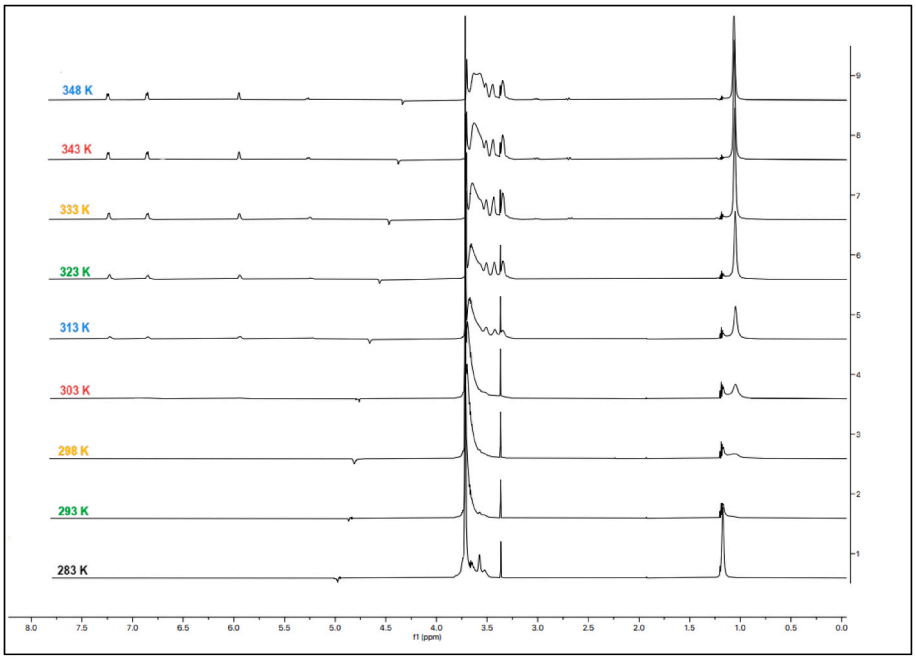

Fig. 6. ^{1}H NMR spectra of NAR loaded Pluronic P84 (1% w/v) micelles with temperature variation from 283 to 348 K.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jddst.2023.104403.

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