



RESVERATROL ENHANCE LOADING CAPACITY AND SOLUBILITY OF DIMETHYLCURCUMIN IN PLURONIC F-127 NANOMICELLES

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Abstract

Cancer is the leading cause of death worldwide and is still increasing nowadays. Several natural products and their derivatives have been of interest for their anti-cancer activities and have been intensively studied for their applications. In this study, we focused on developing a curcumin analog ([*(1E, 6E)*-1, 7-*bis* (3, 4-dimethoxyphenyl) hepta-1, 6-diene-3, 5-dione or dimethylcurcumin (DMC)], resveratrol (RES), and co-loading of both compounds in nanomicelles to improve the loading capacity and water solubility. Pluronic F-127 was utilized as a nanomicelles construction by thin film hydration method. Preparations of DMC, Res, and co-loaded DMC and Res nanomicelles were optimized by varying the ratios between the loaded drug(s) and Pluronic F-127. The stable nanomicelles gave clear solutions, and the obtained freeze-dried powders could re-disperse in water. Their sizes were in a range of 20- 50 nm, in spherical form with zeta potentials in a range of -9 – -12 mv. All formulations with 200 mg of pluronic-F-127 in a final solution of 4 ml could encapsulate Res and DMC up to 23.17 ± 0.88 mg and 1.50 ± 0.03 mg, respectively. However, when DMC was co-loaded with 20 mg of Res, a clear solution with a much higher loading capacity of 3.74 ± 0.07 mg (about 2.5 times) was observed which may be induced, by π - π interaction and H-bonding. The FT-IR, DSC, and p-XRD results demonstrated that DMC and Res were dispersed in dried powder in amorphous forms. The obtained nanomicelles are useful for further investigation for anti-breast cancer treatment.

Keywords: Nanomicelles, dimethylcurcumin, resveratrol, loading capacity, water solubility, improvement.

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INTRODUCTION

Cancer accounts for nearly ten million deaths, or nearly one in six deaths in 2020, resulting in the leading cause of death across the world.^[1] Breast cancer is one of the most common cancers in the world, and many patients are affected by it. In Thailand, breast cancer is the top leading causes of new cancer cases in women.^[2] Therefore, new anti-breast cancer agents or novel targeted drug delivery systems are required.^[3, 4] Current breast cancer treatment is a combination of surgery and radiation, which may be accompanied by hormone treatment. A chemotherapy and combined drug therapies, either before or after surgery are common in the treatment.^[5, 6] Chemotherapy is one of the most common treatment for cancers; however, it may cause many side effects due to the process of systemic treatment, which kills all dividing cells, non-selectively leading to cell damage and killing both healthy and cancer cells at the same time. This is resulting in the most common side effects such as hair loss, fatigue, diarrhea, nausea, and vomiting.^[7, 8] All undesired side effects cause non-compliance in cancer patients and may result in unsuccessful treatment. Therefore, a selective chemotherapeutic agent that kills only cancer cells without or with minimal adverse effects on the healthy cells is currently needed.^[9]

There are several strategies for increasing the efficacy of chemotherapeutic drugs while reducing side effects. For example, targeted therapy includes: kinase inhibitors and monoclonal antibodies, Chimeric-antigen receptor-T cell therapy,^[10] immune check point inhibitors^[11] and others.^[12] Nanotechnology is a promising drug delivery approach that is gaining much interest in anticancer therapeutics.^[13-15] It could improve the pharmacological effect by enhancing the specificity towards cancer cells, resulting in fewer side effects in cancer treatment. A growing number of applications of nanotechnology in breast cancer treatment have been reported, including the use of liposomes,^[16, 17] polymeric micelles,^[18] polymeric nanoparticles,^[19] polymer-drug conjugate,^[20] nanostructured lipid carriers and solid lipid nanoparticles.^[21] Nanomicelles are one of the nanotechnology delivery systems that, are self-assembled, biocompatible, and are biodegradable normally constructed from amphiphilic block polymers biodegradable. Nanomicelles with sizes ranging from 10-200 nm are ideal for improve anticancer activity.^[22] Nanocarriers for anticancer drugs promote the targeting of cancer cells by enhancing drug molecules through the leaky

vascular system and allowing them to across the tumor endothelium, leading to drug accumulation in the tumors.^[23-25] A number of natural and synthetic polymers have been successfully employed for the development of nanomicelles.

Pharmaceutical and clinical applications have long described the use of amphiphilic block copolymers, such as pluronics. Pluronic are the block copolymers of hydrophilic, poly(ethylene oxide) (PEO) and hydrophobic, poly(propylene oxide) (PPO).^[22, 26] A number of reports demonstrated that tumor therapy has vastly improved by utilizing pluronic-based multi-functional drug carriers. Since, they have been designed to fit the characteristics of tumor microenvironments.^[9, 27] Natural products and their derivatives become significant options in breast cancer treatment. Among them, a synthetic curcumin analog (*[(1E, 6E) -1,7-bis (3, 4-dimethoxyphenyl) hepta-1, 6-diene-3, 5-dione* or; dimethylcurcumin (DMC), [Figure 1] is structurally similar to that of curcumin.^[28]

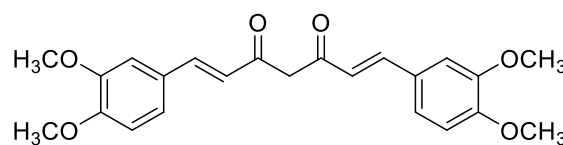


Figure 1 Structure of DMC

DMC has been shown to exhibit anti-breast, anti-prostate, anti-leukemia cancers, anti-trypanosomal, anti-leishmanial, anti-oxidation, and anti-mycobacterial activities.^[28, 29] Furthermore, DMC showed higher activity in anti-cancer, antioxidant, and anti-mycobacterial than curcumin.^[29, 30] Resveratrol (Res) is a naturally occurring polyphenol that has been reported as a chemopreventive, a neuroprotective, a cardioprotective agents, as well as anti-ageing properties.^[31, 32] They are also reported to be safe and effective anticancer agents and can hinder the growth of breast cancer by inhibiting cancer cell proliferation, angiogenesis, metastasis, and promoting apoptosis. However, the use of DMC and resveratrol is limited by their pharmacokinetic properties since they are hydrophobic and have poor solubility in water. In addition, Res has a rapid metabolism, resulting in a short plasma half-life.^[33, 34] The preparation of nano-micelles of curcumin has been reported. However, there is no report on the development of curcumin analog (DMC) and Res co-loaded nanomicelles. Herein, the preparations of Pluronic nanomicelle formulation encapsulating DMC, Res, and DMC coloaded with Res for further utilization in breast cancer treatment are reported.^[35, 36] The

improvements in the loading capacity of DMC were investigated by varying the amount of resveratrol. The physicochemical properties of the resulting co-loaded nanomicelles were characterized and reported.

MATERIALS AND METHODS

Materials

Resveratrol was purchased from Carbosynth Limited, Compton, Berkshire, UK. Pluronic F-127 was purchased from Sigma-Aldrich, Germany. Analytical grade acetonitrile was bought from Fluka, Germany. DMC was synthesized in our laboratory with a purity of over 99%. A nylon-membrane syringe filter with pore size 0.22 μm was purchased from Vertical Chromatography, Thailand. Analytical grade dimethyl sulfoxide (DMSO) was purchased from Labscan, Thailand.

Methods

Preparation of Pluronic-F-127-Res loaded nanomicelles, Pluronic-F-127-DMC loaded nanomicelles, and Pluronic-F-127-DMC-Res co-loaded nanomicelles

Pluronic F-127-Res loaded, Pluronic-F-127-DMC loaded, and Pluronic-F-127-DMC-Res co-loaded nanomicelles were prepared by thin film hydration method according to the method previously described by Carlson, et al. (2014)^[37] with a slight modification. First, a stock solution of Pluronic F-127 (100 mg/ml) was prepared. Stock solutions of Res (25 mg/ml) and DMC (5 mg/ml) were separately prepared in acetone and further used in micelles preparation. Four formulations of Res loaded pluronic F-127 nanomicelles (R1-R4), 8 formulations of DMC-loaded pluronic F-127

nanomicelles (D1-D8), and 18 formulations of DMC-Res-co-loaded pluronic F-127 nanomicelles were prepared according to Tables 1, 2, and 3, respectively.

In the preparation process, 2 ml of the stock solution of Pluronic F-127, and varied volumes of stock solutions of Res or DMC were separately added to each 125 ml round bottom flask and well mixed. Acetone was removed from the mixtures by using a rotary evaporator (Heidolph, Hei-CHILL 350, England) under vacuum at 60 $^{\circ}\text{C}$, resulting in a homogeneous drug polymer film. The micelles were obtained by rehydrating of the film in 4 ml of warm deionized water with gentle agitation, followed by vortex and sonication for 10 min. The resulting micelles solutions were filtered through a nylon syringe filter (pore size 0.22 μm) to remove any non-encapsulated drug. Res-Pluronic F-127 loaded, DMC-Pluronic F-127 loaded nanomicelles, and Res-DMC-loaded pluronic F-127 nanomicelles dried powders, respectively were obtained by adding glycine (1% w/v) to the resulting nanomicelles solutions, and subjected to a freeze dryer (Telstar Cryodos, JOSEP TAPIOLAS 120, Spain). The entrapment efficiency of Res and DMC was determined using a HPLC system [HITACHI, chromaster, Japan] with a UV-Vis detector (HITACHI, chromaster 5430 Diode array detector, Japan) at $\lambda = 304$ nm for Res and fluorescence detector (HITACHI, chromaster 5440 FL detector, Japan) for DMC (excited $\lambda = 420$ and emission $\lambda = 470$ nm). The entrapment efficiency (%EE) and loading capacity (%LC) of Res and DMC were quantified and interpretation concentrations were obtained from the calibration curves of standard solutions of Res and DMC using the following equation:

$$\%EE = \left(\frac{\text{wt of each drug entrapped in the micelles samples}}{\text{wt of drugs added in micelles preparation}} \right) \times 100$$

$$\%LC = \left(\frac{\text{wt of each drug entrapped in the micelles samples}}{\text{wt of total amount of micelles}} \right) \times 100$$

Table 1 Formulations for the preparation of Res loaded Pluronic F-127 nanomicelles in 4 ml final volume.

Formulation code	Volume (ml) of Pluronic F-127 (100 mg/ml)	Volume (ml) of Res stock solution 25 mg/ml
R1	2	0.4
R2	2	0.8
R3	2	1.0
R4	2	1.2

Table 2 Formulations for the preparation of DMC-loaded Pluronic F-127 nanomicelles in 4 ml final volume

Formulation code	Volume (ml) of Pluronic F-127 (100 mg/ml)	Volume (ml) of DMC stock solution 5 mg/ml)
D1	2	0.08
D2	2	0.16
D3	2	0.24
D4	2	0.32
D5	2	0.40
D6	2	0.48
D7	2	0.60
D8	2	0.80

Table 3 Formulations for the preparation of DMC-Res-co-loaded Pluronic F-127 nanomicelles in 4 ml final volume.

Formulation code	Volume (ml) of Pluronic F-127 (100 mg/ml)	Volume (ml) of Res stock solution 25 mg/ml)	Volume (ml) of DMC stock solution 5 mg/ml)
R2D8	2	0.8	0.8
R3D8	2	1.0	0.8
R4D8	2	1.2	0.8
R0.75D9	2	0.3	1.0
R1D9	2	0.4	1.0
R5D9	2	0.6	1.0
R2D9	2	0.8	1.0
R3D9	2	1.0	1.0
R0.75D10	2	0.3	1.2
R1D10	2	0.4	1.2
R5D10	2	0.6	1.2
R2D10	2	0.8	1.2
R3D10	2	1.0	1.2
R0.75D11	2	0.3	1.4
R1D11	2	0.4	1.4
R5D11	2	0.6	1.4
R2D11	2	0.8	1.4
R3D11	2	1.0	1.4

Nano-micelles characterizations

Functional groups attached were analyzed using FT-IR spectrometer (Spectrum One, Perkin Elmer Ltd., UK). Thermal characteristics of samples were determined by using a Differential Scanning Calorimeter (Perkin Elmer DSC 8000, USA). The physical states of samples were characterized by Powder X-ray diffractometer (Philips X'Pert MPD, Netherlands).

Size and zeta potential determination

The size, size distribution, and zeta potential of the obtained Res-, DMC-, and DMC-Res-co-loaded Pluronic F-127 nanomicelles were measured using a Zetasizer and dynamic light scattering techniques (Zetasizer Nano ZS, Malvern, England). Before the determination, all samples were freshly prepared and filtered through a 0.22 µm syringe filter.

Differentials Scanning Calorimetry (DSC)

Thermal characteristics of Res, DMC, glycine, dried powder of blank Pluronic F-127 nanomicelles, Res-loaded nanomicelles (formulation R1), DMC-loaded nanomicelles (formulation D3), and DMC-Res-co-loaded

Pluronic F-127 nanomicelles (formulation R3D8) freeze dried samples were determined by using a Differential Scanning Calorimeter (Perkin Elmer DSC 8000, USA). Each sample (0.6 - 6 mg) was weighed and placed in a sealed aluminium pan. Samples were heated at a rate of 10°C/min under nitrogen purge at a rate of 50 mL/min.

Powder X-ray diffractometry (PXRD)

The physical states of Res, DMC, and Res-DMC loaded Pluronic F-127 nanomicelles dried powder were obtained by PXRD technique. The X-ray powder diffraction spectra of all samples were determined at room temperature by using an X-ray diffractometer (Malvern Panalytical, Netherlands) with Cu as an anode material and a graphite monochromator. The experiment was operated at a voltage of 40 kV. The samples were analyzed in a 2θ angle range from 5°- 90°.

Statistical analysis

One-way ANOVA was used to compare the results of all the studies. When $p < 0.05$ present the differences were purposed statistically significant. Statistical Package for the Social Sciences (SPSS)

Version 22.0 software was used for the statistical analysis.

Results and Discussions

Preparation of Res-, DMC, and Res-DMC loaded Pluronic F-127 nanomicelles.

The low water solubility of resveratrol is one of the key problems, limiting the uptake from foods or in pharmaceutical form.^[38, 39] Therefore, improving its water-dispersibility in order to increase its chemical stability, and bioavailability is still in focused.^[40, 41] Clear solutions of Res-loaded Pluronic F-127 nanomicelles were obtained as displayed in Figure 2. Both R1 and R2 gave clear solutions after preparation and standing for 1 day after preparation, indicating stable nanomicelles. Formulations using Res at 25 (R3) and 30 (R4) mg per 200 mg pluronic F-127 gave clear solutions after preparation, however, precipitation was observed at 36 and 2 h after preparation and standing at room temperature, respectively. It could be due to the maximum loading capacity of Pluronic.

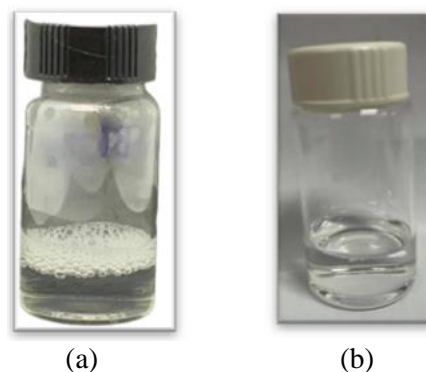


Figure 2 Representative pictures of Res-loaded Pluronic F-127 nanomicelles
(a) Formulation R1 (b) Formulation R2

The Pluronic F-127-DMC nanomicelles products were obtained as displayed in Figure 3. DMC-loaded Pluronic F-127 nanomicelles were prepared using 0.4 (D1, a), 0.8 (D2, b), 1.2 (D3, c), 1.6 (D4, d), 2.0 (D5, e), 2.4 (D6, f), 3.0 (D7, g), and 4.0 (D8, h) mg of DMC per 200 mg of Pluronic F-127. All formulations were obtained as clear solutions after preparation. However, formulations D5 and D6 precipitated 2 h and 20 min after standing at room temperature, respectively. On the other hand, precipitation in D7 and D8 formulations was observed immediately after preparation. The results indicated that Pluronic F-127 could enhance water solubility of DMC, however Pluronic F-127 could take only maximum of 1.2 mg per 100 mg by thin film hydration method.

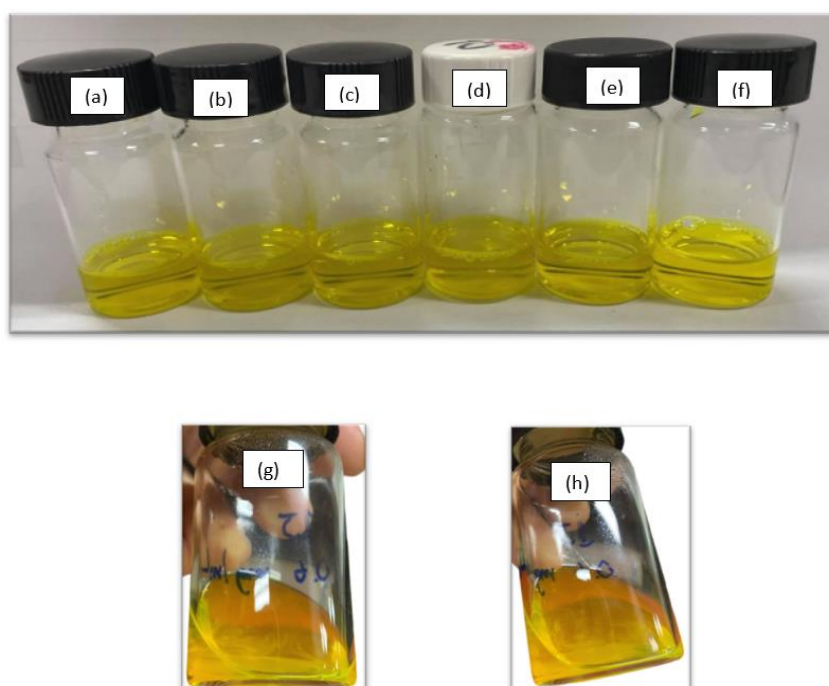


Figure 3 Appearances of DMC-loaded Pluronic F-127 micelles using DMC 0.4 mg (a), 0.8 mg (b), 1.2 mg (c), 1.6 mg (d), 2 mg (e), 2.4 mg (f), 3.0 (g) and 4.0 (h) per 200 mg of Pluronic F-127 in 4 ml, respectively.

Only DMC-Res loaded Pluronic F-127 micelles using 4 mg of DMC and 20 (R2D8), 25 (R3D8), 30 (R4D8) mg of resveratrol per 200 mg Pluronic F-127 were obtained as clear solutions after preparation (Figure 4). However, formulation R4D8 showed precipitation after standing at room temperature for 24 h. DMC-Res loaded Pluronic F-127 micelles using 5 mg of DMC and 7.5 (R0.75D9), 10 (R1D9), 15 (R5D9), 20 (R2D9), 25 (R3D9) mg of Res per 200 mg Pluronic F-127 did not give desired nanomicelles products. R1D9 formulations precipitated after 10 min. R5D9 and R2D9 formulation precipitated after 15 min. Both R0.75D9 and R3D9 formulations precipitated immediately. DMC-Res loaded Pluronic F-127 micelles using 6 mg of DMC and 7.5 (R0.75D10), 10 (R1D10), 15 (R5D10), 20 (R2D10), 25 (R3D10) mg of Res per 200 mg Pluronic F-127 were not stable. R1D10 formulation precipitated 10 min after preparation, while formulations R0.75D10, R5D10, R2D10 and R3D10 precipitated immediately after adding pluronic solution. DMC-Res loaded Pluronic F-127 micelles using 7 mg of DMC and 7.5 (R0.75D11), 10 (R1D11), 15 (R5D11), 20 (R2D11), 25 (R3D11) mg of Res per 200 mg Pluronic F-127 were not formed during preparation process. The result indicated that DMC and Res co-loaded in Pluronic-F-127 for water solubility improvement, however, there are still some limitations in loading capacity. It is interesting that using co-loaded strategy, stable and clear solution of DMC was obtained at higher amount (2 mg: 100 mg Pluronic-F-127) than using only DMC (1.2 mg: 100 mg Pluronic-F-127) as mentioned above.

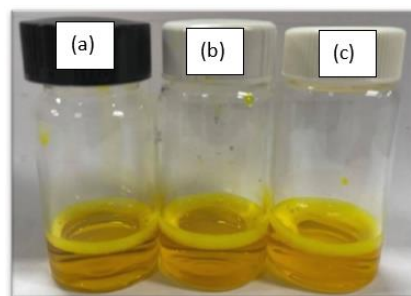


Figure 4 Appearances of DMC -Res loaded Pluronic F-127 micelles using DMC 4 mg and Res 20 mg (a:R2D8), 25 mg (b:R3D8), 30 mg (c:R4D8), per 200 mg of Pluronic F-127 respectively at 15 minutes after preparation.

Freeze dried products of the obtained Res-, DMC, Res-DMC loaded Pluronic F-127 nanomicelles.

The freshly prepared blank Pluronic-F127 nanomicelles, Pluronic F-127-Res loaded nanomicelles (Formulations R1, R2 and R3), Pluronic F-127-DMC loaded nanomicelles (formulations D1, D2, D3 and D4) and Pluronic F-127-Res-DMC loaded nanomicelles (formulation R2D8, R3D8 and R4D8) were dried by freeze-drying process. Glycine 1% w/v was added to all formulations before using for freeze-drying (Telstar Cryodos, DOSEP TAPIOLAS 120, Spain). The dried products were weighed and calculated the % recovery. The appearances of all samples from formulations D1, D2, D3 and D4 were yellow color in fluffy form. Freeze-dried powder of co-loaded Res and DMC in Pluronic F-127 nanomicelles were successfully obtained from only 3 formulations of R2D8, R3D8 and R4D8 resulting in FR-R2D8, FR-R3D8, and FR-R4D8, respectively. All dried samples were obtained as orange, light and bulky powder in high yields (85.02 – 94.30%) indicating less loss during preparation process.

Table 4 Appearances and % recovery of the dried nanomicelles powder after freeze drying process

Formulation code	% Recovery \pm SD	Appearance observation
Blank Pluronic F-127	92.41 \pm 1.17	bright white, light-fluffy powder
FR-R1	85.02 \pm 1.58	
FR-R2	86.19 \pm 1.16	light-fluffy powder in beige
FR-R3	87.49 \pm 1.43	
FR-D1	90.56 \pm 1.73	bright yellow color in fluffy form
FR-D2	89.61 \pm 1.53	bright yellow color in fluffy form
FR-D3	90.14 \pm 1.05	dark yellow color in fluffy form
FR-D4	91.66 \pm 0.98	dark yellow color in fluffy form
FR-R2D8	94.30 \pm 1.05	
FR-R3D8	93.37 \pm 0.92	orange, light and bulky powder
FR-R4D8	91.35 \pm 1.06	

The entrapment efficiencies (%EE) and loading capacities (%LC) of Res, DMC, Res-DMC loaded Pluronic F-127 nanomicelles.

Table 5 summarized the percentage of encapsulation efficiencies (%EE) and the percentage of loading capacities (%LC) of the obtained Res loaded Pluronic F-127 nanomicelles. All formulations were prepared by using 200 mg of Pluronic F-127 in a final solution of 4 mL water. However, amount of Res in the preparation was varied including 10 mg (R1), 20 mg (R2), 25 mg (R3) and 30 mg (R4), respectively. All products after being freshly prepared were subjected to HPLC analysis. The results demonstrated that using Pluronic F-127 200 mg with Res 10 mg, 20 mg, 25 mg, and 30 mg could entrap 8.86 ± 0.13 mg, 18.19 ± 0.69 mg, 20.90 ± 0.77 mg and 23.17 ± 0.88 mg, respectively. The results when expressed as %

encapsulation efficiencies (%EE) were 88.62 ± 1.32 , 90.93 ± 3.44 , 83.59 ± 3.10 and 77.25 ± 2.94 %, respectively.

%EE of formulations R1 and R2 were not significantly different, indicating that increasing Res from 10 to 20 mg did not provide higher encapsulation efficiency. However, when increasing Res to 25 and 30 mg found reduction in % entrapment efficiencies which reduced from $88.62 - 90.93\%$ (R1 and R2) to 83.59 ± 3.10 (R3) and $77.25 \pm 2.94\%$ (R4), respectively. This may be due to the saturation of the Res in Pluronic F-127. Res was entrapped within the core of the micelles by self-assembly of the tri block – copolymer in aqueous medium. The main interaction site for the hydrophobic drug and micelle core is provided by the $-\text{CH}_2-$ group in the PPO chain of Pluronic F-127.^[42]

Table 5 The entrapment efficiencies (EE) and loading capacities (LC) of Res loaded Pluronic F-127 nanomicelles.

Formulation code	Weight ratio pluronic (mg) : Res (mg) in 4 mL of the obtained micelles	Entrapped Res (mg)	%EE \pm SD	%LC \pm SD
R1	200 : 10	$8.86 \pm 0.13^*$	$88.62 \pm 1.32^*$	$4.21 \pm 0.06^*$
R2	200 : 20	$18.19 \pm 0.69^*$	$90.93 \pm 3.44^*$	$8.25 \pm 0.31^{**}$
R3	200 : 25	$20.90 \pm 0.77^{**}$	$83.59 \pm 3.10^{**}$	$9.29 \pm 0.34^{***}$
R4	200 : 30	$23.17 \pm 0.88^*$	$77.25 \pm 2.94^{**}$	$10.07 \pm 0.38^{***}$

*, **, ***, indicating significant different determined by using one-way ANOVA at p-value = 0.05

The nanomicelles obtained from the preparation process using Res 5% of Pluronic F-127 (R1) resulting in $4.21 \pm 0.66\%$ loading capacity and when increasing Res to 10% (R2), double loading capacity (8.25 ± 0.31 %) was observed. Pluronic F-127 contains PPO block MW of 3600 and PEO block MW of 8820, therefore it could incorporate drug(s) into its core during micelles preparation. It is known that Pluronic nanomicelles help increase solubility, and circulation time of hydrophobic drugs.^[43, 44] We found that Pluronic F-127 improved water solubility of Res similar to previous results.^[45, 46]

The loading capacity of Res in the obtained nanomicelles was not improved when increasing Res to 12.5 (R3) and 15 % (R4) by wt. Using Res 12.5% and 15% by weight provided slightly higher loading capacities of Res to $9.29 \pm 0.34\%$ (R3) and $10.07 \pm 0.38\%$ (R4), respectively. It is therefore indicated that the internal core of the micelles has limited space and can only uptake a limited amount of Res. As a result, when increasing the amount of Res more than 10% by weight per polymer, the percentage of loading capacity did not significantly increase.

The entrapment efficiencies (EE) and loading capacities (LC) of the prepared DMC loaded PluronicF127 nanomicelles.

The percentage of entrapment efficiencies (%EE) and the percentage of loading capacities (%LC) of the obtained DMC loaded Pluronic F-127 nanomicelles are listed in Table 6. All formulations were prepared by using 200 mg of Pluronic F-127 in 4 mL final solution in water. However, the amount of DMC in the preparation process was varied including 0.4 mg (D1), 0.8 mg (D2), 1.2 mg (D3), 1.6 mg (D4), 2.0 mg (D5), 2.4 mg (D6), 3 mg (D7), and 4 mg (D8), respectively. Only formulations D1 – D5 after freshly prepared were subjected to HPLC analysis since formulations D6–D8 precipitated immediately after hydration process which indicating unstable micelles. The results demonstrated that using Pluronic F-127 200 mg with DMC 0.4 mg (D1), 0.8 mg (D2), 1.2 mg (D3), 1.6 mg (D4), and 2.0 mg (D5) could entrap 0.33 ± 0.013 mg, 0.65 ± 0.025 mg, 0.98 ± 0.013 mg, 1.29 ± 0.025 mg and 1.50 ± 0.031 mg, respectively. The results when expressed as % encapsulation efficiencies (%EE) were 81.26 ± 3.20 , 81.28 ± 3.17 , 81.47 ± 1.12 , 80.37 ± 1.58 and 74.23 ± 1.58 %, respectively.

Table 6 Pluronic F127-DMC nanomicelles formulations and their entrapment efficiencies (%EE) and loading capacity (%LC)

Formulation code	Weight ratio Pluronic F-127 (mg) :DMC (mg) in 4 ml of the obtained micelles	Entrapped DMC (mg)	%EE \pm SD	%LC \pm SD
D1	200 : 0.4	0.33 \pm 0.013*	81.26 \pm 3.20*	0.16 \pm 0.007*
D2	200 : 0.8	0.65 \pm 0.025*	81.28 \pm 3.17*	0.38 \pm 0.006**
D3	200 : 1.2	0.98 \pm 0.013*	81.47 \pm 1.12*	0.49 \pm 0.007***
D4	200 : 1.6	1.29 \pm 0.025*	80.37 \pm 1.58*	0.64 \pm 0.010****
D5	200 : 2.0	1.50 \pm 0.031**	74.23 \pm 1.58**	0.56 \pm 0.009*****
D6	200 : 2.4	ND	ND	ND
D7	200 : 3	ND	ND	ND
D8	200 : 4	ND	ND	ND

*, **, ***, indicating significant different determined by using one-way ANOVA at p-value = 0.05, ND = Not determined due to the observation of precipitation after preparation.

%EE of formulations D1, D2, D3 and D4 were not significantly different, it is therefore increasing DMC from 0.4 to 1.6 mg did not provide higher encapsulation efficiency. However, when increasing DMC to 2.0 mg (D5) resulting in reduction in % entrapment efficiencies which reduced from 80.37 – 81.47% of D1 – D4 to 74.23 \pm 1.58 in D5 formulation. DMC was entrapped within the core of the micelles by self-assembly of the tri block – copolymer in aqueous medium. The main interaction site for the hydrophobic drug and micelle core is provided by the -CH₂- group in the PPO chain of Pluronic F-127.^[42]

Moreover, the nanomicelles obtained from the preparation process using DMC 0.2% per wt of Pluronic F-127 (D1) resulting in 0.16 \pm 0.007 % loading capacity, when increasing DMC in the preparation process to 0.4 % by weight (D2), double loading capacity (0.38 \pm 0.006%), to 0.6 % by weight (D3), 3-times loading capacity (0.49 \pm 0.007%), and to 0.8% by weight (D4), 4-times loading capacity (0.64 \pm 0.010 %) were observed. However, a decrease in loading capacity of DMC in the obtained nanomicelles was observed. We found that DMC loaded in Pluronic F-127 also assisted water solubility, akin to curcumin which has similar chemical structure as DMC.^[42, 47] However, increasing DMC to 1 % (D5) which providing loading capacity only at 0.56 \pm 0.009%. As a result, micelles provided maximum encapsulation because the internal core of the micelles has limited space. These findings are comparable to curcumin.^[42]

The entrapment efficiencies (EE) and loading capacities (LC) of Res -DMC loaded Pluronic F-127 nanomicelles.

The entrapment efficiencies of Res and DMC in Res-DMC nanomicelles were determined only samples of formulations R2D8, R3D8 and R4D8 since other formulations gave unstable

nanomicelles and the results are summarized in Table 7.

Formulation R2D8, used 20 mg and 4 mg of Res and DMC, respectively in the preparation process, provided 17.86 \pm 0.084 mg of Res and 3.74 \pm 0.066 mg of DMC entrapped in the nanomicelles. %EE of Res and DMC in R2D8 were 89.32 \pm 0.42% and 93.59 \pm 1.66%, respectively. The results indicated that almost all Res and DMC could be entrapped in nanomicelles during the preparation process. Similar results were observed with the formulation R3D8 which uses 25 mg and 4 mg of Res and DMC in nanomicelles preparation. R3D8 could entrap 21.77 \pm 0.077 mg and 3.69 \pm 0.056 mg of Res and DMC, respectively in the obtained nanomicelles. %EE of resveratrol and DMC in R3D8 were 87.09 \pm 0.31% and 92.37 \pm 1.41%, respectively, showed that almost all used active compounds were well encapsulated in the obtained nanomicelles and gave stable nanomicelles after preparation. This suggests that the improvement in solubility found in our study is like those found in the use of curcumin combined with Res in Carlson 's study (2014).^[37]

In the preparation of R4D8 nanomicelles, 30 mg and 4 mg of resveratrol and DMC were used respectively. R4D8 could entrap 25.29 \pm 0.426 mg and 3.73 \pm 0.060 mg of resveratrol and DMC resulting in 84.29 \pm 1.42% and 93.34 \pm 1.50% entrapment efficiencies, respectively. However, R4D8 was observed to have precipitation after preparation of 48 h.

All formulations provided the same number of % loading capacities of DMC (1.81-1.83%), since the same amount of DMC 4 mg were used in the preparation process. However, increasing amount of DMC in the nanomicelles preparation process from 4 to 5, 6 and 7 mg, unstable nanomicelles were obtained since precipitates were observed

after preparation process. It is interesting that co-loaded Res and DMC in the nanomicelles preparation gave an effect on the loading capacities of both compounds in the obtained nanomicelles. As the results indicated in Table 7 that using only Res in the nanomicelles preparation at 30 mg provided 77.25 ± 2.94 %EE and 10.07 ± 0.38 %LC, however when using Res at the same amount co-loaded with 4 mg DMC, the obtained nanomicelles provided slightly higher %EE (84.29 ± 1.42 %) and %LC (11.01 ± 0.19 %). The results indicated that DMC may play an important role to assist the encapsulation of Res in the nanomicelles.

Similar results were also observed with the loading efficiency of DMC when co-loaded with Res. As the result indicated in Table 7 that the maximum amount that could give stable DMC-nanomicelles without precipitation was 2 mg (for 200 mg Pluronic F-127). When 4 mg of DMC was co-loaded with Res either 20, 25 or 30 mg, stable nanomicelles without precipitation were obtained. %LC increased from 0.56 ± 0.009 % to 1.81-1.83% when DMC co-loaded with Res. The result showed that higher drug loading capacity could be improved due to interactions between DMC and Res such as hydrogen bonding, π -stacking, or both interactions.^[48] However, using DMC more than 4 mg either used the same amount of Res (R2D9,

R3D9, R2D10, R3D10, R2D11 and R3D11) or lower amount of Res (R0.75D9, R1D9, R5D9, R0.75D10, R1D10, R5D10, R0.75D11, R1D11, R5D11) could not improve entrapment efficiencies of DMC. All the latter formulations were not stable and precipitate after preparation. The stable co-loaded nanomicelles were obtained from the formulations using 4 mg of DMC co-loaded with 20 and 25 mg Res per 200 mg Pluronic F-127 in 4 ml water. Therefore, these formulations were used for further investigations.

Curcumin has been shown to be an effective preventative and therapeutic agent against many types of cancer. However, there are problems of stability, solubility, and cytotoxicity to normal cells. Therefore, derivatives of curcumin were developed. The one used in this research was DMC. DMC has many anticancer qualities and is more stable than curcumin, it still has poor solubility and is cytotoxic to normal cells. A drug delivery system using pluronic F-127 as a carrier increases the solubility of DMC and targets the delivery of DMC to cancer cells.

The loading of DMC into pluronic F-127 could be enhanced in this study by co-loaded with Res caused by π - π interaction and H-bonding with Res.

Table 7 Pluronic F-127 Res-DMC nanomicelles formulations and their entrapment efficiencies (EE) and loading capacity (LC) (mean + S.D.)

Formulation code	Weight ratio pluronic (mg) : RES (mg) : DMC (mg)	Entrapped RES (mg)	%EE of RES	%LC of RES	Entrapped DMC (mg)	%EE of DMC	%LC of DMC
R2D8	200 : 20 : 4	$17.86 \pm 0.084^*$	$89.32 \pm 0.42^*$	$8.13 \pm 0.04^*$	$3.74 \pm 0.066^*$	$93.59 \pm 1.66^*$	$1.83 \pm 0.03^*$
R3D8	200 : 25 : 4	$21.77 \pm 0.077^*$	$87.09 \pm 0.31^*$	$9.69 \pm 0.03^{**}$	$3.69 \pm 0.056^*$	$92.37 \pm 1.41^*$	$1.81 \pm 0.03^*$
R4D8	200 : 30 : 4	$25.29 \pm 0.426^{**}$	$84.29 \pm 1.42^{**}$	$11.01 \pm 0.19^{***}$	$3.73 \pm 0.060^*$	$93.34 \pm 1.50^*$	$1.83 \pm 0.03^*$
R0.75D9	200 : 7.5 : 5	ND	ND	ND	ND	ND	ND
R1D9	200 : 10 : 5	ND	ND	ND	ND	ND	ND
R5D9	200 : 15 : 5	ND	ND	ND	ND	ND	ND
R2D9	200 : 20 : 5	ND	ND	ND	ND	ND	ND
R3D9	200 : 25 : 5	ND	ND	ND	ND	ND	ND
R0.75D10	200 : 7.5 : 6	ND	ND	ND	ND	ND	ND
R1D10	200 : 10 : 6	ND	ND	ND	ND	ND	ND
R5D10	200 : 15 : 6	ND	ND	ND	ND	ND	ND
R2D10	200 : 20 : 6	ND	ND	ND	ND	ND	ND
R3D10	200 : 25 : 6	ND	ND	ND	ND	ND	ND
R0.75D11	200 : 7.5 : 7	ND	ND	ND	ND	ND	ND
R1D11	200 : 10 : 7	ND	ND	ND	ND	ND	ND
R5D11	200 : 15 : 7	ND	ND	ND	ND	ND	ND
R2D11	200 : 20 : 7	ND	ND	ND	ND	ND	ND
R3D11	200 : 25 : 7	ND	ND	ND	ND	ND	ND

*, **, ***, indicating significant different determined by using one-way ANOVA at p-value = 0.05, ND = Not determined due to the observation of precipitation after preparation

Size and zeta potential determination of Res, DMC, Res-DMC loaded Pluronic F-127 nanomicelles.

Pluronic blank nanomicelles were prepared using thin-film hydration method similar to the drug-loaded nanomicelles. The sizes of the blank Pluronic F-127 micelles were 39.3 ± 2.55 nm with PDI value of 0.394 ± 0.052 and zeta potentials of -10.37 ± 0.40 mV.

All stable formulations were subjected to analyze for their size, PDI and zeta potentials. The results (Table 8) indicated that the obtained micelles were in nano sizes range (about 25 – 50 nm). When the preparation used varied amount of Res and DMC in the processes provided slightly different in sizes among samples. All samples have narrow size distribution (PDIs = 0.06 – 0.29). Slightly smaller sizes of the Res-Pluronic F-127 micelles than blank micelles were observed. It may be due to the hydrophobicity property of Res which was entrapped in the core of the obtained nanomicelles induce the shrinking of the micelles. Formulations D1 – D5 gave micelles in nano size (39.40 – 50.30

nm) with PDI in a range of 0.142 – 0.290. DMC loaded pluronic nanomicelles gave significantly bigger sizes than blank Pluronic nanomicelles. This could be due to the molecular distribution between Pluronic chain of DMC inside the obtained micelles. Co-loaded formulations using 4 mg of DMC in the preparation process with variation amount of gave similar sizes about 25 nm. The result demonstrated that the varying amount of Res in the preparation process did not affect their sizes. The obtained mixed compounds loaded nanomicelles gave smaller sizes than blank nanomicelles, which could be due to the major contribution of the entrapped Res in the central core. All formulations gave nano-sized micelles with less than 100 nm indicated that the obtained products were suitable for drug delivery to the cancer cells.^[49] Zeta potential values of all stable nanomicelles were in a range of (-9.09) to (-11.17) mV which were not significantly different among the samples. The same zeta potential values were the contribution of the Pluronic F-127 rather than the encapsulated compounds.

Table 8 Sizes, PDI and zeta potential values of the obtained Res-loaded Pluronic F-127 nanomicelles.

Formulation code	Size (nm) \pm SD	PDI \pm SD	Zeta (mV) \pm SD
R1	$26.12 \pm 0.49^*$	$0.187 \pm 0.006^*$	$-10.30 \pm 0.12^*$
R2	$26.50 \pm 0.47^*$	$0.155 \pm 0.025^*$	$-9.03 \pm 0.34^*$
R3	$24.98 \pm 0.62^{**}$	$0.096 \pm 0.030^*$	$-9.41 \pm 0.22^*$
R4	$27.32 \pm 0.41^{***}$	$0.134 \pm 0.017^*$	$-11.3 \pm 0.45^*$
D1	$41.84 \pm 1.34^*$	$0.142 \pm 0.014^*$	$-9.09 \pm 0.19^*$
D2	$39.40 \pm 0.19^*$	$0.246 \pm 0.048^{**}$	$-11.17 \pm 0.25^*$
D3	$50.30 \pm 0.60^{**}$	$0.281 \pm 0.103^{**}$	$-9.39 \pm 0.77^*$
D4	$48.09 \pm 2.18^{**}$	$0.290 \pm 0.050^{**}$	$-9.75 \pm 0.43^*$
D5	$49.09 \pm 1.05^{**}$	$0.262 \pm 0.030^{**}$	$-9.76 \pm 0.56^*$
R2D8	$25.34 \pm 0.38^*$	$0.082 \pm 0.009^*$	$-12.87 \pm 0.95^*$
R3D8	$25.77 \pm 0.52^*$	$0.074 \pm 0.012^*$	$-11.00 \pm 1.01^*$
R4D8	$25.73 \pm 0.76^*$	$0.065 \pm 0.017^*$	$-11.17 \pm 0.55^*$

*, **, *** indicating significant different determined by using one-way ANOVA at p-value = 0.05

FOURIER TRANSFORM INFRARED SPECTROSCOPY (FT-IR)

FT-IR transmission spectrum of DMC, Pluronic F-127, and DMC-loaded Pluronic F-127 nano-micelles are displayed in Figure 5. The spectrum of Pluronic F-127 shows three major characteristic peaks at 963.8 , $1,112.4$ cm^{-1} of -C-O symmetrical structure and -C-O- asymmetrical of either group and $1,281.2$ cm^{-1} of -CH₂ vibration with -OH stretching peak $3,424.2$ cm^{-1} . In DMC spectrum displays absorption bands at $1,596.8$, 1580.0 and 1511.2 cm^{-1} corresponded to the C=C stretching of the aromatic groups. Strong absorption bands at $1,621.7$ were observed which corresponding to the -C=O stretching of the ketone groups. Weak bands at $3,003.5$, $2,934.0$ and $2,835.3$ cm^{-1} of -CH stretching of all alkyl groups and medium absorption band at 965.4 cm^{-1} belongs to the -C-H bending trans disubstituted alkenes were observed. The FT-IR spectrum of the nano-micelles confirmed the interaction of DMC with Pluronic micelles (Figure 5). The major peak at $3,413.8$ cm^{-1} of hydroxyl (-OH) vibration of Pluronic F-127 is still being observed. The -CO symmetrical structure and C-O asymmetrical stretching vibration of the ether groups in the middle formation are shifted to 963.4 and $1,108.0$ cm^{-1} respectively. Slightly shifted of -CH₂ vibration peak to $1,281.4$ cm^{-1} was noticed. The characteristic peaks of DMC in the nano-micelles spectra almost disappeared since the concentration of DMC in the nano-micelles was much smaller than Pluronic F-127 (only 1% w/w), however stronger and broader peak at $1,610.9$ cm^{-1} was

observed. The shifts in the peak positions and appearance of DMC peaks in the nano-micelles spectra indicated the encapsulation of DMC in the nano-micelles.

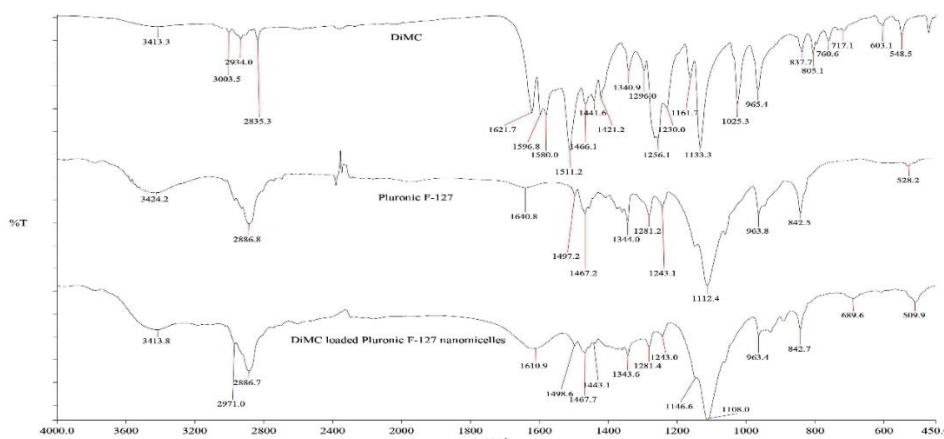


Figure 5 FT-IR transmission spectrum of DMC, Pluronic F-127, DMC-loaded Pluronic F-127 nano-micelles

FT-IR spectra of Res, Pluronic F-127, and Res loaded Pluronic F-127 nano-micelles were determined by KBr disc method. FT-IR spectrum of Pluronic F-127 (Figure 6) shows characteristic bands at 3,424.2 cm⁻¹ (-OH), 2,886.8 cm⁻¹ (-C-H), 1,112.4 and 963.8 cm⁻¹ (-C-O). FT-IR spectrum of Res shows characteristic peaks at 3,247.2 cm⁻¹ (phenolic -OH), 1,606.3 cm⁻¹ (-C=O), 1,586.5 cm⁻¹ (aromatic -C=C-), and 1,147.3 cm⁻¹ (-C-O stretching). In FT-IR spectrum of Res-loaded Pluronic F-127 nano-micelles, only the major characteristic peaks of Pluronic F-127 were observed, no characteristic bands of Res were present in the spectrum which may be due to low amount of Res was encapsulated in the nano-micelles as well as the shift in band of phenolic-OH of Res and the absence of band of -C-O group. This

suggested that Res was encapsulated within the nano-micelles possibility due to the hydrogen bonding between Res and Pluronic F-127. This interaction was confirmed by the FT-IR spectrum of physical mixture of Res-Pluronic F-127. Since in physical mixing (1:1 ratio), Res has no evidence of interaction with Pluronic F-127 found in the FT-IR spectrum. Most of the major characteristic bands of both Res and Pluronic F-127 were still remained in the physical mixture spectrum. However, the major bands of Res such as peaks at 1,606.3 cm⁻¹ (-C=O), 1,586.5 cm⁻¹, C-C=C- aromatic) and 1,147.3 cm⁻¹ (-C-O, stretching) were not observed in the Res-loaded nano-micelles spectrum indicate that Res was encapsulated in the nano-micelles. The possible interactions could be by hydrogen bonding.

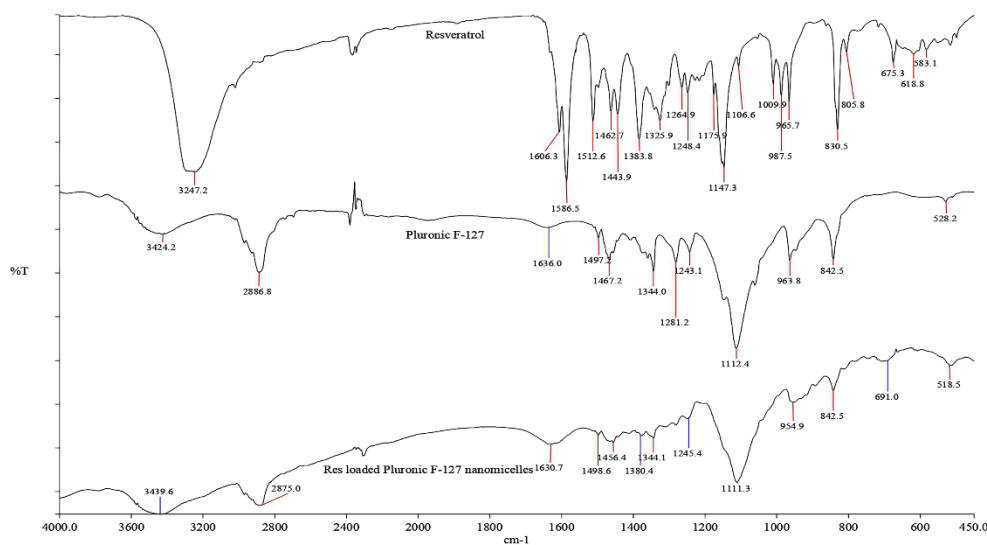


Figure 6 FT-IR spectra of resveratrol, Pluronic F-127 and resveratrol loaded Pluronic F-127 nanomicelles

Differentials Scanning Calorimetry (DSC)

The DSC thermograms of Res, and Res loaded Pluronic F-127 nano-micelles are shown in Figure 8. DSC thermogram of Res showed a melting endotherm at about 260.24°C. This indicates the melting point of Res which is in a good agreement with the reported value in the literature.^[48] Single sharp peak of Res indicates the crystallinity form. In the thermogram of Res-loaded Pluronic F-127 nano-micelles, no sharp endothermic peaks of Res were observed at 260.24°C but two peaks at 47.72°C and 242.52°C were observed. The peak of Res in the dried powder Res-loaded nano-micelles was still observed as a single sharp peak but at a

lower temperature and intensity at 242.52°C. It is therefore Res in the nano-micelles dried powder presents in crystalline form.

DSC thermogram of DMC showed a sharp peak of melting endotherm at about 125.22°C indicated melting point of DMC.^[50] However, no melting point peak of DMC was observed in the DSC thermogram of DMC loaded Pluronic F-127 nano-micelles. The result demonstrated that DMC was well dispersed in the nano-micelles dried powder in an amorphous form. It is therefore nano-micelles preparation could change DMC crystallinity state and may improve DMC water solubility.

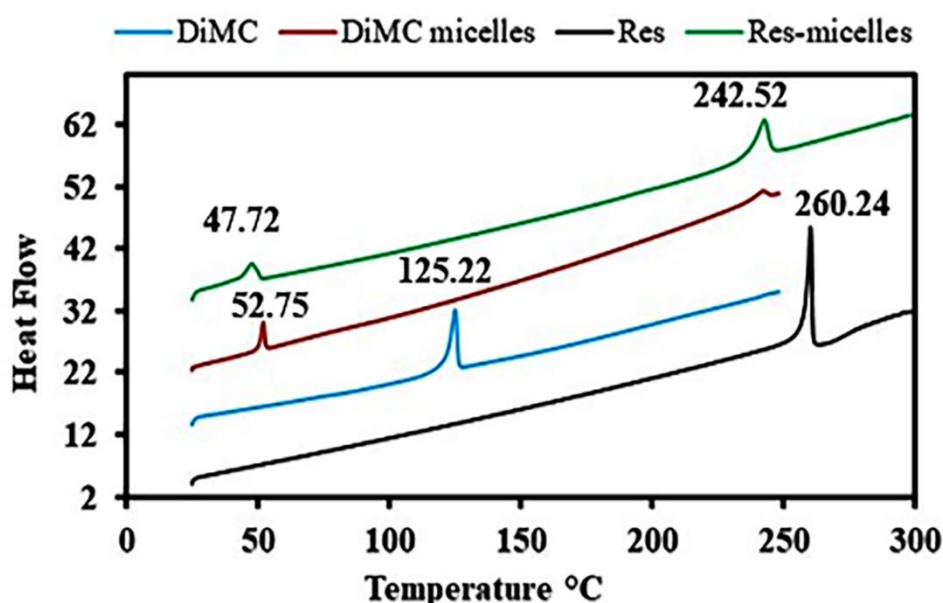


Figure 7 DSC analysis of DMC, DMC loaded nano-micelle, Res and Res loaded nano-micelles

Powder X-Ray Diffractometry (PXRD)

The PXRD pattern of DMC showed a number of distinctive sharp peaks at the position (2θ) 13.54°, 14.44°, 15.51°, 17.29°, 18.53°, 20.79°, 22.71°, 23.66°, 26.37°, 26.59° and 28.19° indicating crystallinity property of DMC. The characteristic crystallinity peaks of DMC disappeared in the PXRD pattern of DMC loaded Pluronic F-127 dried powder nano-micelles. Additionally, the two major peaks of PEO groups of Pluronic F-127 also remained intact with high intensity, indicating the nano-micelles preparation process reduced the crystallinity of DMC.

The PXRD pattern of Res showed a number of distinctive sharp peaks at 6.64°, 13.28°, 16.39°, 19.22°, 22.39°, 23.63°, 25.27° and 28.32° peak position (2θ) similar to the previous reports.^[51, 52]

These sharp peaks of Res were still observed in the PXRD patterns of physical mixtures prepared by mixing resveratrol with Pluronic F-127 in a weight ratio of 1 : 1. Therefore, Res in physical mixtures remain in crystalline state indicating no intermolecular interaction. In contrast to the PXRD patterns of Res in the dried powder nano-micelles no distinctive sharp peaks of resveratrol are presented. The major peaks belong to the Pluronic F-127 and glycine in the formulation. The results demonstrated that encapsulated Res in Res-Pluronic F-127 nano-micelles powder was dispersed in an amorphous form.

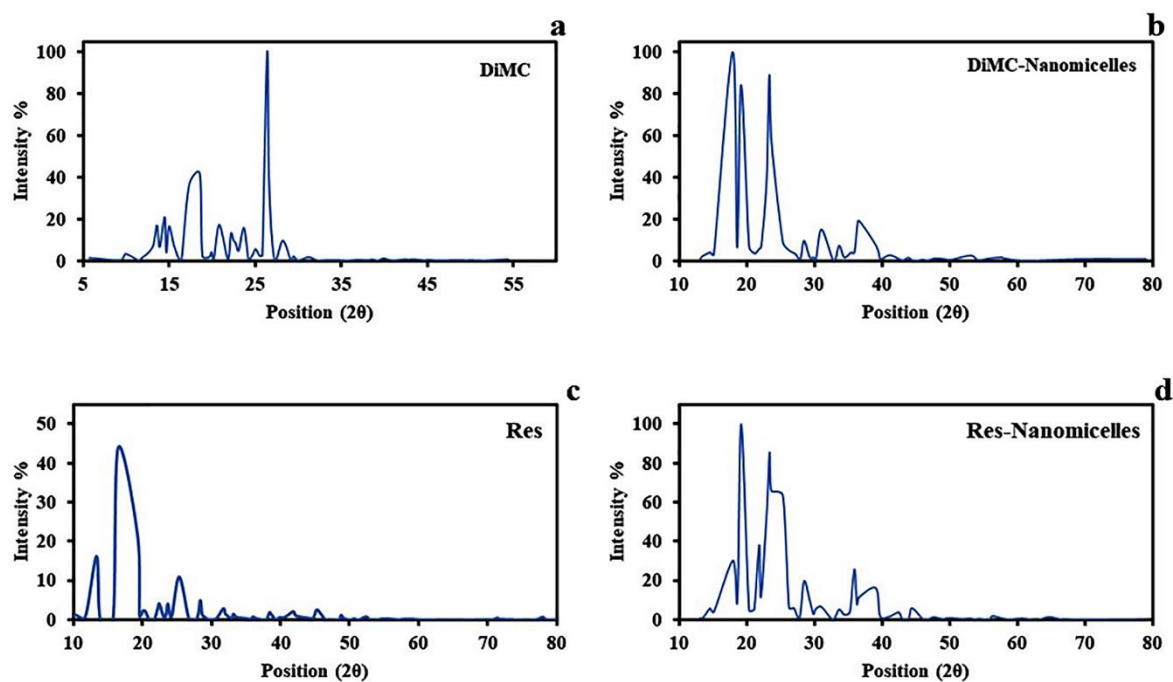


Figure 8 XRD analysis of a. DMC, b. DMC loaded nano-micelles, c. Res, and d. Res loaded nanomicelles

CONCLUSION

This work Res, DMC and co-loaded Res-DMC Pluronic F-127 nanomicelles prepared by thin-film hydration method were successfully obtained in high yields and encapsulation efficiencies. The enhanced drug loading capacities and solubility of DMC were observed by co-loading with Res. They were obtained in less than 100 nm size in spherical shapes which are suitable for anti-cancer improvement. The cytotoxicity and cell uptake against cancer cells are needed to prove these strategies.

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