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Development and evaluation of propolis loaded mixed micellar gel: Formulation, optimization and anticancer potential

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ABSTRACT

To increase the effectiveness of the anticancer natural product propolis extract (PE), mixed micellar gel formulation composed of Pluronic F127 and Tween 80 was investigated. Cold technique was used to formulate mixed micellar gel loaded with propolis extract (PE-MMG). 3^2 factorial design was employed to optimize formulation. PE-MMG was characterized for micellar size, surface morphology, surface charge, drug loading, entrapment, release study and *in vitro* cytotoxicity. Developed optimized formulation showed desirable formulation characteristics Optimized formulation showed desirable characteristics for improved therapeutic performance. Enhanced cytotoxicity potential revealed through MTT Assay by PE-MMG as compared with PE showed enhanced permeability and chemosensitization. In overall developed formulation further studied as a promising carrier for cancer therapy.

Keywords: mixed micellar gel, propolis, Pluronic F127, Tween 80, MCF 7

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INTRODUCTION

The sticky resinous bee product well known as propolis masticated from a combination of beeswax and resin that is gathered by Indian stingless bees from plant parts. Propolis is composed of different chemical constituents including flavonoids, polyphenols, amino acids, minerals etc., due to which it shows various biological activities. Because of the anti-inflammatory, immunomodulatory, and antiviral properties of propolis, it is widely used in traditional medicine[1]. Propolis is investigated for its antiproliferative impact on cancer cells in addition to these benefits. Its anticancer activity of propolis is also well proven through several investigations[2-5]. Propolis is thought to have an anticancer impact by causing apoptosis and cell cycle arrest. Propolis and its active ingredients, primarily CAPE, blocks cell cycle progression and trigger apoptosis in a variety of cancer types[6-9]. The region (where propolis was gathered) and dose, which both affect the chemical composition of propolis, are the primary determinants of its antiproliferative effects. The chemical compositions of propolis changes based on the bee species, area, and types of plants etc.[10–14]. Previous studies[6,15-19] have demonstrated the impact of propolis on breast cancer cell lines, prostate cancer cell lines, and leukemia cell lines. Propolis, however, may have distinct impacts on various cancer cells[4,20]. Apoptosis induction is shown to be substantially dependent on the concentration of propolis extract consist of phenolic acids and flavonoids[21-23] as well as the cytotoxic components of propolis that causes anticancer effects[23-25].

Propolis from different geographical locations are well proven for anticancer potential with apoptosis and some other mechanisms[25]. CAPE a propolis constituent also plays a role in the modulation of breast cancer through an epigenetically mediated pathway[26]. Propolis nanoparticles have antibacterial and anticancer properties *in vitro,* and cancer cells internalize the nanoparticles. Nanoparticle formulation of propolis demonstrated excellent anticancer potential with safety and biocompatibility[26-27]. However, their clinical application is limited by their low bioavailability, poor targeting, and low aqueous solubility. To increase therapeutic efficacy, safety, and patient acceptability for a variety of natural bioactives, nanomedicine-based drug delivery systems have been created[24-27]. Solubility of poorly soluble drugs can be enhanced encapsulation in inner hydrophobic core of mixed micelles due to their distinctive self-assembled core-shell structure, which improves solubility, stability, and bioavailability. The pharmacokinetic and biodistribution behavior of the micelles is significantly influenced by the outside hydrophilic shell[28]. In addition, the improved permeability and retention (EPR) effect of mixed micellar

nanostructures enables passive targeting into solid tumors[28]. Literature study reports that Pluronic® F127 micelles have the limitation in terms of physical stability, drug release and loading of lipophilic moieties. Mixed micelles composed of Pluronic F127 (PF127) and Tween 80 overcame these issues and shows improved stabilities and biopharmaceutical properties of dosage form[29]. Additionally, Pluronic F127 and Tween 80 in a fixed combination so as to provide the desired thermos reversibility for the skin application[30]. These systems have been used in conjunction with a number of anticancer medications, including doxorubicin, propofol, docetaxel, and gambogic acid[31-36]. This study aims for design, development and evaluation of mixed micellar gel formulation of propolis extract PE-MMG to improve drug loading, drug solubilization, stability, better release profile and enhanced cytotoxicity and cancer therapy.

METHODOLOGY

Material

Authenticated Indian propolis was procured from CBRTI, Pune. Tween 80 (Polysorbate 80, Mw=1309) and Pluronic F127 (Mw=12,600, PEO99-PPO67-PEO99) were bought from Sigma-Aldrich Bangalore, India. Analytical Reagent (AR) grade was used for all other reagents and solvents in the investigation.

Methods

Extraction and characterization of propolis

Authenticated crude propolis was purchased from CBRTI, Pune and extracted by following the previously developed method from removal of wax, debris matter etc. by giving hexane pretreatment. Further ethanolic extract of propolis was prepared by maceration technique to obtain enriched ethanolic extract of Indian propolis which was characterized for physical appearance, color, odor, melting point, UV absorbance, total balsam content etc.[20,28].

Preparation of propolis mixed micellar gel

Propolis mixed micellar gels was formulated using “cold” technique, followed by method previously described with slight modifications[29]. PF127 (20%, w/w) and Tween 80 were added to doubled distilled water. The liquid mixture was gently stirred with the magnetic stirrers to dissolve all the PF127 granules overnight at 4°C. The transparent mixed micelle solution at 4°C obtained. Further drug was added at 4°C and mixed until a clear solution was obtained.

The procedure and the process variables that affected the properties and usefulness of the mixed micelles were better understood with preliminary trials. Pluronic F127 concentration (X1) and Tween 80 concentration (X2) were

shown to be important variables in defining the characteristics of the micelles. In order to explore the impact of X1 and X2 (independent variables) on micelle size and EE (dependent variables) a 3^2 factorial design was used as shown in Table 1. This allowed for the creation of a response surface plot[28].

Table 1. Details of formulation batches 3^2 factorial design

Batches	X1 Amt. of PF127	X2 Amt. of Tween 80	X1 Amt. of PF127 (mg)	X2 Amt. of Tween 80 (ml)
F1	-1	-1	100	2
F2	-1	0	100	1.5
F3	-1	1	100	1
F4	0	-1	75	2
F5	0	0	75	1.5
F6	0	1	75	1
F7	1	-1	50	2
F8	1	0	50	1.5
F9	1	1	50	1

Micelles size & surface charge

Size of PE-loaded mixed micelles was assessed on Horiba SZ 100, at 90° scattering angle, measurements of particle size were made. The samples' average particle size was calculated in nanometer after being dispersed in distilled water. Electrophoretic mobility was also determined at a temperature of 25°C.

Drug Loading (DL) & Entrapment Efficiancy (EE)

UV-VIS spectrophotometer was used to measure PE's absorbance at 314 nm, the concentration of PE in the mixed micelles was discovered. Prior to measurement, the micellar solution was suitably diluted with alcohol and by following procedures as described by Bothiraja C et al. drug loading and entrapment efficiancy was evaluated[28].

$$DL\ (\%) = \frac{\text{Weight of the drug in micelles}}{\text{Weight of the feeding phospholipid and drug}} \text{ X } 100$$

$$EE\ (\%) = \frac{\text{Weight of the drug in micelles}}{\text{Weight of the feeding drug}} \text{ X } 100$$

Surface morphology

Transmission electron microscopy technique was used to examine the morphology of the mixed micelles that were loaded with PR. Zeiss EM 109 TEM was used at 80 kV accelerating voltage was to inspect and take pictures of the sample after any surplus solution had beendrained[27].

In vitro release of PE from mixed micelles

in vitro dialysis bag technique was used to study release of PE from mixed micelles using phosphate-buffer saline (pH 6.8) as release medium. Dialysis bag MW cut-off 12,000 Da was used for formulation (1 mg equivalent) as well pure drug as control. Study was carried out under continuous magnetic stirring at 100 rpm/min and 37°C + 0.5°C. UV-VIS spectrophotometer was used to measure the absorbance of PR[26].

In vitro cytotoxicity studies

In vitro cytotoxicity study for propolis extract and developed formulation was carried out by MTT assay as with 96 well plate technique as shown in schematic presentation in Figure 1. ELISA plate reader was used to record the intensity at 540 nm[20]. Schematic presentation of assay format has been given in Figure 1.

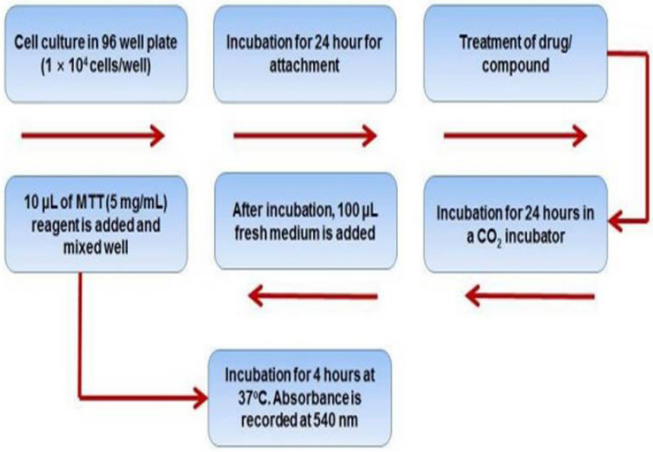

Figure 1. Schematic diagram of MTT assay

RESULTS and DISCUSSION

Attempt has been made to formulate Propolis-loaded Mixed Micelle Gel (PE-MMG). Over the past few decades, it has been well demonstrated that drug delivery methods based on micellar nanotechnology can increase the solubility, effectiveness, and safety of a variety of active ingredients. In order to increase its solubility and ensure prolonged release, PE-MMG has been formulated and explored as a nano-carrier. Design of Experiments (DoE) methodology was used to investigate the impact of the Propolis loaded Mixed Micelle Gel composition on particle size and encapsulation effectiveness.

Preliminary evaluation and characterization of Propolis

At room temperature propolis extract was sticky and yellow to brownish red in color. Propolis was found to have a melting point of 70-71°C.

Propolis extract UV absorbance maxima was observed at 314 nm. Propolis calibration curves was plotted in phosphate buffer saline pH 6.8 was found to follow Beer-Lambert's law over this range. Propolis was found to exhibit high linearity (r2=0.998) over the concentration range of 10-80 micro gram/mL.

Formulation development and optimization

Cold technique method was employed for formulation of Mixed Micelle Gels (MMG). Impact of formulation variables are studied on the particle size and encapsulation as crucial evaluation parameters.

Initial trial batches studied to understand effect of formulation variable subsequently based on results obtained detailed 3^2 factorial design was used for optimization of material attributes. Drug concentration was kept constant, and nine batches were formulated using a 3^2 factorial design. Using Design Expert® Version 12.0, multiple regression analysis was performed. The equation obtained from multiple regression analysis are as below;

Equation 1 Particle Size = +165.00-11.67*A+18.17*B+2.25*AB+8.00A2-15.50*B2

Equation 2 Encapsulation Efficiency = +82.83+1.83*A-4.17*B-1.25*AB-0.40*A2+2.60*B2

Study results revealed that as shown in Figure 1, and Table 1; PF127 and Tween 80 had a favorable effect on the particle size and encapsulation efficiency was shown by the positive coefficients of the major terms X1 and X2. According to the surface plot, the effects of PF127 and Tween 80 on encapsulation efficiency and particle size were curvilinear as shown in Figure 2. The results obtained

from data analysis taken for the determination of optimized batch. From the Design Expert® Version 12.0 software the optimized batch was determined at level of PF127 as (0) and that of Tween 80 as (+1). These values were best fitted with F5 batch of mixed micelle formulation. So F5 batch was considered as an optimized batch based on data in Table 2 and Table 3.

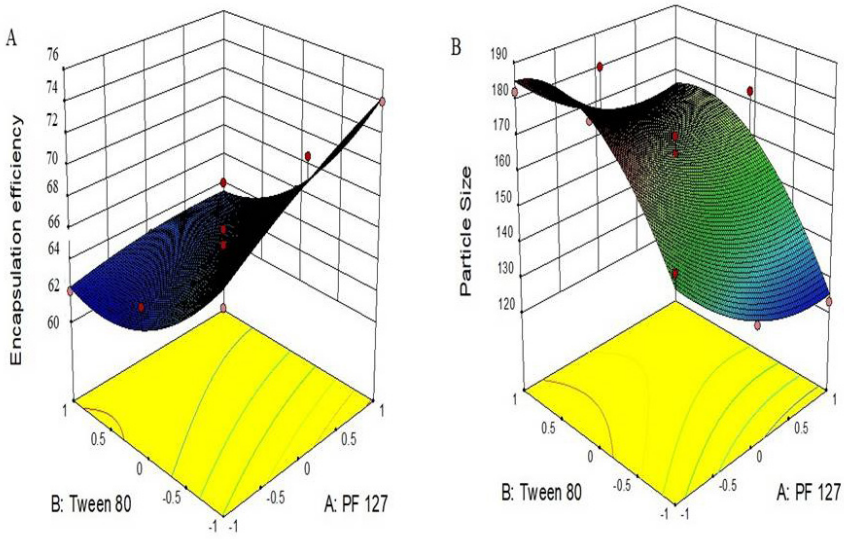

Figure 2. Response surface plots, (A) Encapsulation efficiency (B) Particle size

Table 2. Formulation characterization of PE-MMG

Batch	Particle Size (nm)	Zeta Potential (mV)	Entrapment Efficiency (%)
F1	123 ± 0.85	5.04 ± 1.02	62.52 ± 0.75
F2	130 ± 0.73	5.56 ± 1.13	61.33 ± 0.83
F3	157 ± 1.01	5.88 ± 1.06	65.22 ± 0.76
F4	157 ± 0.96	5.01 ± 1.24	70.65 ± 0.73
F5	162 ± 0.94	5.37 ± 1.04	76.45 ± 0.96
F6	170 ± 1.02	5.09 ± 0.85	71.69 ± 1.22
F7	173 ± 0.71	5.05 ± 0.98	73.75 ± 1.01
F8	180 ± 0.80	5.92 ± 0.70	72.31 ± 1.13
F9	182 ± 0.83	5.89 ± 0.90	62.52 ± 0.96

Table 3. Optimized batch solution

Sr. No	PF127 mg	Tween 80 Ml	Particle Size nm	Encapsulation Efficiency %	Desirability (R2)	Remark
01	72	1.5	160	75%	0.9924	Selected

Characterization of formulation

Particle size

As shown in Table 2 increase in particle size was seen as the concentration of PF127 and Tween 80 was raised. The optimum particle size 162 + 0.5 nm as shown in Figure 3 with single peak of size distribution observed. Micellar size was strongly affected by concentration of selected variables.

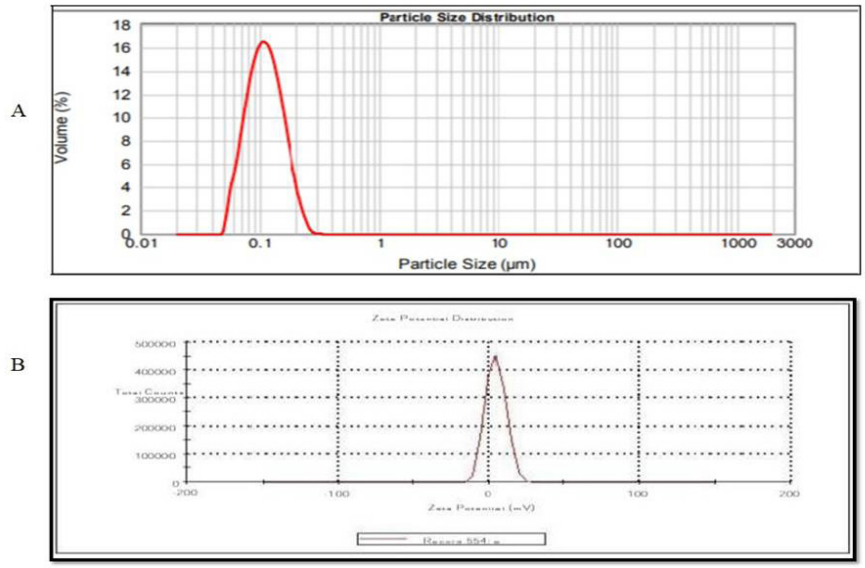

Figure 3. Particle size (A) and zeta potential analysis (B) of F5 formulation of mixed micelle

Zeta potential

Zeta potential is a crucial metric that provides data on surface charges, which directly affect colloidal stability and interaction with bodily cells. The mixed micelle formulation was evaluated for zeta potential from the value it is observed that slight positive values as shown in Table 2 which may be due to presence of PF127 on surface. The values are within acceptable limit and ensure stability. The optimized formulation showed positive zeta potential 5.37 + 1.04 mV as shown in Figure 3.

Encapsulation efficiency

The percent encapsulation efficiency in mixed micelle formulation was observed as shown in Table 2. The F5 batch showed 76.45 + 0.96 % the higher amount of encapsulation efficiency in formulation, with optimal concentration of PF127 and Tween 80.

Surface morphology

The typical spherical core-shell architecture of mixed micelles, which were spherical in shape and homogenous in size, was seen in TEM image (Figure 4). Into the dark core area Propolis is forming the micellar core which is hydrophobic in nature whereas hydrophilic PF127 shows their presence in outer corona shell structure.

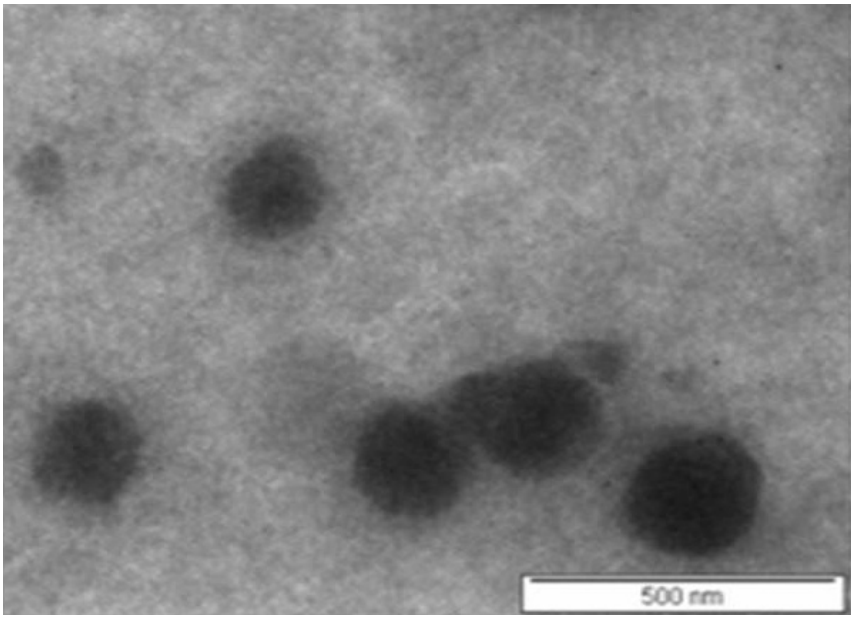

Figure 4.TEM image of Mixed Micelles

***In-vitro* drug release study**

Release pattern of propolis from a mixed micellar gel formulation is observed as shown in Figure 5. Result pattern shows that propolis diffuses freely in solution at about 97.12 + 0.55% in 8 hours. However, the Propolis release from formulation burst release of 46.20 + 0.48% at initial phase in first 2 hrs and sustained release 97.12 + 0.55% up to 8 hours. Three fundamental mechanisms erosion, diffusion, and degradation help a loaded medication release from a mixed mi-

celle. Any one of the three mechanisms or all three can contribute to drug release in a mature system. The first burst release observed during the *in vitro* release may be due to adsorbed drug on micelles, whereas a sustained release may have been brought on by the drug's diffusion. The sustained release of entrapped drug from mixed micelles is a crucial factor in the production of the desired formulation because it keeps a steady level of drug at the site of action over time.

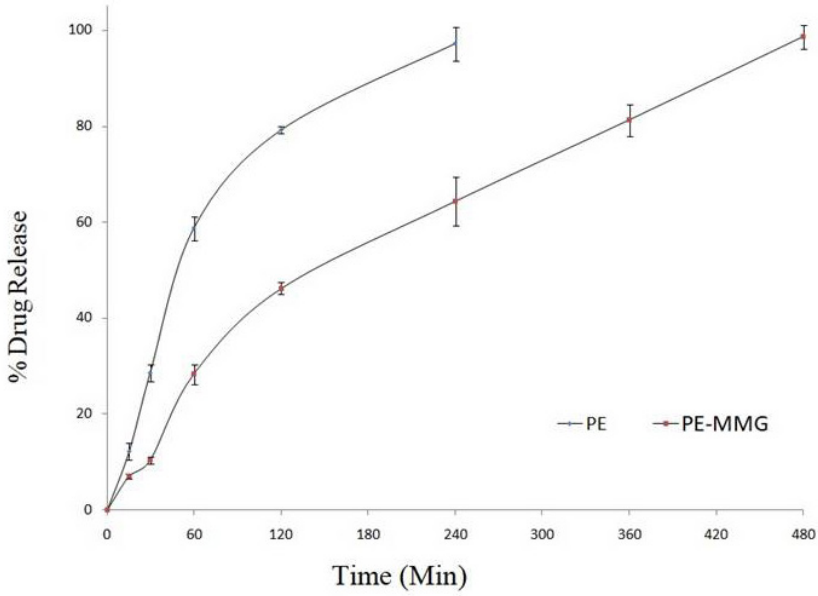

Figure 5. *In vitro* drug release study

Cytotoxicity study

The cytotoxicity study is performed by the MTT Assay method. In this study different concentration of formulation was used to carried cytotoxicity 10, 20, 40 and 80 ug/ml. The *in vitro* anticancer activity of drug and formulation was investigated against MCF-7 cells. The results illustrated in Figure 6 (a–d), Table 4 indicated that PE-MMG showed comparatively better GI50 and TGI as cell growth inhibition parameters to propolis extract.

The TGI value of mixed micellar formulation and propolis extract was found to be 19.2 ± 0.12 µg/mL and 30.10 ± 0.49 µg/mL, respectively. Whereas GI50 value of mixed micellar formulation and propolis extract were obtained less than 10µg/mL. Improved cytotoxicity of formulation as compared to pure drug form may be due to increased cellular uptake through phagocytosis or micellar solubilization.

Table 4. *In vitro* cytotoxicity study

Samples	MCF-7cell line TGI (µg/mL)	MCF-7cell line GI50 (µg/mL)
PE	30.10 ± 0.49	<10
PE-MMG	19.2 ± 0.12	<10
ADR	<10	<10
Blank MMG	74.8 ± 0.11	27.2 ± 0.05

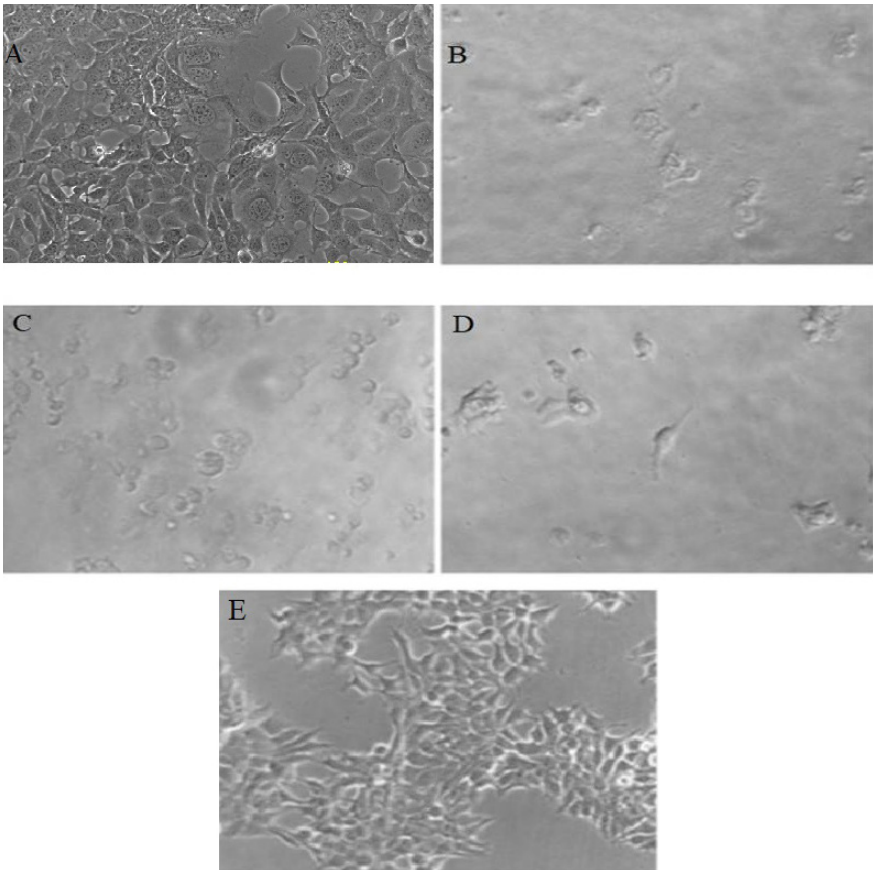

Figure 6. *In vitro* cytotoxicity study (A- MCF 7 Breast cancer cells-Control, B- Treatment of standard, C- Treatment with ethanolic extract of propolis, D- Treatment with Formulation PE-MMG, E- Treatment with blank MMG without drug)

Overall, developed propolis mixed micellar gel formulation displayed desirable formulation characteristics. Optimized formulation showed micellar size 134 nm, spherical shape, 5.04 mV zeta potential, 91.21 + 0.56 % drug loading and encapsulation efficiency was obtained 74 + 0.42 %. The developed formulation showed 46.20 + 0.48% burst release in first 2 hr followed by sustained release 97.12 + 0.55% up to 8 hrs. In overall PE-MMG also showed high encapsulation efficiency which may also give good physical as well chemical stability at various physiological pH. Desirable drug release pattern and improved cytotoxicity potential on MCF-7 cells as compared with propolis extract may improve biopharmaceutical performance. In overall present formulations strategy may be a promising for the delivery of propolis in cancer therapy.

STATEMENT OF ETHICS

Not applicable as no human or animal subjects were involved in the study.

CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest associated with this study.

AUTHOR CONTRIBUTIONS

Concept – H.K, S.R; Design – H.K, S.R,V.P; Supervision – H.K, S.R; Resources – H.K, N.P; Materials – H.K, S.R, N.P.; Data Collection and/or Processing – H.K, S.G, S.R, N.P, V.P.; Analysis and/or Interpretation – H.K, S.G, S.R, N.P, V.P.; Literature Search – H.K, S.G, N.P.; Writing – H.K, N.P; Critical Reviews – H.K, S.G, N.P, V.P, S.R.

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